**30 May 2001**
**15/01**

# INQUIRY REPORT AND REGULATORY IMPACT STATEMENT

## APPLICATION A406

## PERMISSION FOR USE OF NEOTAME

## EXECUTIVE SUMMARY

- ANZFA received an application on 14 December 1999 from Food Liaison Ltd to amend the *Food Standards Code* so as to permit the use of Neotame as an intense sweetener and flavour enhancer by amending Standard A8 – Artificial Sweetening Substances and Standard A6 –Flavourings and Flavour enhancers.

- An assessment, by ANZFA of scientific evaluations at Full Assessment indicated that there were no public health and safety concerns with the use of Neotame as an intense sweetener and flavour enhancer at the levels proposed for use for the general population, and its use was technologically justified. Consequently this Application will, if approved, require an amendment to Volume 1 (Standard A1, A11 and 1.3.1) and Volume (Standard 1.2.4, 1.3.1 and 1.3.4) 2 of the *Food Standards Code*, rather than Volume 1 (Standard A6 and A8), based on the basis that Neotame posed no health and safety concerns and could consequently be approved as a general additive into Volume 1 (Standard 1.3.1) and Volume 2 (Standard 1.3.1) of the *Food Standards Code*.

- Neotame is a dipeptide methyl ester derivative with a sweetness potency 7000-13000 times that of sugar. The applicant claims that Neotame has a clean, sweet taste with no undesirable taste characteristics and exhibits functionality and stability in a wide range of beverages and foods.

- Eleven submissions were received in response to the Preliminary Assessment (section 14 notice under the *Australia New Zealand Food Authority Act 1991*). Seven of these supported the application while the other four did not support the application and expressed concern that the toxicological evaluation was based upon studies provided by the applicant.

- A further 18 submissions were received following Full Assessment, of which, 4 did not support the application. Supporting submissions highlighted the technological benefits

for approval of Neotame, in particular, the smaller quantities and lower concentrations needed for food applications, compared to other intense sweeteners. Non-supporting submissions again raised concerns over public health and safety, in particular, specific aspects of the toxicological data provided by the applicant. However, these points had been addressed at Full Assessment and in addition, were revisited in ANZFA's review of the toxicology report at Inquiry.

- The scientific evaluations indicated that there are no public health and safety concerns with the use of Neotame as an intense sweetener and flavour enhancer at the levels proposed for use for the general population and its use is technologically justified. Neotame can be generally permitted in Volume 1 and 2 of Standard 1.3.1 (Schedule 2) of the *Food Standards Code*.

- The proposed changes at Inquiry are to Volume 1 (Standard A1, A11 and 1.3.1) and Volume 2 (Standard 1.2.4, 1.3.1 and 1.3.4) of the *Food Standards Code.* The proposed changes are consistent with ANZFA's section 10 objectives. The requested changes should be implemented and come into force on gazettal.

- The Regulatory Impact Statement supports the requested amendments and concludes that the preferred option is Option 2 - amend Volume 1 (Standard A1 and 1.3.1) and Volume 2 (Standard 1.2.4 and 1.3.1) of the *Food Standards Code* to permit the use of Neotame as an intense sweetener and flavour enhancer, and provide a specification for Neotame in Volume 1 (Standard A11) and Volume 2 (Standard 1.3.4) of the *Food Standards Code*.

**OBJECTIVES AND BACKGROUND OF THE APPLICATION**

The Authority had before it an Application (A406) received from Food Liaison Ltd on 14 December 1999 seeking a variation to the list of approved artificial sweetening substances in Standard A8 –Artificial Sweetening Substances, and a variation to the list of permitted flavour enhancers in Standard A6 – Flavourings and Flavour enhancers, to include Neotame.

As indicated at Full Assessment, a scientific evaluation of Neotame indicated that there were no public health and safety concerns with the use of Neotame as an intense sweetener and flavour enhancer at the levels proposed for use for the general population, and that its use is technologically justified. Consequently this Application will, if successful, require an amendment to Volume 1 (Standard A1, A11 and 1.3.1) and Volume 2 (Standard 1.2.4, 1.3.1 and 1.3.4) of the *Food Standards Code,* rather than Volume 1 (Standard A6 and A8) on the basis that Neotame posed no health and safety concerns and could consequently be approved as a general additive into Volume 1 and 2.

The applicant claims that Neotame has a clean, sweet taste with no undesirable taste characteristics and exhibits functionality and stability in a wide range of beverages and foods. Neotame can be used alone or blended with other sweeteners. Permission has been requested for Neotame to be used broadly as a sweetener in food, as Neotame has exhibited greater stability in baked goods and dairy foods compared to some other intense sweeteners such as aspartame.

Neotame is a dipeptide methyl ester derivative with a sweetness potency 7000-13000 times that of sugar. This will result in smaller quantities and lower concentrations of Neotame

being used for food applications compared to other intense sweeteners. Neotame is not metabolised to phenylalanine. Therefore, no special labelling provisions that apply to other intense sweeteners will be needed to alert consumers with phenylketonuria, since the food product will not contain phenylalanine. However, general additive labelling requirements are still required for foods containing Neotame.

## RELEVANT PROVISIONS

There is no current permission for the use of Neotame as an artificial sweetening substance or as a flavour enhancer in Australia or New Zealand.

## AUSTRALIA

The initial application sought the addition of Neotame into Volume 1 of the *Food* (Standard A6 and A8) –

### Standard A6 – Flavouring and Flavour Enhancers.

Standard A6 provides for the appropriate use of flavour enhancers as food additives.

### Standard A8 – Artificial Sweetening Substances.

Seven other intense sweeteners (saccharin, cyclamate, aspartame, acesulphame potassium, thaumatin, sucralose and alitame) are approved for use in a range of foods and beverages.

### Volume 1 and Volume 2 of the *Food Standards Code* – Standard 1.3.1

An assessment, by the Authority, of scientific evaluations at Full Assessment indicated that there were no public health and safety concerns with the use of Neotame as an intense sweetener and flavour enhancer at the levels proposed for use for the general population, and its use was technologically justified. It was determined that Neotame posed no health and safety concerns and could consequently be approved as a general food additive into Volume 1 (Standard 1.3.1) and Volume 2 (Standard 1.3.1) of the *Food Standards Code*. Consequently this Application will, if approved, require an amendment to Volume 1 (Standard 1.3.1) and Volume 2 (Standard 1.3.1) of the *Food Standards Code,* to include Neotame as a general food additive, rather than an amendment to Volume 1 (Standard A6 and A8).

Standard 1.3.1 in both Volume 1 and Volume 2 of the *Food Standards Code* regulates the use of food additives in the production and processing of food – and contains Schedules specifying:

Schedule 1 - Permitted uses of food additives by food type;
Schedule 2 - Miscellaneous additives permitted to GMP in processed foods specified in Schedule 1;
Schedule 3 - Colours permitted to GMP in processed foods specified in Schedule 1;
Schedule 4 - Colours permitted to specified levels in processed foods specified in Schedule 1; and
Schedule 5 -Technological functions, which may be performed by food additives.

Food additives included in 1.3.1 include flavour enhancers and artificial sweetening substances such as aspartame, in addition to others.

**NEW ZEALAND**

*New Zealand Food Regulations 1984.*

251. Artificial sweeteners

(1) In these regulations "artificial sweetener" means any substance that, when added to food, is capable of imparting sweetness to that food, and that is not a saccharide, sugar alcohol, or carbohydrate sweetener.

(2) The following substances shall be artificial sweeteners for the purposes of these regulations:

Saccharin and its sodium, calcium, and ammonium compounds;

Sodium cyclamate and calcium cyclamate;

Aspartame;

Alitame;

Acesulphame K; and

Thaumatin.

International

Neotame has not been approved for use in other countries and there are no specific Codex Standards for Neotame. The United States Food and Drug Administration is currently in the process of reviewing Neotame as a food additive.

**PROPOSED CHANGES**

Volume 1 and Volume 2 of the *Food Standards Code*

Since receiving application A406, the Australia New Zealand Food Standards Council (ANZFSC) adopted the *Australia New Zealand Food Standards Code* (known as Volume 2 of the *Food Standards Code*). Consequently, the Inquiry Report includes drafting for both Volume 1 and Volume 2 of the Code.

Standard 1.3.1 - Food Additives, contained in Volume 1 and Volume 2 of the *Food Standards Code,* provides permissions for the addition of additives including intense sweeteners. An assessment by ANZFA of scientific evaluations at Full Assessment revealed that there were no public health and safety concerns with the use of Neotame as an intense sweetener and flavour enhancer at the levels proposed for use for the general population, and its use was technologically justified. Consequently this Application will, if approved, require an amendment for Neotame to be permitted as a food additive in Volume 1 (Standard A1, A11 and 1.3.1) and Volume 2 (Standard 1.2.4, 1.3.1 and 1.3.4) of the *Food Standards Code*

(Attachment 1), rather than an amendment to Volume 1 (Standard A6 and A8) on the basis that Neotame poses no health and safety concerns and could consequently be approved as a general additive into Volume 1 (Standard 1.3.1) and 2 (Standard 1.3.1).
A review of the proposed drafting after Full Assessment for the Inquiry Report resulted in minor changes to the Full Assessment drafting including -

- **Full Assessment Report - Proposed amendment to Standard T1 of the *Food Standards Code***

An amendment to the Transitional Standard (T1) was proposed in the drafting at Full Assessment. The proposed amendment attempted to ensure that manufacturers refer to product specifications in Volume 1 (Standard A11) and Volume 2 (Standard 1.3.4) for food additives contained in Volume 1 (Standard 1.3.1) and Volume 2 (Standard 1.3.1), where applicable.

A review of the Full Assessment draft variations revealed that an amendment to Standard T1 would not achieve this aim and was not required in Volume 2 (Standard 1.3.1) as the *Purpose* commentary to that standard alerted manufacturers to the fact Volume 2 (Standard 1.3.4) prescribes standards for the identity and purity of food additives. In addition to the above, Standard 1.3.4 is a standard of general application, and as specified in clause 1 "applies to substances added to food in accordance with this Code, and to such substances sold for use in food".

During the transition period, manufacturers may elect to manufacture to either Volume 1 or Volume 2. The reference in the *Purpose* commentary in Volume 1 (Standard 1.3.1) to "Standard 1.3.4 prescribes standards for the identity and purity of food additives" requires minor amendment to read Standard A11 in the place of Standard 1.3.4. The basis for this changed is that manufacturers who elect to manufacture to Volume 1 (Standard 1.3.1, must comply with the requirements of Volume 1. Food produced by manufactures may not comply with a combination of parts of Volume 1 and Volume 2 (and in New Zealand parts of the Food Regulations). Consequently, Volume 1 (Standard 1.3.1) will need to have a minor amendment to it so that it refers to Standard A11, the correct standard containing specifications for identity and purity of food additives for Volume 1.

<u>Conclusion</u>

The draft amendment to Standard T1 at Full Assessment has been removed from drafting attached to the Inquiry Report and drafting has been included for the minor amendment of Volume 1 (Standard 1.3.1) to replace the reference to Standard 1.3.4 in the *Purpose* commentary of that Standard with a reference to Standard A11 as detailed above.

- **Inquiry Report – Additional draft variation to Volume 1 (Standard A11) of the *Food Standards Code***.

During the transition period manufacturers may elect to manufacture to either Volume 1 or Volume 2. However, food produced by manufactures may not comply with a combination of parts of Volume 1 and Volume 2 (and in New Zealand parts of the Food Regulations).

Drafting was included at Full Assessment for the inclusion of product specifications for Neotame into Volume 2 (Standard 1.3.4 – Identity and Purity), but did not incorporate

product specifications into Volume 1 (Standard A11 – Specifications for identity of food additives, processing aids, vitamins, minerals and other added nutrients).
As manufacturers who elect to manufacture to Volume 1are unable to produce food utilising standards from Volume 2, specifications for Neotame needed to be included in both Volume 1 (Standard A11) and Volume 2 (Standard 1.3.4).

Conclusion:

An additional draft amendment to Volume 1 (Standard A11) has been included in the Inquiry Report to incorporate product specifications for Neotame into this standard, thereby rectifying the omission in the drafting at Full Assessment.

## PUBLIC CONSULTATION

### *Preliminary Assessment*

A notice requesting public comment was posted on 23 February 2000 and submissions closed on 5 April 2000.

Submissions were received from the Confectionery Manufacturers of Australasia, New Zealand Dairy Board, Mr Arnold Ward, National Council of Women of Australia, Goodman Fielder, Dietitians Association of Australia, Australian Food and Grocery Council, Australasian Soft Drink Association, InforMed Systems, Ms Barbara Baragwanath, and Ms Natalie Baragwanath. The main issues raised are summarised below.

- **Support technological benefits of Neotame over existing permitted sweetening substances (subject to satisfactory toxicology and safety evaluation).**

Confectionery Manufacturers of Australasia, Goodman Fielder, InforMed Systems Ltd, Australian Food and Grocery Council, New Zealand Dairy Board, Australasian Soft Drink Association Ltd, Dietitians Association of Australia.

These submissions highlighted the technological benefits for approval of Neotame, in particular, the smaller quantities and lower concentrations needed for food applications, compared to other intense sweeteners

- **Concern about safety and that the safety evaluation is based on studies provided by the applicant.**

National Council of Women of Australia, Ms Barbara Baragwanath, Ms Natalie Baragwanath, and Mr Arnold Ward

These submissions highlighted concerns over the use of toxicological studies provided by the applicant as not being independent of the company, that Neotame was more toxic than aspartame and that there was overwhelming evidence that Neotame is associated with adverse effects. This data had been provided and sourced via the Internet.

### *Full Assessment*

The Board of the Authority approved the Full Assessment Report on 14 December 2000 and the draft standard was released for public comment on 20 December 2000.

**Summary of new submissions received at Inquiry (Attachment 6)**

At Inquiry, 18 submissions were received of which the majority supported the application (14/18); and four submissions did not support the application. The later submissions raised public health and safety concerns, which ANZFA had already addressed at Full Assessment. Additionally, ANZFA reviewed the toxicology report at Inquiry in order to revisit these concerns and to resolve the issues raised in key submissions below.

The two most extensive submissions, which raised specific points that ANZFA needed to address, and respond to, are considered below.

**NutraSweet Company**

NutraSweet submitted a detailed submission, which focused on the following areas:

- In the toxicological assessment, there was too much emphasis placed on the results obtained in the shorter dose-ranging studies which were not substantiated by longer-term studies;
- In the toxicological assessment, conclusions drawn for some studies, particularly in relation to the no-observed-effect levels, were not supported by the data; and
- A need to clarify some comments on labelling, stability of Neotame and some of the assumptions made in the dietary modelling.

**Holland and Knight LLP**

Presented data which suggested that significant safety issues remain unaddressed and must be resolved before approval. The points raised were in respect of a submission to the USA FDA from NutraSweet for approval of Neotame.

This related to the following:

- Effects in two long-term dog studies were due to Neotame toxicity to the liver which were not reversible as implied by the petitioner; and
- A Neotame-induced effect on implantation loss, foetal size and limb development in the rabbit teratology study was masked by the quality of the studies and the high background incidences of effects.

**Evaluation of Issues Raised in Public Submissions**

***Preliminary Assessment***

- **Assessment of Neotame technological functions as an intense sweetener and flavour enhancer.**

A comparison of Neotame against other intense sweeteners was made in the Food Technology Report (Attachment 4). This report concluded that Neotame was a viable alternative to other available intense sweeteners and flavour enhancers and its use is technologically justified.

- **Safety of Neotame and need for independent public health and safety assessment**

A toxicological and dietary exposure report concluded that there are no toxicological concerns from the use of Neotame as an intense sweetener and flavour enhancer (Attachments 3 and 5).

***Full Assessment***

*NutraSweet Submission*

ANZFA has considered in detail the points made by Nutrasweet and has revised the report where it was considered appropriate.

Key changes to the original safety assessment report are as follows:

- The report places less emphasis on some of the minor isolated findings in the short-term range-finding studies when these findings were not repeated in longer-term studies;
- A revision of the no-observed-effect levels (NOELs) in the sub-chronic and chronic studies on the basis that, on reconsideration, the observed bodyweight changes in the animals at the higher dose levels were related to decreased palatability of the Neotame-containing diet rather than to toxicity *per se*; and
- A new ADI of 2 mg/kg bw/day has been set based on a revised overall NOEL of 200 mg/kg bw per day observed in a 1-year study in dogs.

ANZFA has corrected the missing word 'not' from the previous version of the Explanatory Notes, thus concurring, as originally intended, that no special labelling provisions are needed to alert consumers with phenylketonuria (PKU).

ANZFA has also clarified the claims with respect to stability of Neotame in baked goods and dairy foods in the Food Technology Report, and has revised the Dietary Modelling Report to include market share data to more accurately reflect exposure to Neotame for the general population (see Attachment 4 and 5).

*Holland and Knight LLP submission*

ANZFA considered the data in the submission and found that these specific issues were already addressed in the toxicological report. Analysis of the sub chronic and long-term study in dogs showed that the increases in liver alkaline phosphatase activities at high-dose were reversible and no other histopathological changes or increases in other liver enzymes were observed. With respect to the criticism of the rabbit teratology study, ANZFA examined these studies in detail and has concluded that the studies were adequate and conformed to current international toxicological guidelines with respect to quality of the studies. The conclusion is that Neotame is not teratogenic in rabbits up to doses of 1000 mg/kg bw/day (Attachment 3).

## SCIENTIFIC ASSESSMENT

### Toxicological Report (Refer to Attachment 3)

A comprehensive set of toxicology data has been provided to support the safety of Neotame.

Neotame is very stable under the conditions of use. The major degradation product, NC-00751 is also the major metabolite in animal and human studies. It is formed by hydrolysis of the methyl ester group of Neotame. There are other very minor metabolites.

Metabolic studies in rats indicate rapid absorption of around 20-30% of orally administered Neotame followed by hydrolysis to form NC-00751 and rapid excretion via the urine and faeces. There is no evidence of tissue accumulation of either Neotame or its metabolites.

The available short-term, subchronic and chronic studies indicate that Neotame is well tolerated in all species (rats, mice and dogs) with little evidence of treatment-related adverse effects. The most significant finding in these animal species was a decrease in bodyweight and bodyweight gain at the higher dose levels that is accompanied by a decrease in food consumption. These findings are considered to be related to decreased palatability of the Neotame-containing diet rather than to toxicity. Specific studies conducted to examine palatability of the Neotame diet at various dose levels demonstrated marked preference by rats for the control diet than for one containing Neotame. The variety of treatment-related changes in clinical pathology parameters and from histopathological examinations does not indicate any particular Neotame-related toxicity.

There is no evidence of adverse effects in reproduction studies in rats, developmental toxicity studies in rabbits and rats and in a range of *in vitro* and *in vivo* genotoxicity studies.

In human studies, Neotame is well tolerated at dose levels of 1.5 mg/kg bw/day. Plasma glucose levels and insulin levels in non-insulin dependent diabetes mellitus patients were normal following treatment with Neotame at 1.5 mg/kg bw/day.

The chronic studies conducted with Neotame in mice, rats and dogs demonstrate no evidence of adverse effects other than an increase in alkaline phosphatase activity at the highest dose in a long-term dog study. The toxicological significance of this change is unclear, since the change was reversible, no other liver enzymes were elevated and there was no histopathological changes observed. While some lower no-observed-effect levels (NOELs) were found in the subchronic studies, the effects upon which they were based were not seen in the chronic studies. The lowest NOEL, therefore, is 200 mg/kg bw per day established for the 52-week dog study.
After applying a 100-fold safety factor, the acceptable daily intake (ADI)[1] for humans is 2 mg/kg bw per day.

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[1]The ADI is an estimate of the amount of a chemical that can be consumed every day over a lifetime without appreciable health risk.

**Food Technology Report (Refer to Attachment 4)**

Neotame is a viable alternative to other available intense sweeteners and flavour enhancers and its use is technologically justified for use in food and beverages. It has the properties required of an intense sweetener and flavour enhancer. It offers the advantages of greater stability in certain applications and lower usage levels compared to other permitted intense sweeteners and flavour enhancers.

**Dietary exposure assessment report (Refer to Attachment 5)**

The dietary modelling results indicate that for the whole population for both Australia and New Zealand, the estimated dietary exposures to Neotame were well below the ADI for mean respondents and consumers, and were 3 to 6% of the ADI for high consumers. Population results, as opposed to results for smaller age groups, generally give the best indication of dietary exposures over a lifetime. These results are much lower than those derived at Full Assessment, due to data on market share of specific food groups and a revised ADI being included in the revised dietary exposure calculations.

The revised results do not change the overall conclusion made at Full Assessment that Neotame could be generally permitted in Volume 1 and 2 (Standard 1.3.1) of the *Food Standards Code*.

As Neotame is a new intense sweetener, ANZFA proposes to monitor consumption patterns via an intense sweetener consumption survey in the near future to provide base line data on individual sweetener use. As dietary modelling is based on the assumption of market share, monitoring would test the market share values used.

## REGULATORY IMPACT ANALYSIS

## OPTIONS

1. Maintain the *status quo* and not permit the use of Neotame as an intense sweetener and flavour enhancer.
2. Amend Volume 1 (Standard A1 and 1.3.1) and Volume 2 (Standard 1.2.4 and 1.3.1) of the *Food Standards Code* to permit the use of Neotame as an intense sweetener and flavour enhancer, and provide a specification for Neotame in Volume 1 (Standard A11) and Volume 2 (Standard 1.3.4) of the *Food Standards Code*.

### 1. Issue identification

Alternatives to regulation are not considered appropriate for the use of Neotame as an intense sweetener and flavour enhancer. Intense sweeteners for use in Australia are listed in Standard A8 and flavour enhancers in Standard A6 or in Standard 1.3.1 Food Additives. New entries for food additives in Standard 1.3.1 are required to undergo an evaluation to determine efficacy and to ensure that there are no apparent public health and safety concerns with permitting their use. The standard is intended to reflect current use and to prohibit inappropriate use of intense sweeteners and flavour enhancers.

Parties likely to be affected by the possible options as listed above are consumers, manufacturers and State/Territory and New Zealand Health Departments.

**Option 1**

- Maintain the *status quo* and not permit the use of Neotame as an intense sweetener and flavour enhancer.

| AFFECTED PARTY | BENEFITS | COSTS |
|---|---|---|
| **Government** | No perceived benefits | No perceived costs |
| **Industry** | No perceived benefits | There are other intense sweeteners and flavour enhancer agents permitted for use, such as saccharin, cyclamate, aspartame, acesulphame potassium, thaumatin, sucralose, and alitame which industry can currently use. The use of Neotame compared to aspartame however, may result in lower costs and improved function in baked goods and dairy because of its stability. Maintaining the status quo would deny industry any advantages that the use of Neotame may give. |
| **Consumers** | No perceived benefits | An alternative intense sweetener such as Neotame may be seen as desirable to have available to some consumers as it may provide advantages by virtue of its greater stability in baked goods and dairy foods compared to other sweeteners. |

**Option 2**

- Amend Volume 1 (Standard A1 and 1.3.1) and Volume 2 (Standard 1.2.4 and 1.3.1) of the *Food Standards Code* to permit the use of Neotame as an intense sweetener and flavour enhancer, and provide a specification for Neotame in Volume 1 (Standard A11) and Volume 2 (Standard 1.3.4).

| AFFECTED PARTY | BENEFITS | COSTS |
|---|---|---|
| **Government** | No perceived benefit | No perceived cost |
| **Industry** | Permitting the use of Neotame would provide food manufacturers with an alternative intense sweetener and flavour enhancer, which may result in lower costs in all foods and improved function in baked goods and dairy products. | Providing industry with a greater choice of intense sweeteners and flavour enhancer would incur no costs. |
| **Consumers** | Increasing the choice of intense sweeteners and flavour enhancers available may assist in improving food variety and this would be of benefit to consumers. Neotame provides an alternative sweetener for consumers with PKU in that it does not break down to phenylalanine. | No perceived costs apart from the objection some individuals may have to the increase in number of intense sweeteners and flavour enhancers permitted for use on food. |

## 2. Evaluation

Maintaining the *status quo* (Option 1) appears to provide no benefit to government, industry and consumers. Option 1 denies industry access to an intense sweeteners and flavour enhancer, which is of low toxicity, is effective at higher temperatures than other additives, and may contribute to lower production costs.

Option 2, which proposes to amend the joint *Food Standards Code* to permit the use of Neotame as an intense sweetener and flavour enhancer, appears to impose no significant costs on government, industry or consumers and may be of benefit to industry and consumers. Assessment of the costs and benefits of Options 1 and 2 indicates that there would be a net benefit in permitting the use of Neotame.

## ASSESSMENT OF ANZFA'S SECTION 10 OBJECTIVES

### (a) The protection of public health and safety

Toxicological evaluation of Neotame indicates that there are no public health and safety concerns associated with its use as an intense sweetener and flavour enhancer.

### (b) The provision of adequate information relating to food to enable consumers to make informed choices and to prevent fraud and deception

There is a requirement for labelling of food additives in the *Food Standards Code*. Provision of this information would be meaningful to consumers.

### (c) The promotion of fair-trading in food

If approved, all members of the industry may use Neotame, and no issues in relation to fair-trading were raised. To not allow approval may disadvantage manufacturers.

### (d) The promotion of trade and commerce in the food industry

The approval of Neotame will provide industry with an intense sweetener and flavour enhancer that may provide benefits over existing agents. This could facilitate trade and commerce in the food industry.

### (e) The promotion of consistency between domestic and international food standards where these are at variance.

There is currently no approval for use of Neotame as an intense sweetener and flavour enhancer in other countries.

## CONCLUSIONS

Permitting the use of Neotame as an intense sweetener and flavour enhancer is technologically justified and poses no risk to public health and safety at the levels proposed for use. Neotame could be generally permitted in Volume 1 (Standard 1.3.1 – Schedule 2) and Volume 2 (Standard 1.3.1 - Schedule 2).

As Neotame is a new intense sweetener, ANZFA proposes to monitor its use vis an intense sweetener consumption survey in the near future to provide base line data on individual sweetener use. As the dietary modelling is based on the assumption of market share, monitoring would test the market share values used.

Approval of Neotame with a precise specification will provide manufacturers the choice of an alternative intense sweetener and flavour enhancer.

## WORLD TRADE ORGANIZATION (WTO) NOTIFICATION

Australia and New Zealand are members of the WTO and are bound as parties to WTO agreements. In Australia, an agreement developed by the Council of Australian Governments (COAG) requires States and Territories to be bound as parties to those WTO agreements to which the Commonwealth is a signatory. Under the agreement between the Governments of Australia and New Zealand on Uniform Food Standards, ANZFA is required to ensure that food standards are consistent with the obligations of both countries as members of the WTO.

In certain circumstances Australia and New Zealand have an obligation to notify the WTO of changes to food standards to enable other member countries of the WTO to make comment. Notification is required in the case of any new or changed standards which may have a significant trade effect and which depart from the relevant international standard (or where no international standard exists).

The proposed variation to the Code constitutes a minor technical change and is not expected to significantly impact on trade issues for either technical or sanitary or phytosanitary reasons. A notification was not made to the WTO, as approval of Neotame is not expected to significantly impact on trade of member nations. This decision is consistent with the established principles for determining whether notification is required to the WTO.

**Attachments to the Report:**

1. Variations to the *Food Standards Code*
2. Statement of Reasons
3. Toxicological Report
4. Food Technology Report
5. Dietary Assessment Report
6. Summary of submissions received at Inquiry

**ATTACHMENT 1**

**VARIATIONS TO VOLUME 1 AND VOLUME 2 OF THE *AUSTRALIA NEW ZEALAND FOOD STANDARDS CODE***

**APPLICATION A406**

**PERMISSION FOR USE OF NEOTAME**

**To commence: On gazettal**

**[1]** ***Standard A1*** *of Volume 1 of the Food Standards Code is varied by –*

[1.1] *inserting in columns 1 and 2 respectively of the Schedule Part 1 Food Additive Code Numbers (Alphabetical Order), immediately following the entry for Natamycin –*

| | |
|---|---|
| Neotame | Number pending |

[1.2] *inserting in columns 1 and 2 respectively of the Schedule Part 2 Food Additive Code Numbers (Numerical Order), immediately before the entry for Curcumin –*

| | |
|---|---|
| Neotame | Number pending |

**[2]** ***Standard A11*** *of Volume 1 of the Food Standards Code is varied by -*

[2.1] *inserting in columns 1 and 2 respectively of the Schedule, immediately following the entry for Natamycin –*

| | |
|---|---|
| Neotame | Addendum 9 |

[2.2] *inserting immediately following Addendum 8 Specifications for phytosterol esters derived from vegetable oils**,** the following –*

**ADDENDUM 9**

**SPECIFICATION FOR NEOTAME**

Neotame (CAS Number 165450-17-9) is a dipeptide methyl ester derivative, and is prepared by the reductive alkylation of N-L-α-aspartyl-L-phenylalanine 1-methyl ester (aspartame).

| | |
|---|---|
| Formula: | $C_{20}H_{30}N_2O_5$ |
| Molecular Weight: | 378.47 |

Physical Tests

| | |
|---|---|
| Appearance: | Powder |
| Colour: | White to off-white |
| Solubility in water: | 4.75% (w/w) at 60°C, soluble in ethanol and ethyl acetate |
| Refractive index: | 1.3338 |

(0.5% aqueous solution of Neotame at $20^oC$)

| | |
|---|---|
| pH: | 5.80 |
| (0.5% aqueous solution of Neotame at 20°C) | |
| Octanol/$H_2O$ Partition coefficient: $Log_{10}P$=0.917 | |
| $pK_a$: | 3.03/8.08 |

Chemical

| | |
|---|---|
| Melting Range: | 80.9°C – 83.4°C |
| Assay: | Not less than 97.0% and not more than 102% of Neotame calculated on a dry basis. |
| N-(3,3-dimethylbutyl)-L -α-aspartyl-L-phenylalanine: | Not more than 1.5% |
| Lead (Pb): | Not more than 2 mg/kg |
| Other Related Substances: | Not more than 2.0% |
| Water: | Not more than 5.0% |
| Residue on Ignition: | Not more than 0.2% |
| Specific Rotation: | $[\alpha]^{20^\circ}$: between –40.0° and –43.4°, calculated on a dried basis. |

**[3]** ***Standard 1.3.1*** *of Volume 1 of the Food Standards code is varied by deleting the Purpose commentary and substituting –*

> A food additive is any substance not normally consumed as a food in itself and not normally used as an ingredient of food, but which is intentionally added to a food to achieve one or more of the technological functions specified in Schedule 5. It or its by-products may remain in the food. Food additives are distinguishable from processing aids and vitamins and minerals added to food for nutritional purposes.
>
> This Standard regulates the use of food additives in the production and processing of food. A food additive may only be added to food where expressly permitted in this standard. Additives can only be added to food in order to achieve an identified technological function according to Good Manufacturing Practice.
>
> Standard A11 prescribes standards for the identity and purity of food additives.

**[4]** ***Standard 1.3.1*** *of Volume 2 of the Food Standards code is varied by deleting the Purpose commentary and substituting –*

> A food additive is any substance not normally consumed as a food in itself and not normally used as an ingredient of food, but which is intentionally added to a food to achieve one or more of the technological functions specified in Schedule 5. It or its by-products may remain in the food. Food additives are distinguishable from processing aids (see Standard 1.3.3) and vitamins and minerals added to food for nutritional purposes (see Standard 1.3.2).
>
> This Standard regulates the use of food additives in the production and processing of food. A food additive may only be added to food where expressly permitted in this standard. Additives can only be added to food in order to achieve an identified technological function according to Good Manufacturing Practice.

**[5]** ***Standard 1.3.1*** *of Volume 1 and 2 of the Food Standards Code is varied by –*

[5.1] *inserting in columns 1 and 2 respectively of Schedule 2 (Alphabetical listing), immediately following the entry for Monostarch phosphate –*

- Neotame

[5.2] *inserting in columns 1 and 2 respectively of Schedule 2 (Numeric listing), immediately before the entry for Calcium carbonates –*

- Neotame

**[6]** ***Standard 1.2.4*** *of Volume 2 of the Food Standards code is varied by –*

[6.1] *inserting in columns 1 and 2 respectively of Schedule 2, Part 1 Food Additive Code Numbers (alphabetical order), immediately following the entry for Natamycin or pimaricin –*

Neotame **-**

[6.2] *inserting in columns 1 and 2 respectively of Schedule 1, Part 2 Food Additive Code Numbers (numerical order), immediately before the entry for Curcumin –*

Neotame -

**[6]** ***Standard 1.3.4*** *of Volume 2 of the Food Standards Code is varied by inserting in the Schedule, immediately following Addendum 8 Specifications for phytosterol esters derived from vegetable oils –*

**Specification for Neotame**

Neotame (CAS Number 165450-17-9) is a dipeptide methyl ester derivative, and is prepared by the reductive alkylation of N-L-α-aspartyl-L-phenylalanine 1-methyl ester (aspartame).

| | |
|---|---|
| Formula: | $C_{20}H_{30}N_2O_5$ |
| Molecular Weight: | 378.47 |

Physical Tests

| | |
|---|---|
| Appearance: | Powder |
| Colour: | White to off-white |
| Solubility in water: | 4.75% (w/w) at 60°C, soluble in ethanol and ethyl acetate |
| Refractive index: (0.5% aqueous solution of Neotame at 20°C) | 1.3338 |
| pH: (0.5% aqueous solution of Neotame at 20°C) | 5.80 |
| Octanol/$H_2O$ Partition coefficient: | $Log_{10}P=0.917$ |
| $pK_a$: | 3.03/8.08 |

Chemical

| | |
|---|---|
| Melting Range: | 80.9°C – 83.4°C |
| Assay: | Not less than 97.0% and not more than 102% of Neotame calculated on a dry basis. |

| | |
|---|---|
| N-(3,3-dimethylbutyl)-L -α-aspartyl-L-phenylalanine: | Not more than 1.5% |
| Lead (Pb): | Not more than 2 mg/kg |
| Other Related Substances: | Not more than 2.0% |
| Water: | Not more than 5.0% |
| Residue on Ignition: | Not more than 0.2% |
| Specific Rotation: | $[\alpha]^{20^\circ}$: between –40.0° and –43.4°, calculated on a dried basis. |

**ATTACHMENT 2**

**STATEMENT OF REASONS**

**APPLICATION A406**

**PERMISSION FOR USE OF NEOTAME**

The Australia New Zealand Food Authority (ANZFA) had before it an Application (A406) received on 14 December 1999 from Food Liaison Ltd to amend the *Food Standards Code* so as to permit the use of Neotame as an intense sweetener and flavour enhancer by amending Standard A8 – Artificial Sweetening Substances and Standard A6 –Flavourings and Flavour enhancers.

Since receiving Application A406, the Australia New Zealand Food Standards Council (ANZFSC) adopted the *Australia New Zealand Food Standards Code* (now known as Volume 2 of the *Food Standards Code*). Consequently, the Inquiry Report includes drafting for both the Volume 1 (previously known as the Australian *Food Standards Code*) and Volume 2. Since Full Assessment, minor drafting changes to the drafting at Full Assessment have occurred (see Changes to drafting after Full Assessment below).

At Full Assessment of the Application, scientific evaluations indicated that there were no public health and safety concerns with the use of Neotame as an intense sweetener and flavour enhancer for the general population and its use is technologically justified. Neotame could thus be generally permitted in Volume 1 and in Standard 1.3.1 (Schedule 2) of Volume 2. Draft variations to Standard 1.2.4 – Labelling of Ingredients, Standard 1.3.1 - Food Additives and Standard 1.3.4 - Identity and Purity were prepared at Full Assessment.

Neotame has a clean, sweet taste with no undesirable taste characteristics and exhibits functionality and stability in a wide range of beverages and foods. Neotame can be used alone or blended with other sweeteners. Neotame is to be provided broadly as a sweetener in food. It is claimed that Neotame has greater stability in baked goods and dairy foods compared to some other intense sweeteners such as aspartame. Neotame is a dipeptide methyl ester derivative with a sweetness potency 7000-13000 times that of sugar. This will result in much smaller quantities and lower concentrations of Neotame being used for food applications compared to other intense sweeteners.

An extensive toxicological evaluation of Neotame indicates that there are no public health and safety concerns associated with its use as an intense sweetener and flavour enhancer.
The dietary modelling results indicate that for the whole population for both Australia and New Zealand, the estimated dietary exposures to Neotame were well below the acceptable daily intake (ADI[2]) for mean respondents and consumers, and were 3 to 6% of the ADI for high consumers.

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[2] The ADI is an estimate of the amount of a chemical that can be consumed every day over a lifetime without appreciable health risk.

Population results, as opposed to results for smaller age groups, generally give the best indication of dietary exposures over a lifetime. These results are much lower than those derived at Full Assessment, due to the inclusion of precise market share data for selected food commodities and a revised ADI being included in the revised model.

As Neotame is not metabolised to phenylalanine the special labelling provisions that apply to aspartame will not be needed to alert consumers with phenylketonuria, since the product will not contain phenylalanine. However, general additive labelling requirements are still required for Neotame.

It is concluded that permitting the use of Neotame as an intense sweetener and flavour enhancer is technologically justified and poses no increased risk to public health and safety. It is proposed that Neotame be generally permitted as a food additive in Volume 1 (Standard 1.3.1) and Volume 2 (Standard 1.3.1).

## REGULATION IMPACT ANALYSIS

ANZFA develops food regulations suitable for adoption in Australia and New Zealand. It is required to consider the impact, including compliance costs to business, of various regulatory (and non-regulatory) options on all sectors of the community that includes the consumers, food industry and governments in both countries. The regulation impact assessment will identify and evaluate, though not be limited to, the costs and benefits of the regulation, and its health, economic and social impacts. In the course of assessing the regulatory impact, ANZFA is guided by the *Australian Guide to Regulation* (Commonwealth of Australia 1997) and New Zealand *Code of Regulatory Practice*.

Consideration of the Regulatory Impact for this application concludes that the amendment to the Code is cost effective, of benefit to both producers and consumers, and is the preferred regulatory option.

## CHANGES TO DRAFTING AFTER FULL ASSESSMENT

Since receiving application A406, the Australia New Zealand Food Standards Council (ANZFSC) adopted the *Australia New Zealand Food Standards Code* (now known as Volume 2 of the *Food Standards Code*). Consequently, the Inquiry Report includes drafting for both Volume 1 and Volume 2 of the *Food Standards Code*.

Standard 1.3.1 - Food Additives, contained in Volume 1 and Volume 2 of the *Food Standards Code,* provides permissions for the addition of additives including intense sweeteners. An assessment by the Authority of scientific evaluations at Full Assessment revealed that there were no public health and safety concerns with the use of Neotame as an intense sweetener and flavour enhancer at the levels proposed for use for the general population, and its use was technologically justified. Consequently this Application will, if approved, require an amendment for Neotame to be permitted as a food additive in Volume 1 of the *Food Standards Code* (Standard A1, A11 and 1.3.1) and Volume 2 (Standard 1.2.4, 1.3.1 and 1.3.4) of the *Food Standards Code* (Attachment 1), rather than an amendment to Volume 1 (Standard A6 and A8) on the basis that Neotame poses no health and safety concerns and could consequently be approved as a general additive into Volume 1 (Standard 1.3.1) and 2 (Standard 1.3.1) of the *Food Standards Code*. A review of the proposed drafting after Full Assessment for the Inquiry Report resulted in minor changes to the Full Assessment drafting including -

- **Full Assessment Report - Proposed amendment to Standard T1 of the *Food Standards Code***

An amendment to the Transitional Standard (T1) was proposed in the drafting at Full Assessment. The proposed amendment attempted to ensure that manufacturers refer to product specifications in Volume 1 (Standard A11) and Volume 2 (Standard 1.3.4) for food additives contained in Volume 1 (Standard 1.3.1) and Volume 2 (Standard 1.3.1), where applicable.

A review of the Full Assessment draft variation to the Code revealed that an amendment to Standard T1 would not achieve this aim and was not required in Volume 2 (Standard 1.3.1) as the *Purpose* commentary to that standard alerted manufacturers to the fact Volume 2 (Standard 1.3.4) prescribes standards for the identity and purity of food additives. In addition to the above, Standard 1.3.4 is a standard of general application, and as specified in Clause 1 "applies to substances added to food in accordance with this Code, and to such substances sold for use in food".

During the transition period manufacturers may elect to manufacture to either Volume 1 or Volume 2 of the Code. The reference in the *Purpose* commentary in Volume 1 (Standard 1.3.1) to "Standard 1.3.4 prescribes standards for the identity and purity of food additives" requires minor amendment to read Standard A11 in the place of Standard 1.3.4. The basis for this changed is that manufacturers who elect to manufacture to Volume 1 (Standard 1.3.1), must comply with the requirements of Volume 1. Food produced by manufactures may not comply with a combination of parts of Volume 1 and Volume 2 (and in New Zealand parts of the Food Regulations). Consequently, Volume 1 (Standard 1.3.1) will need to have a minor amendment to it so that it refers to Standard A11, the correct standard containing specifications for identity and purity of food additives in Volume 1.

Conclusion

The draft amendment to Standard T1 at Full Assessment has been removed from drafting attached to the Inquiry Report and drafting has been included for the minor amendment of Volume 1 (Standard 1.3.1) to replace the reference to Standard 1.3.4 in the *Purpose* commentary of that Standard with a reference to Standard A11 as detailed above.

- **Inquiry Report – Additional draft variation to Volume 1 (Standard A11) of the *Food Standards Code***.

During the transition period manufacturers may elect to manufacture to either Volume 1 or Volume 2 of the *Food Standards Code*. However, food produced by manufactures may not comply with a combination of parts of Volume 1 and Volume 2 (and in New Zealand parts of the Food Regulations).

Drafting was included at Full Assessment for the inclusion of product specifications for Neotame into Volume 2 (Standard 1.3.4 – Identity and Purity), but did not incorporate product specifications into Volume 1 (Standard A11 – Specifications for identity of food additives, processing aids, vitamins, minerals and other added nutrients). As manufacturers who elect to manufacture to Volume 1 are unable to produce food utilising standards from Volume 2, specifications for Neotame needed to be included in both Volume 1 (Standard A11) and Volume 2 (Standard 1.3.4) of the Code.

Conclusion:

An additional draft amendment to Volume 1 (Standard A11) has been included in the Inquiry Report to incorporate product specifications for Neotame into this standard, thereby rectifying the omission in the drafting at Full Assessment.

## WORLD TRADE ORGANIZATION (WTO) NOTIFICATION

Australia and New Zealand are members of the WTO and are bound as parties to WTO agreements. In Australia, an agreement developed by the Council of Australian Governments (COAG) requires States and Territories to be bound as parties to those WTO agreements to which the Commonwealth is a signatory. Under the agreement between the Governments of Australia and New Zealand on Uniform Food Standards, ANZFA is required to ensure that food standards are consistent with the obligations of both countries as members of the WTO.

In certain circumstances Australia and New Zealand have an obligation to notify the WTO of changes to food standards to enable other member countries of the WTO to make comment. Notification is required in the case of any new or changed standards which may have a significant trade effect and which depart from the relevant international standard (or where no international standard exists).

This matter does not need to be notified to the WTO as a Sanitary or Phytosanitary (SPS) notification because it does not impact on human or animal health or a Technical Barrier to Trade (TBT) as it is not expected to significantly impact on trade of other member nations.

## FOOD STANDARDS SETTING IN AUSTRALIA AND NEW ZEALAND

The Governments of Australia and New Zealand entered an Agreement in December 1995 establishing a system for the development of joint food standards. On 24 November 2000, Health Ministers in the Australia New Zealand Food Standards Council (ANZFSC) agreed to adopt the new *Australian New Zealand Food Standards Code*. The new Code was gazetted on 20 December 2000 in both Australia and New Zealand as an alternate to existing food regulations until December 2002 when it will become the sole food code for both countries. It aims to reduce the prescription of existing food regulations in both countries and lead to greater industry innovation, competition and trade.

Until the joint *Australia New Zealand Food Standards Code* is finalised the following arrangements for the two countries apply:

- **Food imported into New Zealand other than from Australia** must comply with either Volume 1 (known as Australian *Food Standards Code)* or Volume 2 (known as the joint *Australia New Zealand Food Standards Code)* of the Australian *Food Standards Code*, as gazetted in New Zealand, or the New Zealand *Food Regulations 1984,* but not a combination thereof. However, in all cases maximum residue limits for agricultural and veterinary chemicals must comply solely with those limits specified in the New Zealand *(Maximum Residue Limits of Agricultural Compounds) Mandatory Food Standard 1999.*

- **Food imported into Australia other than from New Zealand** must comply solely with Volume 1 (known as Australian *Food Standards Code)* or Volume 2 (known as the joint

*Australia New Zealand Food Standards Code)* of the Australian *Food Standards Code*, but not a combination of the two.

- **Food imported into New Zealand from Australia** must comply with either Volume 1 (known as Australian *Food Standards Code)* or Volume 2 (known as *Australia New Zealand Food Standards Code)* of the Australian *Food Standards Code* as gazetted in New Zealand, but not a combination thereof. Certain foods listed in Standard T1 in Volume 1 may be manufactured in Australia to equivalent provisions in the New Zealand *Food Regulations 1984.*

- **Food imported into Australia from New Zealand** must comply with Volume 1 (known as Australian *Food Standards Code)* or Volume 2 (known as *Australia New Zealand Food Standards Code)* of the Australian *Food Standards Code*, but not a combination of the two. However, under the provisions of the Trans-Tasman Mutual Recognition Arrangement, food may **also** be imported into Australia from New Zealand provided it complies with the New Zealand *Food Regulations 1984.*

- **Food manufactured in Australia and sold in Australia** must comply with Volume 1 (known as Australian *Food Standards Code)* or Volume 2 (known as *Australia New Zealand Food Standards Code)* of the Australian *Food Standards Code* but not a combination of the two. Certain foods listed in Standard T1 in Volume 1 may be manufactured in Australia to equivalent provisions in the New Zealand *Food Regulations 1984.*

In addition to the above, all food sold in New Zealand must comply with the New Zealand *Fair Trading Act 1986* and all food sold in Australia must comply with the Australian *Trade Practices Act 1974*, and the respective Australian State and Territory *Fair Trading Acts.*

Any person or organisation may apply to ANZFA to have the *Food Standards Code* amended. In addition, ANZFA may develop proposals to amend the Australian *Food Standards Code* or to develop joint Australia New Zealand food standards. ANZFA can provide advice on the requirements for applications to amend the *Food Standards Code*.

**FURTHER INFORMATION**

**Submissions**: No submissions on this matter are sought as the Authority has completed its assessment and the matter is now with the Australia New Zealand Food Standards Council for consideration.

**Further information** on this and other matters should be addressed to the Standards Liaison Officer at the Australia New Zealand Food Authority at one of the following addresses:

PO Box 7186
Canberra Mail Centre ACT 2610
AUSTRALIA
Tel (02) 6271 2258
email: slo@anzfa.gov.au

PO Box 10559
The Terrace WELLINGTON 6036
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Tel (04) 4739942
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Requests for copies of the full Inquiry Report or other information papers should be addressed to the Authority's Information Officer at the above address, or Email info@anzfa.gov.au

**ATTACHMENT 3**

**SAFETY ASSESSMENT REPORT**

**APPLICATION A406**

**PERMISSION FOR USE OF NEOTAME**

## SUMMARY

### Introduction

The NutraSweet Company is seeking approval for the use of a new artificial sweetener (Neotame) in Australia and New Zealand. The proposed uses include, but are not limited to, soft drink beverages (both carbonated and non-carbonated), beverage concentrates, beverage mixes, dairy beverages, fruit juice products, alcoholic drinks, non-dairy desserts, gelatin, ice cream, breakfast cereals and as a tabletop sweetener for use in hot beverages such as tea or coffee.

Neotame (L-phenylalanine, N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-,1-methyl ester; or NC-00723) is a dipeptide methyl ester derivative, chemically-related to aspartame. It has a sweetness potency of 7000 to 13,000-times sweeter than sucrose, and 30 to 60-times that of aspartame, depending on the use.

Neotame was very stable in mock beverage solutions simulating commercial formulations. The only degradation product formed at >1% was L-phenylalanine, N-[N-3,3-dimethylbutyl)-L-α-aspartyl (NC-00751), which is also the major metabolite in both animals and humans and other minor degradation products (each <1%) which are not expected to be detectable at actual use levels of Neotame.

### Metabolism and toxicokinetics

A number of studies on the metabolism and toxicokinetics were performed in rats and dogs, mainly via the oral route, although the intravenous route was also investigated.

Neotame was rapidly absorbed following oral dosing, with around 14% of the administered dose absorbed in a pilot metabolism study and around 20-30% absorbed in the definitive rat metabolism study. Hydrolysis of Neotame to L-phenylalanine, N-[N-3,3-dimethylbutyl)-L-α-aspartyl (NC-00751) occurred rapidly in all species tested. Excretion of Neotame and its metabolites was also relatively rapid and complete in all species tested. After oral dosing with radioactive Neotame, high levels were found in the stomach, the gastrointestinal tract, liver, kidney and bladder. There was no evidence of accumulation in any tissue. The major route of excretion was via the faeces, either without absorption or following absorption and biliary excretion (approximately 6% of the administered dose in the rat). Hydrolysis of Neotame to NC-00751 occurred during passage through the gastrointestinal tract.

Oral treatment of rats with NC-00751 lead to absorption of around 4% of the administered dose. The majority of orally administered NC-00751 is excreted unchanged in the faeces within 48hrs.

## Acute Toxicity

### *Parent compound*

No acute toxicity studies were conducted with Neotame, however the levels administered in the short-term studies indicate that Neotame is likely to be of very low acute oral toxicity.

### *Degradation products*

Single-dose oral gavage studies were conducted in rats on the degradation products, NC-00764 and NC-00777 at up to 6.0 mg/kg bw/day and also on NC-00779 at up to 3.0 mg/kg bw/day. No deaths were seen over a 14-day observation period and no treatment related abnormal clinical signs were seen throughout the studies.

## Short-term studies

Range-finding studies were conducted in mice, rats and dogs in order to establish dose regimes for long-term studies and to assess any initial signs of toxicity and identify target organs for toxicity at high dose levels. Diet palatability studies were also conducted in rats.

Two dietary studies in mice were conducted at dose levels up to 300 mg/kg bw/day and 8000 mg/kg bw/day, respectively, for 14 days. In the first study, there was no evidence of treatment-related effects. In the second study, the only effect observed was a decrease in bodyweight gain at 500 mg/kg bw/day and above in females and at 4000 mg/kg bw/day and above in males. There was a decrease in food consumption seen at the highest dose level (8000 mg/kg bw/day).

Two dietary studies in rats were conducted at dose levels up to 300 mg/kg bw/day and 6000 mg/kg bw/day, respectively, for 14 days. In the first study, the only effect observed was a decrease in bodyweight gain in both males and females at the 30 and 300 mg/kg bw/day dose levels. Food consumption was also significantly reduced in both sexes at these dose levels. In the second study, there were sporadic clinical and pathological changes that were not considered treatment-related. There was a significant decrease in bodyweight in males at 4000 mg/kg bw/day and above and in females at 6000 mg/kg bw/day. Bodyweight gain was decreased in males and females at 2000 mg/kg and above. Food consumption was decreased from 2000 mg/kg bw/day in males and from 600 mg/kg bw/day in females.

A dietary study in dogs was conducted at dose levels up to 300 mg/kg bw /day for 14 days. The only effect observed was a decrease in bodyweight gain from 30 mg/kg bw/day in males and from 300 mg/kg bw/day in females. This was accompanied by a decrease in food consumption. Other observed changes were considered incidental.

Two palatability studies were conducted in rats to examine the effect of increasing concentrations of Neotame in the diet up to 15000 ppm (equivalent to 1000 mg/kg bw/day). Preference for an untreated diet was observed at dose levels of 150 ppm Neotame and above in the diet (equivalent to 10 mg/kg bw/day). At the highest dose level, virtually none of the treated diet was eaten by preference. When offered treated diet only, total food consumption was decreased from 5000 ppm (equivalent to 100 mg/kg bw/day). Bodyweight gain was decreased at 5000 ppm in the diet and above.

**Subchronic studies**

Subchronic studies have been conducted in mice, rats and dogs.

A dietary study in mice was conducted at dose levels up to 8000 mg/kg bw/day for 13 weeks. Bodyweight gain was decreased in both sexes in the early part of the study at 1000 mg/kg bw/day and in males throughout the study at 4000 (intermittently) and 8000 mg/kg bw/day. These decreases are considered to be related to a reduction in palatability as food consumption was decreased from 1000 mg/kg bw/day in males and in both sexes at higher dose levels. There was a treatment-related increase in absolute and relative liver weight in both males and females. Absolute liver weight was increased at 8000 mg/kg bw/day, while the relative liver weight was increased at 4000 and 8000 mg/kg bw/day. These changes were not accompanied by histopathological changes or changes to clinical chemistry parameters. The NOEL, based on liver weight changes, was 1000 mg/kg bw/day.

A dietary study in rats was conducted at dose levels of up to 3000 mg/kg bw day for 13 weeks followed by a 4 week reversibility period. There was a decrease in bodyweight and bodyweight gain in both sexes at 3000 mg/kg bw/day. These decreases are considered to be related to a reduction in palatability as food consumption was decreased from 1000 mg/kg bw/day in males and 100 mg/kg bw/day in females. There was a treatment-related decrease in plasma cholesterol levels at 1000 mg/kg bw/day and above. A number of organ weight changes were observed at 3000 mg/kg bw/day. The NOEL, based on the changes in plasma cholesterol, was 300 mg/kg bw/day.

A dietary study in dogs was conducted at dose levels of up to 2000 mg/kg bw/ day (reduced to 1200 mg/kg bw/day after 3 weeks) for 13 weeks followed by a 4 week reversibility period. There was a significant decrease in bodyweight in both sexes at the high dose level and in bodyweight gain from 600 mg/kg bw/day. These decreases are considered to be related to a reduction in palatability as food consumption was decreased also at these dose levels. There were significant treatment-related changes in some clinical chemistry parameters from the 600 mg/kg bw/day dose level. Absolute and relative liver weight was increased from 600 mg/kg bw/day in females and at high-dose in males but was not accompanied by histopathological changes (absolute weight increases not statistically significant). The NOEL, based on clinical pathology changes and on relative liver weight changes, was 200 mg/kg bw/day.

**Chronic toxicity / carcinogenicity studies**

Chronic toxicity/carcinogenicity studies have been conducted in mice, rats and dogs.

A dietary study in mice was conducted at dose levels up to 4000 mg/kg bw/day for 104 weeks. There was a decrease in bodyweight in both sexes at dose levels of 400 mg/kg bw/day and above. Bodyweight gain was decreased in males from 400 mg/kg bw/day and in females from 50 mg/kg bw/day. These decreases are considered to be related to a reduction in palatability as food consumption was decreased also at these dose levels. There were no treatment-related organ weight changes. The small non-significant increase in adenomas in male mice at the high dose level was not accompanied by an increase in pre-neoplastic lesions and was not considered treatment-related. The NOEL, based on the absence of adverse effects at the highest dose tested, was 4000 mg/kg bw/day.

A dietary study in rats was conducted at dose levels up to 1000 mg/kg bw/day for 52 weeks followed by a 4-week reversibility period. There was a decrease in bodyweight in both sexes at dose levels of 100 mg/kg bw/day and above. Bodyweight gain was decreased from 300 mg/kg bw/day in females. These decreases are considered to be related to a reduction in palatability as food consumption was decreased also at these dose levels. The ophthalmoscopic lesions observed in male rats were not considered to be treatment-related. The changes observed in clinical pathology parameters and in histopathological examination were small and sporadic and not considered to be treatment-related. The NOEL, based on the absence of adverse effects at the highest dose tested, was 1000 mg/kg bw/day.

A second dietary study in rats was conducted at dose levels up to 1000 mg/kg bw/day for 104 weeks. There was a decrease in bodyweight in both sexes at dose levels of 50 mg/kg bw/day and above. Bodyweight gain was significantly decreased at all dose levels. These decreases are considered to be related to a reduction in palatability as food consumption was decreased also at these dose levels. The changes observed in clinical pathology parameters and in histopathological examination were small and sporadic and not considered to be treatment-related. There was no treatment-related increase in the incidence of tumours. The NOEL, based on the absence of adverse effects at the highest dose tested, was 1000 mg/kg bw/day.

A dietary study in dogs was conducted at dose levels up to 800 mg/kg bw/day for 52 weeks. There was a slight decrease in bodyweight in both sexes at the high dose level. In males, but not females, there was also a slight decrease in bodyweight gain at all dose levels. These decreases are considered to be related to a reduction in palatability as food consumption was also significantly decreased at the high dose level. There were significant treatment-related increases in alkaline phosphatase activity at 800 mg/kg bw/day. There were no significant treatment-related organ weight changes and no significant treatment-related gross or histopathological changes. The NOEL, based on the alkaline phosphatase activity changes was 200 mg/kg bw/day.

**Reproduction studies**

A series of reproduction studies were performed in rats. One-generation studies were performed in conjunction with the 52-week and 104-week chronic studies at dose levels up to 1000 mg/kg bw/day. Two range-finding studies were conducted at dose levels up to 1000 and 3000 mg/kg bw/day, respectively. A two-generation reproduction study was conducted at dose levels up to 1000 mg/kg bw/day. In all of these studies, there were no treatment-related changes in reproductive parameters in the first or second generations. The NOEL for reproductive effects was 1000 mg/kg bw/day.

**Developmental studies**

A series of teratology/developmental studies were performed in rats and rabbits.

Female rats were fed diet containing Neotame, which resulted in dose levels of 0, 100, 300 or 1000 mg/kg bw/day for 28 days before pairing and until day 20 of gestation. There were no treatment-related deaths or abnormal clinical signs, no effects on reproductive parameters, no visceral abnormalities were seen in foetuses and on post-mortem examination no treatment-related maternal findings were seen. Neotame was not teratogenic at doses up to 1000mg/kg bw/day.

Female rabbits were dosed by oral gavage with Neotame on gestation days 6 to 19 in two separate studies up and including doses of 1000 mg/kg bw/day. There were no treatment-related effects on reproductive parameters, and no treatment-related macroscopic abnormalities found on post-mortem examination in dams or foetuses. Neotame was not teratogenic in rabbits at doses up to 1000mg/kg bw/day.

**Genotoxicity studies**

Neotame was negative in a range of genotoxicity studies, including bacterial reverse gene mutation assay and forward gene mutation assay using mouse lymphoma cells. Neotame did not produce chromosome damage in Chinese hamster ovary cells, and a mouse micronucleus assay was negative.

The main metabolite of Neotame, NC-00751, was negative in a bacterial reverse gene and forward gene mutation assay. A number of degradants, NC-00764, NC-00777 and NC-00779 were negative in forward and reverse gene mutation assays and mouse micronucleus studies. Overall, there was no evidence for NEOTAME or any of the metabolites or degradants producing any genotoxic damage.

**Human studies**

A series of human studies were performed to examine the pharmacokinetic profile in humans following single or multiple oral doses and the potential to tolerate Neotame for 2 weeks or 13 weeks of oral exposure.

The pharmacokinetic studies indicate that Neotame is rapidly absorbed with maximum plasma levels seen at approximately 30-45 minutes with the plasma level linearly related to the dose administered. Urinary excretion of Neotame accounted for approximately 1% of the administered dose while NC-00751 accounted for approximately 20%. When radioactively-labelled Neotame is administered orally, approximately 30% of the radioactivity is excreted in the urine while approximately 70% is excreted in the faeces. NC-00751 is the major metabolite in both urine and faeces. A similar pattern of excretion was noted following multiple oral treatments.

Neotame was well tolerated in both a 2-week and 13-week toleration study at dose levels of 0.5 or 1.5 mg/kg bw/day. There were no treatment-related changes in physiological parameters during the study. Adverse effects reported during the study were not considered to be related to treatment. There were no treatment-related effects on plasma glucose or on insulin levels in non-insulin dependent diabetes mellitus patients at dose levels of 1.5 mg/kg bw/day.

**Pharmacology studies**

A number of studies investigating the pharmacological activity of Neotame were performed, using a range of test methodologies. There was no effect on the progress of charcoal through rat intestine following Neotame administration, indicating that it did not affect gastric motility. Hexobarbitol sleeping times were not affected by Neotame treatment. The contractility of isolated guinea pig ileum was not affected by Neotame. In anaesthetised dogs, there was no change in cardiovascular, respiratory or renal function following Neotame administration.

## DISCUSSION

A comprehensive set of toxicology data has been provided to support the safety of Neotame.

Neotame is very stable under the conditions of use. The major degradation product, NC-00751 is also the major metabolite in animal and human studies. It is formed by hydrolysis of the methyl ester group of Neotame. There are other very minor metabolites.

Metabolic studies in rats indicate rapid absorption of around 20-30% of orally administered Neotame followed by hydrolysis to form NC-00751 and rapid excretion via the urine and faeces. There is no evidence of tissue accumulation of either Neotame or its metabolites.

The available short-term, subchronic and chronic studies indicate that Neotame is well tolerated in all species with little evidence of treatment-related adverse effects. The most significant finding was a decrease in bodyweight and bodyweight gain at the higher dose levels that is accompanied by a decrease in food consumption. These findings are considered to be related to decreased palatability of the Neotame-containing diet rather than to toxicity. Specific studies conducted to examine palatability of the Neotame diet at various dose levels demonstrated marked preference by rats for the control diet than for one containing Neotame. The variety of treatment-related changes in clinical pathology parameters and from histopathological examinations does not indicate any particular Neotame-related toxicity.

There is no evidence of adverse effects in reproduction studies in rats, developmental toxicity studies in rabbits and rats and in a range of *in vitro* and *in vivo* genotoxicity studies.

In human studies, Neotame is well tolerated at dose levels of 1.5 mg/kg bw/day. Plasma glucose levels and insulin levels in non-insulin dependent diabetes mellitus patients were normal following treatment with Neotame at 1.5 mg/kg bw/day.

The chronic studies conducted with Neotame in mice, rats and dogs demonstrate no evidence of adverse effects other than an increase in alkaline phosphatase activity at the highest dose in a long-term dog study. However, this elevation in alkaline phosphatase was reversible, no other liver enzymes were elevated and there was no histopathological changes observed. While some lower NOELs were found in the subchronic studies, the effects upon which they were based were not seen in the chronic studies. The lowest NOEL, therefore, is 200 mg/kg bw/day established for the definitive 52-week dog study. Applying a 100-fold safety factor, the acceptable daily intake (ADI) for humans in 2 mg/kg bw/day.

## NEOTAME

## INTRODUCTION

The NutraSweet Company is seeking approval for the use of the new artificial sweetener, Neotame, in Australia and New Zealand. The proposed uses include, but are not limited to, soft drink beverages (both carbonated and non-carbonated), beverage concentrates, beverage mixes, dairy beverages, fruit juice products, alcoholic drinks, non-dairy desserts, gelatin, ice cream, breakfast cereals and as a tabletop sweetener for use in hot beverages such as tea or coffee.

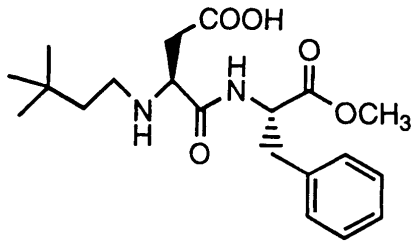
Neotame is a dipeptide methyl ester derivative, chemically related to aspartame. It has a sweetness potency of 7000 to 13,000-times sweeter than sucrose, and 30 to 60-times that of aspartame, depending on the use. Neotame is very stable under conditions of use. The major degradation product, NC-00751, is also the major metabolite in animal and human studies. It is formed by hydrolysis of the methyl ester group, and is present in minimal amounts in commercial conditions. Under conditions of higher temperature and humidity, the level of NC-00751 increased to around 3% after 52 weeks storage, while other metabolites were present at levels of less than 0.19%.

*Chemical structure*

**Parent compound**
Common name: Neotame
Chemical names: L-phenylalanine,N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-1-methyl ester

N-[N:(3,3-dimethylbutyl) L-α-aspartyl]-L-phenylalanine 1-methyl ester

CAS registry number: 165450-17-9
Manufacturer's code numbers and/or synonym(s): NC-00723
Empirical formula: $C_{20}H_{30}N_2O_5$; Molecular weight: 378.47
Structural formula:




**Note:** Further details of the physical and chemical properties and impurity profiles for Neotame are shown in Appendix A.

*Stability/major degradation products*

Neotame degradation was evaluated in mock beverage solutions simulating formulations used in commercial cola soft drinks (pH 2.8 and 3.2), lemon-lime soft drink (pH 3.8), and root beer soft drinks (pH 4.5), lacking the flavour components. Mock beverage samples were formulated at 200 ppm, a concentration 12–13-times higher than the anticipated concentration in soft drinks (to increase the ability to detect degradation products). For samples prepared at pH 3.2 and stored at 20ºC for 8 weeks, the major route of degradation was the hydrolysis of the methyl ester moiety to form NC-00751 (see Figure 1), which was the only degradation product formed at >1%. The minor degradation products (each <1%) were NC-00764, NC-00777 and NC-00779 (see Figure 1). These degradation products are not expected to be detectable at actual use levels of Neotame. Two further products, NC-00769 (N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine) and NC-00754 (N-(3,3-dimethylbutyl)-L-aspartic acid) were only detected after high concentrations of Neotame were subjected to more extreme conditions of pH, temperature and time.

Neotame was also stable under conditions relevant to hot beverages such as tea or coffee. Beverages containing Neotame (pH 3.2 to 6.5) were subjected to high temperature, short time process and then cooled, with no significant loss of Neotame (< 0.5%).

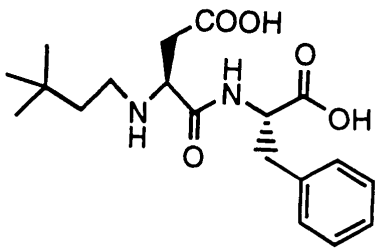
**NC-00751**

Chemical name: L-phenylalanine, N-[N-(3,3-dimethylbutyl)-L-α-aspartyl

CAS registry number: 190910-14-6

Empirical formula: $C_{19}H_{28}N_2O_5$; Molecular weight: 364.44

Structural formula:




***NC-00764***

Chemical name: N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine 1-methyl ester

***NC-00777***

Chemical name: N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine 1-methyl ester

***NC-00779***

Chemical name: N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine

See Figure 1

*Possible nitrosation of Neotame*

As Neotame contains a secondary amine, there is a potential for N-nitrosation at this site. This can occur as a result of interaction with dinitrogen trioxide (formed from nitrite under conditions of low pH). Using the rate constant for the nitrosation of dimethylamine after adjustment for differences in pKa values between the protonated amine of dimethylamine and Neotame, the hypothetical amounts of N-nitrosated Neotame exposure from soft drink consumption have been calculated, assuming consumption of Neotame at the 90$^{th}$ percentile after storage to be orders of magnitude less than exposure to nitrosated amines from other sources.

Calculations prepared by Monsanto indicates that the hypothetical risk posed by nitrosation of Neotame is extremely low which was attributed to the low concentration of nitrite in beverages and low concentrations of nitrite in stomach of consumers.

Although there were no studies, which have directly addressed the issue of nitrosation, there was no indication that any of the effects seen in the available toxicological studies could be attributed to nitrosation of Neotame. Therefore, nitrosation is not considered to be of any toxicological significance.

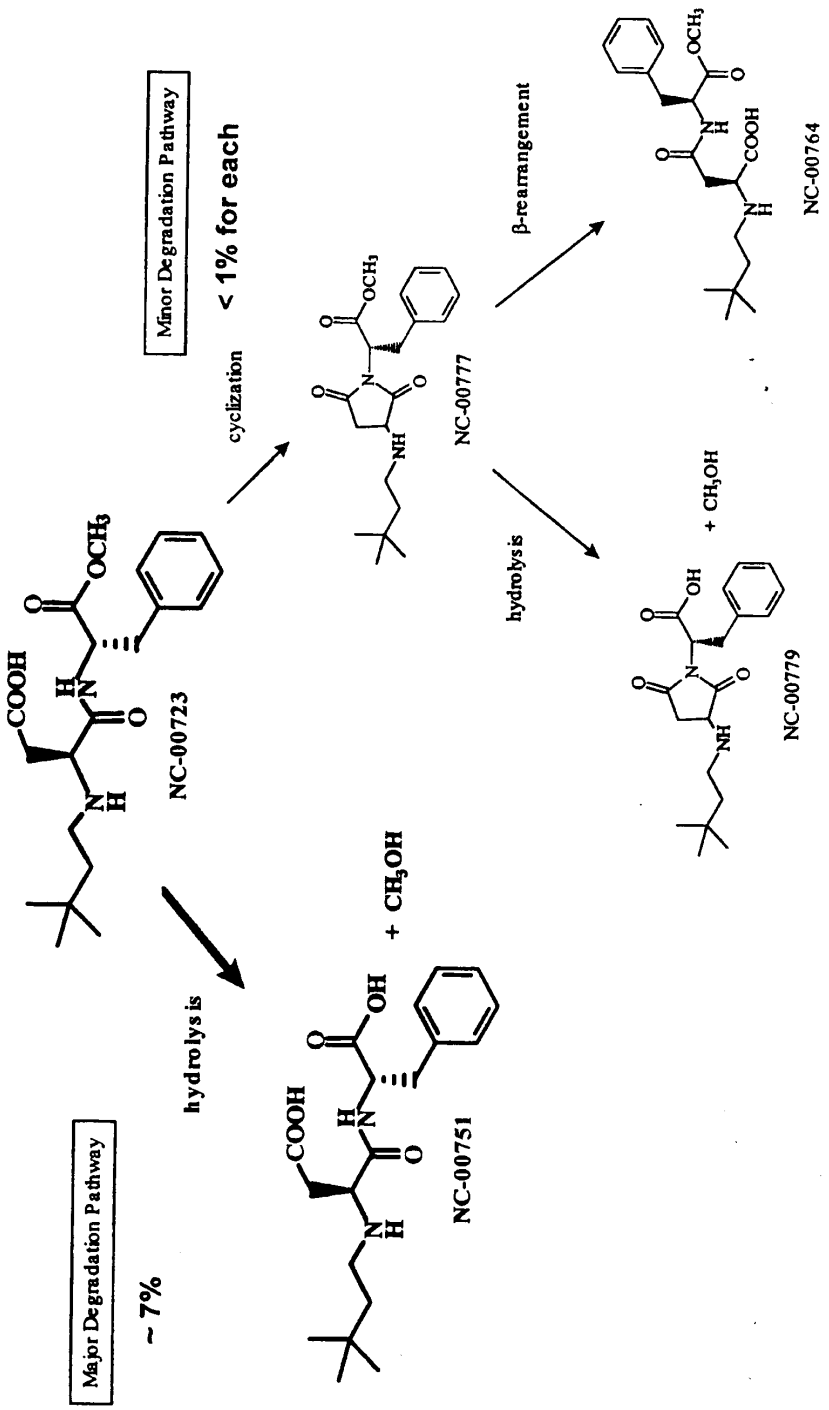



**Figure 1 Neotame degradation pathways in mock beverage under relevant conditions of use (8 weeks storage at 20°C, pH 3.2)**

## METABOLISM AND TOXICOKINETICS

### 1. Hawkins DR, Kirkpatrick D, Aikens PJ & Saxton J (1995) $^{14}$C-NEOTAME Metabolism in the Rat Pilot Investigation. Laboratory Study No: NTS 28/9433150. Sponsor Study No: PCR-957.

| | |
|---|---|
| Laboratory: | Huntingdon Research Centre Ltd, Huntingdon, Cambridgeshire England |
| Date of study: | 23 June 1994–8 October 1994 |
| GLP: | UK, EEC, OECD, USFDA |
| Test chemical: | NC-00723: 3-(3,3-dimethylbutylamino)-N-(α-carboxyphenylethyl) succinamic acid methyl ester. |
| Radiolabelled: | Huntingdon Research Centre Ltd. Batch no. MRH/NTS 19/BK2/31/1. activity: 23.57 μCi/mg, 8.92 mCi/mmol, purity: >98% |
| Non-labelled: | NutraSweet Company, Batch no: DEF-1037-272-2, purity: not specified |
| Reference chem: | NC-00751: 3-(3,3-dimethylbutylamino)-N-(α-carboxyphenylethyl) succinamic acid<br>NC-00754: N-(3,3-dimethylbutyl)-L-aspartic acid |
| Test species: | Crl:CDBR VAF plus Sprague Dawley rats (Charles River UK Ltd, Margate, Kent UK) |

**Study design**

Rats were housed in groups in the acclimatisation period with free access to food and water. All rats were given a single oral gavage dose of radiolabelled $^{14}$C- NC-00723 in 1% (w/v) aqueous sodium carboxymethylcellulose at 15 mg/kg bw. Rats were then divided into four investigation groups. Groups 1 and 3 remained in group housing. Group 2 were housed individually in glass metabolism cages, while group 4 were housed individually in restraining cages.

Group 1 rats (3/sex) were dosed and blood was taken from the tail vein at intervals up to 24 h post-dose, separated into cell and plasma fractions, and a radio assay done on each sample. Rats were killed after the final sampling. Two additional rats (1/sex) were not dosed and provided control plasma.

Group 2 rats (2/sex) were housed in glass metabolism cages for 72 h after dosing with NC-00723 for collection of urine, faeces and expired air. A cagewash was done at 72 h, when the rats were killed, and the carcasses solubilised for analysis of retained radioactivity. Urine and faeces were pooled for analysis of metabolites as well as total radioactivity.

Group 3 rats were anaesthetised at 0.5 and 2 h after dosing, using 2/sex/time period. Blood was collected at these times and the rats were then killed.

Group 4 rats (2 males) were anaesthetised and the bile ducts and stomach cannulated. The radiolabelled NC-00723 was administered via the stomach cannula, along with sodium taurocholate in sodium chloride. Bile was collected for at intervals up to 48 h after dosing. Urine and faeces were collected for 0-24 and 24-48 h after dosing. Rats were killed at 48 h after dosing, the carcasses solubilised and radioactivity determined.

**Results**

**Absorption**
Plasma levels following oral dosing with NC-00723 peaked at 30 min after dosing in females and 1 h after dosing in males. There was a rapid decline over the first 4 h. Maximum levels in males were 0.566 µg equivalent of NC-00723/mL plasma and in females were 0.522 µg equivalent of NC-00723/mL plasma.

**Metabolism**
The major metabolite identified in plasma, urine, faeces and bile was NC-00751.

**Excretion**
The excretion of NC-00723 was determined using Group 2 rats. The excretion, expressed at percentage of total dose, is summarised in the following table.

**Excretion of NC-00723 in the rat (Mean%)**

| | **Males** | **Females** |
|---|---|---|
| Urine (0-72 h) | 8.53 | 10.3 |
| Cagewash (72 h) | 0.05 | 0.05 |
| Faeces (0-72 h) | 92.6 | 90.5 |
| Expired air (0-72 h) | 0.03 | <0.01 |
| Carcass (72 h) | <0.11 | 0.13 |

In males, urinary excretion was virtually complete within 12 h, while in females, excretion continued over the first 24 h. The majority of faecal excretion occurred in both sexes between 6 and 24 h after dosing. Very little of the administered dose was excreted in expired air or retained in the carcass.

In group 4 rats (males only), urinary excretion was similar to that seen above, with around 5-9% of the administered dose excreted. Biliary excretion accounted for approximately 5.7% of the administered dose, while faecal excretion accounted for around 85% of the administered dose. Little radioactivity was retained in the carcass.

Based on these studies, a maximum of approximately 14% of the administered dose was absorbed. Most of the faecal recovery was of unabsorbed material. In all studies around 100% of the administered dose was recovered. There was very low retention of test material or metabolites in the carcass. The test material behaved in a similar manner in both males and females. The major metabolite found was NC-00751. While other minor metabolites were found, these were not positively identified. No parent NC-00723 was found in plasma, urine or faeces, although the study authors recognised that conversion to NC-00751 may have occurred during processing.

**2. Kirkpatrick D, Aikens PJ Nicholson J and Saxton J (1997) $^{14}$C-NC-00723 Metabolism in the rat. Laboratory Study No: MRO/51. Sponsor Study No: PCR-1027.**

Laboratory: Huntingdon Research Centre Ltd, Huntingdon, Cambridgeshire England
Date of study: 19 March 1996–31 August 1996
GLP: UK, EEC, OECD, USFDA
Test chemical: NC-00723: α-N-(3,3-dimethylbutylamino)-L-aspartyl-L-phenylalanine methyl ester

Radiolabelled: Huntingdon Research Centre Ltd. Batch no. MRH/NTS 31/37/2. activity: 52.77 μCi/mg, purity: >98%
Non-labelled: Monsanto Company, Batch no: 95MP-028-3, purity: >98%
Reference chem: NC-00751: α-N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine
NC-00754: N-(3,3-dimethylbutyl)-L-aspartic acid
Test species: Crl:CDBR Sprague Dawley rats (Charles River UK Ltd, Margate, Kent UK)

**Study design**

During the test period, rats were individually housed in metabolism cages. Group 1 rats (6/sex) received a single oral dose of $^{14}$C-NC-00723 at 15 mg/kg bw. Urine and faeces were collected at intervals for 72 h after dosing. Group 2 rats (6/sex) were given an IV dose of $^{14}$C-NC-00723 at 15 mg/kg bw, and urine and faeces were collected as for group 1, while group 3 rats received a single oral dose of $^{14}$C-NC-00723 at 120 mg/kg bw. In all studies, a cagewash was done at 72 h. All rats were killed at this time and the carcasses retained for analysis of radioactivity. Radioactivity in all samples and the metabolites present in the urine and faeces were determined.

**Results**

**Metabolism**

The major metabolite found in urine after 48 h was NC-00751, independent of the route of administration or the dose. NC-00754 was detected at lower levels (around 10% of the levels of NC-00751 following oral dosing). Parent compound was found only in the urine of females rats after IV dosing (3.7% of the dose); none was detected in the urine of any other groups. A glucuronide metabolite was also detected at low levels (0.4-0.5% of the dose) in the urine, independent of dose or route of administration. Two minor metabolites, each present at <1.6% of the dose were identified.

In the faeces, NC-00751 was the major metabolite (approx. 70-80% of the dose after oral administration). NC-00754 was detected at lower levels, 0.8-2.5% of the dose, while NC-00723 was not detected in any samples. Low levels of an unidentified metabolite were also found, 0.7-1.2% of the dose.

**Excretion**

Urinary excretion after oral high dose (HD) and low dose (LD) and IV dosing is presented in the following table.

**Urinary excretion of radioactivity (as % total dose) following dosing with NC-00723**

| Urine | Oral (15 mg/kg bw) | | IV (15 mg/kg bw) | | Oral (120 mg/kg bw) | |
|---|---|---|---|---|---|---|
| | Male | Female | Male | Female | Male | Female |
| 0-6 h | 6.1 | 4.0 | 32 | 30.7 | 6.2 | 6.7 |
| 6-12 h | 2.3 | 2.6 | 1.8 | 1.5 | 1.2 | 0.9 |
| 12-24 h | 1.5 | 2.1 | 1.5 | 1.5 | 0.7 | 0.8 |
| 24-48 h | 0.8 | 1.7 | 0.4 | 0.8 | 0.4 | 1.0 |
| 48-72 h | 0.1 | 0.2 | 0.2 | 0.2 | ND | 0.1 |
| TOTAL | 10.8 | 10.4 | 35.9 | 34.6 | 8.5 | 9.6 |

There were limited sex differences in urinary excretion, although in both the LD oral and in the IV study, males had higher initial excretion levels. The quantity excreted (as a percentage of total oral dose) was slightly decreased at the HD. Based on comparison of urinary excretion after oral and IV dosing, the absorption of NC-00723 is approximately 30%.

Faecal excretion is presented in the following table.

**Faecal excretion of radioactivity (as % total dose) following dosing with NC-00723**

| **Faeces** | **Oral (15 mg/kg bw)** | | **IV (15 mg/kg bw)** | | **Oral (120 mg/kg bw)** | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** |
| 0-6 h | ND | ND | ND | ND | ND | ND |
| 6-12 h | 10.1 | ND | 3.2 | ND | 38 | ND |
| 12-24 h | 65.9 | 67.2 | 51.4 | 41.9 | 43 | 51.2 |
| 24-48 h | 8.4 | 16.3 | 3.3 | 15.0 | 6.1 | 27.5 |
| 48-72 h | 0.3 | 2.2 | 0.2 | 0.7 | 0.2 | 1.4 |
| TOTAL | 84.6 | 86.3 | 58.1 | 59.2 | 87.2 | 84.5 |

ND=not detected or mean not calculable

Faecal excretion commenced in males between 6 and 12 h after dosing, and peaked in the 12 to 24-h period. Faecal excretion was not detected in females until 12 h after dosing, however the extent of excretion was not significantly different between sexes. Faecal excretion was decreased following IV dosing in comparison to either HD or LD oral dosing. The percentage of the dose excreted in the faeces was not significantly affected by the dose.

There were very low levels found in the carcass or in the cagewash in all groups, and the recovery of radioactivity ranged from 93-98% in all groups.

NC-00723 was rapidly absorbed and excreted, with the majority of the administered dose excreted within 48 h. There was extensive metabolism of the both absorbed and unabsorbed NC-00723, with NC-00751 being the major metabolite found in both urine and faeces. Two minor metabolites were found, but were not identified.

**3. Hawkins DR, Kirkpatrick D, Aikens PJ & Beeby TL (1995) $^{14}$C-NC-00723 Determination of the Distribution in Rats by Whole-Body Autoradiography. Laboratory Study No: NTS 29/943207. Sponsor Study No: PCR-958.**

| | |
|---|---|
| Laboratory: | Huntingdon Research Centre Ltd, Huntingdon, Cambridgeshire England |
| Date of study: | 13 July 1994–13 October 1994 |
| GLP: | UK, EEC, OECD, USFDA |
| Test chemical: | NC-00723: 3-(3,3-dimethylbutylamino)-N-(α-carboxyphenylethyl) succinamic acid methyl ester. |
| Radiolabelled: | Huntingdon Research Centre Ltd. Batch no. MRH/NTS 19/BK 2/31/1.<br>activity: 23.57 μCi/mg, 8.92 mCi/mmol, purity: >98% |
| Non-labelled: | NutraSweet Company, Batch no: DEF-1037-272-2, purity: not specified |
| Test species: | Lister Hooded rats (Harlan Olac Ltd, Bicester Oxon UK) |

**Study design**

Rats were given a single oral dose of radiolabelled NC-00723 at 15 mg/kg bw, using 5/sex. Pairs of rats (1/sex) were killed at 0.5, 2, 6, 12 and 24 h after dosing. After sacrifice, rats were pinned out and frozen rapidly. Sagittal sections were taken through the carcass at 6 levels. The samples included (but were not limited to) the following features: kidney (males) or ovaries (females), intraorbital lacrimal gland, harderian gland, adrenal gland, half brain and/or thyroid and brain and spinal cord. Sections were mounted and examined by autoradiography.

**Results**

Qualitative assessment of radioactivity present in male and female rats indicated that the highest levels of radioactivity were present in rats killed earliest after dosing. Levels decreased rapidly with time. At 0.5 and 2 h after dosing, the highest levels were found in stomach, GI tract, liver, kidneys and bladder, with lower levels of radioactivity distributed throughout the rest of the body. Levels were very low in the central nervous system, and no binding to pigmented skin or the eye was observed. Levels were consistent with the circulation of radioactivity in the bloodstream.

In subsequent time periods (6, 12 and 24 h), the passage of radioactivity through the excretory organs was seen. By 24 h after dosing, only very low levels of radioactivity remained in the animal and there was no evidence of accumulation in any tissue.

**4. Hawkins DR, Aikens PJ & Beeby TL (1996) $^{14}$C-NC-00723 Determination of the Distribution in Pregnant and Non-pregnant Rats by Whole-Body Autoradiography. Laboratory Study No: MTO 43/960202. Sponsor Study No: PCR-1031.**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England |
| Date of study: | 20 September 1995–31 January 1996 |
| GLP: | UK, EEC, OECD, USFDA |
| Test chemical: | NC-00723: α-N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester |
| Radiolabelled: | Huntingdon Life Sciences Ltd. Batch no. MRH/NTS 31/37/2. activity: 52.77 μCi/mg, 19.97 mCi/mmol, purity: >98% |
| Non-labelled: | Monsanto Company, Batch no: 95 MP028-3, purity: not specified |
| Test species: | Female Crl CDBR VAF Plus Sprague Dawley rats (Charles River UK, Kent UK) |

**Study design**

Eight pregnant and 8 non-pregnant rats were used. They were each given a single oral dose of radiolabelled NC-00723 in spring water at 15 mg/kg bw. Pairs of rats (1 pregnant, 1 non-pregnant) were killed at 0.5, 2, 6, 12 and 24 h after dosing. After sacrifice, rats were pinned out and frozen rapidly. Sagittal sections were taken through the carcass at 6 levels. The samples included (but were not limited to) the following features: ovaries, intraorbital lacrimal gland, harderian gland, adrenal gland, half brain and/or thyroid and brain and spinal cord. Sections were mounted and analysed by autoradiography.

**Results**

Tissue distribution of radioactivity was similar in pregnant and non-pregnant rats. Placental levels were low at 0.5 and 2 h after dosing, with levels similar to those seen in other peripheral tissues. These levels were consistent with circulating blood levels. No radioactivity was seen in the foetus at any time.

Highest levels of radioactivity were seen shortly after dosing, with high levels initially in the stomach contents, GI tract, liver, kidneys and bladder, with lower levels in the rest of the body. Over subsequent time periods, the passage of radioactivity through the excretory organs was seen. No tissue accumulation was seen, and levels were very low after 24 h.

**5. Hawkins DR, Kirkpatrick D, Aikens PJ & Saxton J (1995) $^{14}$C-NC-00723 Tissue Distribution in the Rat. Laboratory Study No: NTS 38/951568. Sponsor Study No: PCR-0959**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England |
| Date of study: | 8 November 1994–6 January 1995 |
| GLP: | UK, EEC, OECD, USFDA |
| Test chemical: | NC-00723: 3-(3,3-dimethylbutylamino)-N-(α-carboxyphenylethyl) succinamic acid methyl ester. |
| Radiolabelled: | Huntingdon Research Centre Ltd. Batch no. MRH/NTS 31/37/2. activity: 52.7 μCi/mg, 19.97 mCi/mmol, purity: >98% |
| Non-labelled: | NutraSweet Company, Batch no: 94 UP8-3-4, purity: not specified |
| Test species: | Male Lister Hooded rats ( Harlan UK Ltd, Shaw's Farm Oxon UK) |

**Study design**

Twenty one male rats were given a single oral dose of radiolabelled NC-00723 at 15 mg/kg bw. Three rats were killed at each of 0.25, 1, 2, 6, 12, 24 and 48 h after dosing. The adrenal glands, brain, eyes, heart, kidney, liver, lungs, lymph nodes, pancreas, prostate gland, spleen, submaxillary salivary gland, testes, thymus, thyroid, bladder and gastrointestinal tract were obtained. Samples of bone marrow, fat, muscle, pigmented and non-pigmented skin were also obtained, along with samples of whole blood.
A measure of the haematocrit was obtained and the plasma and whole blood were radio assayed. Organ weights were recorded. The GI tract was separated into the stomach, SI, LI and caecum, the contents were separated from the tissue. All samples were homogenised and a radio assay performed.

**Results**

The radioactivity present in tissues at different time points is presented in the table below.

**Radioactivity in tissues (in μg equivalents/g) after oral dosing at 15 mg/kg bw**

| Tissue | 0.25 h | 1 h | 2 h | 6 h | 12 h | 24 h | 48 h |
|---|---|---|---|---|---|---|---|
| Stomach | 202 | 60.3 | 43.7 | 39.8 | 11.3 | 1.13 | 0.046 |
| stomach contents | 312 | 86.8 | 46.0 | 46.3 | 9.77 | 0.151 | 0.019 |
| small intestine | 81.5 | 132 | 115 | 28.0 | 14.3 | 2.08 | 0.064 |
| SI contents | 69.5 | 201 | 189 | 35.1 | 9.62 | 1.41 | 0.029 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| large intestine | 5.94 | 12.0 | 31.4 | 101 | 115 | 10.7 | 0.107 |
| LI contents | 1.89 | 2.54 | 18.9 | 202 | 62.4 | 6.16 | 0.064 |
| caecum | 7.99 | 9.54 | 70.1 | 207 | 106 | 14.1 | 0.144 |
| liver | 9.83 | 13.2 | 6.83 | 1.20 | 0.489 | 0.122 | 0.025 |
| kidneys | 3.39 | 4.89 | 2.46 | 0.645 | 0.487 | 0.129 | 0.034 |
| bladder | 0.671 | 2.26 | 2.12 | 0.426 | 0.443 | 0.079 | 0.024 |
| plasma | 0.658 | 0.865 | 0.446 | 0.092 | 0.047 | 0.015 | 0.005 |
| whole blood | 0.392 | 0.514 | 0.268 | 0.058 | 0.036 | 0.012 | 0.005 |

The highest levels of radioactivity were therefore seen in the GI tract with moderate levels in the liver and kidneys. Levels in tissues not reported in the table were at <0.3% of the administered dose at all time points after dosing. There was no evidence of accumulation in any tissues at any time period after dosing.

**6. Hawkins DR, Kirkpatrick D, Aikens PJ & Saxton J (1997) NC-00723 Pharmacokinetics of single doses in the rat after oral and intravenous administration. Laboratory Study No: NTS 40. Sponsor Study No: PCR-1028.**

Laboratory: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England
Date of study: 31 July 1995–3 June 1996
GLP: UK, EEC, OECD, USFDA
Test chemical: NC-00723: α-N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester.
Radiolabelled: Huntingdon Life Sciences Ltd. Batch no. MRH/NTS 31/37/2 activity: 52.77 μCi/mg, 19.97 mCi/mmol, purity: >98%
Non-labelled: Monsanto Company, Batch no: 95 MP028-3
Test species: Crl CDBR Vaf Plus Sprague Dawley Rats (Charles River UK Ltd, Margate Kent UK)

**Study design**

Rats were given a single oral dose of radiolabelled NC-00723 in deionised water at 15 or 120 mg/kg bw (30/sex/group) or a single IV dose of radiolabelled NC-00723 in isotonic saline at 15 mg/kg bw using 33/sex/group. An additional 3 rats/sex/group with no dose administered were controls. Following dosing, blood was taken from 3/sex/dose at intervals up to 24 h after dosing. All carcasses were discarded after sampling, without examination. Radio assay of the blood samples was performed and the radioactive plasma constituents were analysed.

**Results**

NC-00723 was found in the plasma of rats at 0.25 h after a dose of 120 mg/kg bw, at levels of 0.016 to 0.019 μg/mL. No parent compound was found in the plasma after any other dose or at any other time period. This indicated very rapid metabolism of NC-00723 in the rat.

The levels of NC-00751 (in μg/mL) in plasma at times after dosing are detailed in the following table.

**Plasma levels of NC-00751 in µg/mL**

| | IV 15 mg/kg | | Oral 15 mg/kg | | Oral 120 mg/kg | |
|---|---|---|---|---|---|---|
| **Time after dosing (h)** | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** |
| 0.1 | 22.3 | 15.7 | | | | |
| 0.25 | 8.31 | 5.69 | 0.545 | .0481 | 4.17 | 2.79 |
| 0.5 | 5.44 | 1.71 | 0.665 | 0.482 | 4.77 | 4.63 |
| 0.75 | 2.18 | 1.10 | 0.526 | 0.320 | 7.98 | 4.05 |
| 1 | 1.38 | 0.810 | 0.399 | 0.206 | 7.73 | 2.73 |
| 2 | 0.493 | 0.048 | 0.304 | 0.112 | 2.81 | 0.740 |
| 4 | 0.036 | <0.011 | 0.179 | 0.019 | 0.870 | 0.197 |
| 6 | <0.011 | <0.011 | 0.025 | <0.011 | 0.112 | 0.071 |
| 8 | <0.011 | <0.011 | <0.013 | <0.011 | 0.036 | <0.011 |
| 12 | <0.011 | <0.011 | <0.011 | <0.011 | <0.011 | <0.011 |

The pharmacokinetic parameters (derived from total plasma radioactivity) are detailed in the following table.

**Pharmacokinetic parameters following oral or IV dosing with NC-00723**

| | IV 15 mg/kg | | Oral 15 mg/kg | | Oral 120 mg/kg | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** |
| Achieved dose (mg/kg ) | 16.7 | 16.7 | 14.6 | 14.7 | 126 | 126 |
| $C_{max}$(µg eq./mL) | 26.5 | 19.3 | 0.831 | 0.695 | 9.28 | 5.99 |
| $T_{max}$(h) | 0.1 | 0.1 | 0.5 | 0.25 | 0.75 | 0.5 |
| $AUC_t$(µg eq.h/mL) | 13.9 | 8.2 | 2.3 | 1.5 | 20.4 | 9.3 |
| $AUC_{24}$(µg eq.h/mL) | 13.9 | 8.2 | 2.4 | 1.5 | 21.9 | 9.9 |

Radioactivity was rapidly absorbed after oral doses, with peak plasma radioactivity at 0.5 h. NC-00723 was rapidly converted to NC-00751 after both oral and IV dosing, with peak mean plasma concentrations at 0.1 h after IV dosing and 0.5 h after oral dosing. Clearance of NC-00751 was rapid and was higher in females than in males. The half life of NC-00751 was about 1 h after either IV or oral dosing. The ratio of the oral AUC to the IV AUC was about 20% for males and 14% for females. This was similar to the oral bioavailability of total radioactivity of around 20%.

**7. Hawkins DR, Kirkpatrick D, Shaw D & Bennett S (1996) $^{14}$C-NC-00751 Metabolism in the Rat. Laboratory Study No: MTO 46. Sponsor Study No: PCR-1119.**

Laboratory: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England
Date of study: 14 November 1995–12 February 1996
Test chemical: NC-00751: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine.
Radiolabelled: Huntingdon Life Sciences Ltd. Batch no. MRH/MTO 44/218, 61 Ci/mol, purity: >98%
Non-labelled: Monsanto Company, Batch no. IP-1123-27-5
Reference: NC-00723: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester
Test species: Crl CDBR Vaf Plus Sprague Dawley Rats (Charles River UK Ltd, Margate Kent UK)

**Study design**

Rats were given a single oral gavage dose of radiolabelled $^{14}$C-NC-00751 in 0.1M sodium phosphate buffer at 15 mg/kg bw. Rats were then divided into four investigation groups. Groups 1 and 3 remained in group housing. Group 2 were housed individually in glass metabolism cages, while group 4 were housed individually in restraining cages.

Group 1 rats (3/sex) were dosed and blood was taken from the tail vein at intervals up to 24 h post-dose, separated into cell and plasma fractions, and a radio assay done on each sample. Rats were killed after the final sampling.

Group 2 rats (2/sex) were housed in glass metabolism cages after dosing with NC-00723. Urine, faeces and expired air were collected until 72 h after dosing. All samples were frozen until analysis, when they were pooled for analysis of metabolites. A cagewash was done at 72 h and rats were killed and the carcasses solubilised for analysis of retained radioactivity.

Group 3 rats were anaesthetised at 0.5 and 2 h using 2/sex/time period after dosing, and blood samples collected.

Group 4 rats (2 males) were anaesthetised and the bile ducts and stomach cannulated. The radiolabelled NC-00751 was administered via the stomach cannula. Bile, urine and faeces were collected until-48 h after dosing. Rats were killed at 48 h after dosing, the carcasses solubilised and radioactivity determined.

**Results**

**Absorption**
Plasma levels of NC-00751 in Group 1 rats peaked at 30 min after dosing in females and 1 h after dosing in males. There was a rapid decline over the first 4 h, with plasma levels below the level of detection from 4 h after dosing. Maximum levels in males were 0.066 µg equivalent of NC-00751/mL plasma and in females were 0.051 µg equivalent of NC-00751/mL plasma.

**Metabolism**
Unchanged NC-00751 was the major component of radioactivity in plasma, bile, urine and faeces. Other metabolites were detected at low levels, but were not identified.

**Excretion**
The excretion of NC-00751 was determined using Group 2 rats, and is summarised in the following table.

**Excretion of NC-00751 in the rat (mean % total dose)**

| | **Males** | **Females** |
|---|---|---|
| Urine (0-72 h) | 1.66 | 1.36 |
| Cagewash (72 h) | 0.05 | 0.02 |
| Faeces (0-72 h) | 98.9 | 100.9 |
| Expired air (0-72 h) | 0.02 | 0.01 |
| Carcass (72h) | <0.01 | <0.01 |

In females, urinary excretion was virtually complete within 12 h, while in males excretion continued over the first 24 h. The majority of faecal excretion occurred in both sexes between 12 and 24 h after dosing, although there was significant excretion in males between 6 and 12 h after dosing. Very little of the administered dose was excreted in expired air or retained in the carcass.

In group 4 rats (males only), urinary excretion was slightly more extensive than that seen above, with 2.32% of the administered dose excreted. Biliary excretion accounted for 1.96% of the administered dose, while faecal excretion accounted for 92.3% of the administered dose. Little radioactivity was retained in the carcass.

Based on these studies, approximately 4% of the administered dose was absorbed. In all groups around 100% of the administered dose was recovered. There was very low retention of test material or metabolites in the carcass. Little metabolism of NC-00751 occurred, with the majority of radioactive material in plasma and excreta being unchanged NC-00751. While other minor metabolites were found, these were not positively identified.

**8. Hall M & Bottomley SM (1997) -NC-00723 Effect on hepatic xenobiotic metabolising enzyme activities in rats by dietary administration for 14 days. Laboratory Study No: MTO 52. Sponsor Study No: PCR-1032.**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England |
| Date of study: | 15 April 1996–25 July 1996 |
| GLP: | UK, EC, OECD, US FDA |
| Test chemical: | NC-00723: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester.<br>Monsanto Company. batch no: 95MP-028-3, purity >97% |
| Test species: | Crl CDBR Sprague Dawley rats Charles River Breeding Laboratories, Margate Kent UK) |

**Study design**

Rats were fed NC-00723 in the diet at 0, 100, 300 or 1000 mg/kg bw/day for 14 days using 6/sex/group. A positive control group were fed phenobarbitol at 75 mg/kg bw/day for days 11 to 14 only, while a separate group of 5/sex were maintained as a health check group. The prepared diets were sampled during the study to determined the level of compound present and the homogeneity.

Rats were observed twice daily for mortality and morbidity, and at least once daily for behavioural changes, reaction to treatment and signs of ill health. The bodyweight was determined at the time of allocation, and the start of treatment, then weekly during the treatment phase. Food consumption was measured weekly, and the test article consumption was calculated from the individual bodyweight, food consumption and concentration of test article in the diet.

At the end of the treatment period, rats were fasted overnight before sacrifice. The liver was removed and weighed, and the liver and kidney sampled for possible future electron microscopy examination.. The liver was homogenised and samples removed and centrifuged. The supernatant was decanted and used to produce a microsomal and cytosolic fraction. The protein concentration of both fractions was determined. The cytochrome P450 content was analysed.

The activity of 7-ethoxyresorufin O-deethylase, testosterone dehydroxylase, lauric acid hydroxylase and p-nitrophenol UDP-glucuronosyltransferase were determined, and the non-protein thiol concentration was assayed.

**Results**

There were no mortalities during the study. Abnormal clinical signs were limited to unsteady gait in the phenobarbitol-dose animals in the first few h after dosing. There were no significant differences in bodyweight between treated groups. Bodyweight gain in males at 1000 mg/kg bw/day NC-00723 was decreased by 21% in comparison to controls ($p \leq 0.05$) during the first week. In females at 1000 mg/kg bw/day, bodyweight gain was decreased by 37% in comparison to controls ($p \leq 0.05$) in the first week and over the whole study period. A decrease in bodyweight gain of 26 % in week 1 and 33% in week 2, which was not statistically significant, was seen in females at 300 mg/kg bw/day. Food consumption was decreased at 1000 mg/kg bw/day in the first week (males 17%, females 14%, $p \leq 0.01$) and over the whole study period (males 10%, females 11%, $p \leq 0.05$), with a non-significant decrease in week 2 of the study (<10% for both sexes). No effects on food consumption were seen at any lower doses.

There were no effects on the terminal bodyweight or absolute liver weight, with exception of the phenobarbitol-dosed females, where a 12% increase in absolute liver weight was seen ($p<0.05$). Liver weight relative to bodyweight was increased in both males (14%) and females (13%) dosed with phenobarbitol, however no changes in relative liver weight were seen in any group dosed with NC-00723.

NC-00723 did not have any effects on the levels of hepatic microsomal protein or cytochrome P450. There were no effects from the administration of NC-00723 on the activity of any of the liver enzymes measured, with the exception of p-nitrophenol UDP-glucuronosyl transferase activity, which was decreased in males at 1000 mg/kg bw/day. The levels seen, however were similar to the median value for untreated historical controls. There were no treatment-related changes in cytosolic protein or non-protein thiol levels. Thus treatment with NC-00723 at doses up to 1000 mg/kg bw/day did not alter hepatic biochemical parameters including xenobiotic metabolising enzymes.

Phenobarbitol administration resulted in increases in hepatic microsomal protein and cytochrome P-450 levels. All liver enzymes analysed showed increased activity following dosing with phenobarbitol, as did the levels of non-protein thiol. There was no change in the level of cytosolic protein following phenobarbitol treatment. These changes were those expected with an enzyme inducer.

**9. Kirkpatrick D, Aikens PJ & Harris RE (1998) $^{14}$C-NC-00723 Metabolite isolation from the Rat. Laboratory Study No: MON 051. Sponsor Study No: PCR-1214.**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England |
| Date of study: | 29 July 1997–20 August 1997 |
| Test chemical: | NC-00723: α-N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester. |
| Radiolabelled: | Huntingdon Life Sciences Ltd. batch: MRH/MTO 56/33/1 57.26 μCi/mg, purity: >98% |
| Non-labelled: | Monsanto Company, Batch no. 95MP-028-3, purity >98% |

Reference: Uncharacterised human metabolite, obtained from study LEC/18 (PCR 1039)
Test species: Crl CDBR Sprague Dawley rats (Charles River UK Ltd, Margate Kent UK)

**Study design**

Rats were group housed by sex during the acclimatisation period and were rehoused in glass metabolism cages for the study. Rats (10/sex) were give 2 oral gavage doses of radiolabelled NC-00723 at 15 mg/kg bw, with doses separated by 8 h. Urine was collected for 0–24 and 24–48 h after the first dose. The radioactivity in the urine was measured and the metabolites characterised.

**Results**

Urinary excretion was mainly complete in the first 24 h after dosing, with males excreting 7.9% of the administered dose, and females excreting 6.6% of the administered dose. In the 24–48 h time period, males excreted 0.41% of the administered dose, while females excreted 0.53% of the administered dose. Analysis of the metabolites indicated that an uncharacterised metabolite found in human urine was present in the urine of female rats, but not of male rats.

**10. Kirkpatrick D, Aikens PJ & Hobbs GR (1998) $^{14}$C-NC-00723 and $^{14}$C-NC-00751. Stability in simulated gastric fluid and intestinal fluid. Laboratory Study No: MON 068. Sponsor Study No: PCR-1218.**

Laboratory: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England
Date of study: 13 October 1997–21 October 1997
Test chemical: NC-00723: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester.
Radiolabelled: Huntingdon Life Sciences Ltd. Batch no. MRH/MTO 56/33/1 57.26 μCi/mg, 21.67 mCi/mmol purity: >98%
Non-labelled: Monsanto Company, Batch no. 95MP-028-3, purity >98%
Second chemical: NC-00751 N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine
Radiolabelled: Huntingdon Life Sciences Ltd. Batch no. MRH/MTO 44/21, 23.63 μCi/mg, 8.61 mCi/mmol purity: >98%
Non-labelled: Monsanto Company, Batch no. IP-1123-27-5, purity 99.24%

**Study design**

Simulated gastric and intestinal fluids were prepared both with and without enzymes, resulting in four test solutions. Gastric fluid was prepared with 2 g NaCl and 3.2 g pepsin in 7 mL HCl and made up to 500 mL with water. Similar gastric fluid without pepsin was also prepared. Intestinal fluid contained 6.8 g potassium phosphate in 125 mL water. Sodium hydroxide (0.2M, 50mL) and 10 g pancreatin were added and the pH was adjusted to 7.5. A similar intestinal fluid without pancreatin was prepared.

Test solutions and simulated gastric or intestinal fluids were warmed to 37$^{o}$C before mixing to contain 50 μg/mL $^{14}$C-NC-00723 or 25 μg/mL $^{14}$C-NC-00751, and the mixture was incubated. Aliquots of 1 mL were removed at 0, 1, 5, 15, 30, 60 and 120 min after mixing.

The reaction in each aliquot was stopped by mixing with 1 mL 1M citric acid and 2 mL acetonitrile and samples were frozen until analysed. The amount of test article and degradants in each sample was calculated as a proportion of the total radioactivity.

**Results**

NC-00723 was stable in simulated gastric fluid both with and without pepsin, and in simulated intestinal fluid without pancreatin. In these solutions, more than 95% of the initially added NC-00723 remained unchanged after 120 min of incubation. In simulated intestinal fluid with pancreatin, rapid hydrolysis occurred, being essentially complete within 15 min. After 120 min only 0.73% of the NC-00723 initially added remained unchanged, with NC-00751 the only detectable product of hydrolysis. The hydrolysis of NC-00723 was therefore enzyme catalysed.

NC-00751 was stable in both simulated gastric and intestinal fluids, both with and without enzymes for 120 min. No discrete degradation products of NC-00751 were detected.

**11. Kirkpatrick D, Aikens PF & Saxton JE (1997) $^{14}$C-NC-00723 and $^{14}$C-NC-00751. Studies of plasma protein binding *in vitro* (rat, dog and human). Laboratory Study No: MON 42. Sponsor Study No: PCR-1208.**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England |
| Date of study: | 6 March 1997–11 October 1997 |
| GLP: | UK, EC, OECD, US FDA |
| Test chemical: | NC-00723: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester. |
| Radiolabelled: | Huntingdon Life Sciences Ltd. Batch MRH/MTO 56/33/1 57.26 μCi/mg, purity: >98% |
| Non-labelled: | Monsanto Company, Batch no. 95MP-028-3, purity >98% |
| Second chemical: | NC-00751: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine |
| Radiolabelled: | Huntingdon Life Sciences Ltd. Batch MRH/MTO 44/21 23.63 μCi/mg: purity: >98% |
| Non-labelled: | Monsanto Company, Batch no. IP-1123-27-5, purity 99.24% |
| Test species: | Male CD Sprague Dawley rats (source: Charles River UK, Margate Kent UK)<br>Male Beagle dogs (source: Harlan Interfauna UK Ltd<br>Male human volunteers |

**Study design**

Whole blood was collected into heparinised tubes from 8 male rats by cardiac puncture and centrifuged. The plasma was harvested and pooled, then stored frozen. Whole blood was collected from 3 male Beagle dogs into heparinised tubes and centrifuged. The plasma was harvested, pooled and frozen. Whole blood was also collected from 9 male human volunteers into EDTA tubes and centrifuged. The plasma was harvested, pooled, and frozen. The plasma was assessed for total protein and albumin content. Human plasma protein components were prepared for comparison.

A membrane binding experiment was conducted to determine the extent of binding of the radiolabelled test compounds to the proposed equipment. In the rat, portions were incubated with $^{14}$C-NC-00751 at 100, 1000 or 10000 ng/mL, with the incubation and analysis conducted in a similar manner as described above. The stability of the test article was not determined, however the pH of the solution was measured. Dog plasma was mixed with $^{14}$C-NC-00723 or $^{14}$C-NC-00751 at 1, 10 or 100 μg/mL. Incubation and analysis of the samples were conducted in a similar manner to the rat samples.

To determine the plasma protein binding of human plasma, 30 mL samples of control plasma or plasma protein component solution was mixed with $^{14}$C-NC-00723 (at 10, 100 or 1000 ng/mL) or $^{14}$C-NC-00751 (at 50, 500 or 5000 ng/mL). Samples were mixed and incubated at 37$^{o}$C for 10 min. A subsample was then removed, and the remainder incubated for a further 20 min, when an additional sample was removed for analysis. All samples were analysed for radioactivity, and the samples were centrifuged in Micropartition devices. The ultrafiltrate was then analysed and the stability of the test article was determined.

**Results**

The membrane-binding test demonstrated that neither test compound bound to the membrane in the ultrafiltration devices used.

***Rat***

NC-00723 was not studied in rats, as it is known to be very unstable in rat plasma. Rat plasma bound between 72.1 and 75.8% of the added radiolabelled NC-00751, with binding occurring very rapidly, with no noted increase in the percentage bound with increasing time. There was a very slight decrease in the percentage of plasma binding with increasing concentration.

***Dog***

In dogs, up to 89% of the radiolabelled NC-00723 was bound at the lowest concentration added. However, at the highest concentration, only 74.6% of the added material was bound. There was a noted decrease in the percentage of the material bound with increasing concentration, and a slight increase in the percentage bound with increased time of incubation. Around 50% of the radiolabelled NC-00751 was bound in dog plasma. The percentage bound decreased with increasing concentration and, at the low and medium concentration, the percentage bound increased with increasing length of incubation.

***Human***

The plasma protein binding of radiolabelled NC-00723 by pooled human plasma was greater than 94% for all doses and time periods. There was a slight increase seen with increased length of incubation, and a slight decrease in the percentage of the material bound with increasing dose. The dissolved albumin bound around 80% of the added NC-00723, while the human $\alpha_1$-acid glycoprotein bound between 8.2 and 14.1% of the added compound. For radiolabelled NC-00751, human plasma bound 85.3% to 90.9% of the added compound, with slight increases seen with increased length of incubation. The percentage bound decreased slightly with increasing concentration at the 10-min incubation, but was not affected at the 30-min incubation.

Albumin bound around 30% of the added NC-00751, while human $\alpha_1$-acid glycoprotein bound less than 10% of the added compound at all concentrations. The percentage of the compound bound to $\alpha_1$-acid glycoprotein increased both with increasing incubation time and with increasing concentration.

Overall, there was little evidence for saturation of plasma binding over the range of concentrations tested. Binding of NC-00751 was lower in human and dog plasma than was NC-00723, with NC-00723 largely bound to albumin.

**12. Hawkins DR, Kirkpatrick D, Shaw D & Bennett S (1996) $^{14}$C-NC-00723 and $^{14}$C-NC-00751. Storage stability in fortified rat and dog urine. Laboratory Study No: MTO 49. Sponsor Study No: PCR-1141.**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England |
| Date of study: | 8 February 1996–18 June 1996 |
| GLP: | UK, OECD, US FDA |
| Test chemical: | NC-00723: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester. |
| Radiolabelled: | Huntingdon Life Sciences Ltd. Batch no. SB/NTS 40/28 19.97 Ci/mol; purity: >98% |
| Non-labelled: | Monsanto Company, Batch no. 95MP-028-3, purity >98% |
| Second chemical: | NC-00751: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine |
| Radiolabelled: | Huntingdon Life Sciences Ltd. Batch MRH/MTO 44/21: 23.63 μCi/mg: purity: >98% |
| Non-labelled: | Monsanto Company, Batch no. IP-1123-27-5, purity 99.24% |
| Test species: | CD Sprague Dawley rats, Beagle dogs |

**Study design**

Urine from non-dosed rats or Beagle dogs was obtained and radiolabelled NC-00723 or NC-00751 at 10 μg/mL added to control tubes or tubes containing rat or dog urine. All samples taken were analysed to determine any breakdown products present. Samples were taken at a range of times as detailed to demonstrate storage stability:

- immediate sampling
- stored frozen for 7 days before analysis
- stored frozen for 7 days, thawed, citric acid added, frozen for additional 7, 28 or 90 days before analysis
- stored frozen for 7 days, thawed, frozen for 7, 28 or 90 days before analysis.

**Results**

Very little degradation of either NC-00723 or NC-00751 was seen following any of the storage conditions. At all times in rat or dog urine, >96% of NC-00723 and >98% of NC-00751 remained in the samples.

**13. Hawkins DR, Kirkpatrick D, Shaw D & Bennett S (1996) $^{14}$C-NC-00723 and $^{14}$C-NC-00751. Storage stability in rat and dog faeces. Laboratory Study No: MTO 50. Sponsor Study No: PCR-1142.**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England |
| Date of study: | 14 February 1996–7 May 1996 |
| GLP: | UK, OECD, US FDA |
| Test chemical: | NC-00723: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester. |
| Radiolabelled: | Huntingdon Life Sciences Ltd. Batch no. SB/NTS 40/28: 19.97 Ci/mol: purity: >98% |
| Non-labelled: | Monsanto Company, Batch no. 95MP-028-3, purity >98% |
| Second chemical: | NC-00751: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine |
| Radiolabelled: | Huntingdon Life Sciences Ltd. Batch. MRH/MTO 44/21: 23.63 μCi/mg,: purity: >98% |
| Non-labelled: | Monsanto Company, Batch no. IP-1123-27-5, purity 99.24% |
| Test species: | CD Sprague Dawley rats, Beagle dogs |

**Study design**

Faeces from non-dosed rats or Beagle dogs was obtained, with 5 g of faeces used in each sample tube. Radiolabelled NC-00723 at 5 μg/g faeces or NC-00751 at 50 μg/g faeces was added to control tubes or tubes containing rat or dog faeces. All samples taken were analysed to determine any breakdown products present. Samples were taken at a range of times as detailed to demonstrate storage stability:

For rat faeces:

- immediate extraction–NC-00723 and NC-00751 with water/acetonitrile
- extracted immediately with citric acid/acetonitrile (NC-00723 only)
- stored at -20$^{o}$C for 7 days citric acid added before thawing, then extracted
- stored at -20$^{o}$C for 7 days, thawed, citric acid added after thawing, then extracted
- stored at -20$^{o}$C for 22 days, citric acid added before thawing ,then extracted (NC-00723 only)

For dog faeces, samples were stored at ambient temperatures in darkness for 6 h before any storage/extraction. This was followed by:

- immediate extraction (NC-00723 and NC-00751)
- addition of 0.2M citric acid, stored frozen for 7 days, then extraction (NC-00723 and NC-00751)
- storage at -20$^{o}$C for 7 days, then extraction (NC-00723 and NC-00751)
- addition of 0.2M citric acid, stored frozen for 28 days, then extracted NC-00751
- storage at -20$^{o}$C for 28 days, then extraction of NC-00751.

Additionally, dog faeces were stored at ambient temperatures in darkness for 16 h before storage/extraction following by:

- immediate extraction (NC-00723 and NC-00751)
- addition of 0.2M citric acid, storage at -20$^{o}$C for 7 days, then extraction (NC-00751)

- storage at -20°C for 7 days, then extraction (NC-00751)
- addition of 0.2M citric acid, storage at -20°C for 28 days, then extraction (NC-00751)
- storage at -20°C for 28 days, then extraction (NC-00751).

Control samples were stored at ambient temperatures in darkness for 6 h, then either extracted or stored at -20°C for 28 days (with and without 0.1M citric acid), or stored at ambient temperatures in darkness for 16 h, followed by extraction.

**Results**

There was no degradation of NC-00751 in rat and dog faeces fortified with this test article in similar experiments to those performed with Neotame. Similarly, there was no degradation in control samples, containing NC-00751 fortification solution alone, that were established as described for NC-00723.

**14. Hawkins DR, Kirkpatrick D, Aikens PJ & Saxton J (1995) $^{14}$C-NC-00723. Metabolism in the Dog. Pilot investigation. Laboratory Study No: NTS-33. Sponsor Study No: PCR-960.**

| | |
|---|---|
| Laboratory: | Huntingdon Research Centre Ltd, Huntingdon, Cambridgeshire England |
| Date of study: | 12 September 1994–17 March 1995 |
| Test chemical: | NC-00723: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester. |
| Radiolabelled: | Huntingdon Life Sciences Ltd. purity >98%<br>Batch no. (oral) MRH/NTS 19/BK2/31/1 activity: 8.92 Ci/mol, 23.57 µCi/mg<br>Batch no.(IV): MRH/NTS 31/37/2; activity 19.97 Ci/mol, 52.77µCi/mg |
| Non-labelled: | NutraSweet Company, Batch no. (oral) 94UP8-3-4, (IV) DEF-1037-272-2 |
| Reference cmpd: | NC-00751: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine<br>NC-00754: N-(3,3-dimethylbutyl)-L-aspartic acid |
| Test species: | Beagle dogs: Consort Ltd, Harewood Park, Harewood End, Hereford UK |

**Study design**

$^{14}$C-NC-00723 was given at 15 mg/kg bw orally in the first study period and IV in the second dosing period. A 2-month recovery phase was allowed between the two dosing periods. Following oral or IV dosing, using 1/sex, urine and faeces were collected for 72 h and frozen. Cage washes were done at the end of each 24-h period. Blood samples were taken pre-dose and for 72 h after oral dosing or 24 h after IV dosing. Radioactivity was measured and metabolites were identified and quantified. Urine collected from 0–24 h after dosing was pooled to allow metabolite and stability analysis. Faecal extracts were pooled after oral dosing (male 6–36 h, female 6–24 h) and after IV dosing (male 2–36 h, female 12–36 h). Faeces were analysed and co-chromatographed with NC-00751.

**Results**

**Absorption**

Based on a comparison of urinary excretion after oral and IV dosing (see excretion section), the absorption following oral dosing was around 47%, with no obvious sex differences.

**Distribution**

Following oral dosing, plasma levels in the male peaked at 0.5 h, and in the female at 0.25 h. Plasma levels were very low by 48 h after dosing. After IV dosing, levels peaked at 5 min after dosing, and at 24 h were similar to those at 24 h after oral dosing.

The pharmacokinetic parameters after oral dosing are set out in the following table. Units for parameters derived from plasma activity are µg equivalents/mL for Cmax and µg equiv.h/mL for AUC. Units for parameters derived from NC-00723 and NC-00751 are µg/mL for Cmax and µg.h/mL for AUC.

**Pharmacokinetics after oral dosing with NC-00723**

| | **Derived from plasma radioactivity** | | **Derived from NC-00723 quantities** | | **Derived from NC-00751 quantities** | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** |
| Cmax | 3.39 | 2.67 | 1.672 | 0.953 | 1.181 | 0.744 |
| Tmax/h | 0.50 | 0.25 | 0.25 | 0.25 | 0.5 | 0.25 |
| AUC | 9.7 | 7.7 | 0.9 | 0.4 | 1.5 | 1.3 |

The pharmacokinetic parameters after IV dosing are set out in the following table.

**Pharmacokinetics after intravenous dosing with NC-00723**

| | **Derived from plasma radioactivity** | | **Derived from NC-00723 quantities** | | **Derived from NC-00751 quantities** | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** |
| Cmax | 15.3 | 17.3 | 10.79 | 12.79 | 3.493 | 6.294 |
| Tmax (h) | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.25 |
| AUC | 18.0 | 20.0 | 3.3 | 3.3 | 5.1 | 7.0 |

The plasma half-life for NC-00723 after IV dosing was 0.4 h in the male, and was not calculated in the female. The plasma half-life of NC-00751 after IV dosing with NC-00723 was 1.2 h in the male and 1.4 h in the female.

**Metabolism**

After oral dosing, plasma radioactivity was initially mainly NC-00723. Over the next 2 h, the amount of NC-00751 in the plasma increased and NC-00723 levels decreased. Other metabolites were present at low levels, and were not identified further. Following IV dosing, levels of NC-00723 dropped rapidly, with very low levels present at 2 h after dosing. Levels of NC-00751 peaked at 5 min after dosing, but were still detectable at 4 h after dosing. Other metabolites were present in low quantities and were not further identified.

**Excretion**

The excretion of radioactivity in urine, faeces and in the cagewash are set out in the following table.

**Excretion of radioactivity (% dose administered) following oral or IV dosing.**

| | Oral dosing (15 mg/kg bw) | | IV dosing (15 mg/kg bw) | |
|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** |
| Urine | 17.9 | 17.9 | 36.6 | 38.1 |
| Cagewash | 3.37 | 5.51 | 1.56 | 3.00 |
| Faeces | 73.4 | 72.3 | 56.9 | 49.5 |

Total recovery in urine, faeces and cagewash varied from 90% to 96% of the administered dose. The majority of the urinary excretion was completed by 24 h after dosing in both the male and female, although excretion continued until 48 h after dosing. Faecal excretion peaked at 6 to 12 h and again at 24 to 36 h after oral dosing in the male, and at 6 to 12 and 12 to 24h after oral dosing in the female. Following IV dosing, faecal excretion peaked at 6 to 12 h after dosing in the male at 12 to 24 h after dosing in the female.

Urine metabolites after oral dosing were mainly NC-00751, with some unidentified minor metabolites. After IV dosing, 15% of the dose was excreted as NC-00751, and approximately 10% was excreted as NC-00723. Faecal metabolites after oral dosing mainly consisted of NC-00751 (47–49% of the dose), with minimal amounts of other metabolites. After IV dosing, around 25% of the dose was excreted in the faeces as NC-00751, with less than 5% of other metabolites present.

**15. Kirkpatrick D, Aikens PJ, Nicholson J Saxton JE & Harris KE (1997) $^{14}$C-NC-00723. Metabolism and Pharmacokinetics in the Dog. Laboratory Study No: MTO/53. Sponsor Study No: PCR-1029.**

Laboratory: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England
Date of study: 10 July 1996–3 March 1997
GLP: UK, EC, OECD, US-FDA
Test chemical: NC-00723: α-N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester.
Radiolabelled: Huntingdon Life Sciences Ltd. purity >98%
Batch no. MRH/NTS 31/37/2; activity 19.97 Ci/mol, 52.77μCi/mg
Non-labelled: Monsanto Company, Batch no. 95MP-028-3, purity>98%
Reference cmpd: NC-00751: α=N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine
NC-00754: N-(3,3-dimethylbutyl)-L-aspartic acid
Test species: Beagle dogsL Harlan Interfauna UK Ltd, Wyton, Cambridgeshire UK

**Study design**

Radiolabelled NC-00723 was given orally at 15 or 120 mg/kg bw, or IV at 15 mg/kg bw, using 3 dogs/sex/dose. Following oral dosing, urine and faeces were collected and frozen for 72 h after dosing. A cage wash was done at 72 h. A 10mL blood sample was taken from the jugular vein pre-dose and at intervals for 24 h after dosing. No post-mortem was performed, and carcasses were discarded at the end of the trial.

One dog vomited after a 15 mg/kg bw dose, and was re-dosed after 67 days. IV dosing was administered over 2–3 min into the cephalic vein. Sampling was done as for oral dosing, with the addition of an extra blood sample at the end of the infusion period. Radioactivity was measured and metabolites identified and quantified.

**Results**

**Distribution**

The concentration of radioactivity in plasma is set out in the following table.

**Plasma levels following dosing with NC-00723**

| | oral 15 mg/kg bw | | **IV 15 mg/kg bw** | | **oral 120 mg/kg bw** | |
|---|---|---|---|---|---|---|
| | **males** | **females** | **males** | **females** | **males** | **females** |
| Max. plasma radioactivity (µg eq NC-00723/mL) | 2.13 | 1.84 | 62.9 | 74.9 | 50.5 | 36.8 |
| Tmax (h) | 0.17 | 1 | end infusion | end infusion | 0.5 | 0.5 |
| Max NC-00723 (ng/mL) | 1498.5 | 1380.8 | 75761.2 | 88680.5 | 27016.3 | 19850.6 |
| Tmax (h) | 0.08 | 0.25 | end infusion | end infusion | 0.5 | 0.5 |
| Max NC-00751 (ng/mL) | 826.6 | 945.7 | 3344.6 | 3636 | 15361.1 | 10694.1 |
| Tmax (h) | 1 | 1 | 0.25 | 0.25 | 0.75 | 0.75 |

**Metabolism**

Urinary metabolites present after oral and IV dosing were mainly NC-00751, with some compound excreted as unchanged NC-00723, particularly after IV dosing. A number of other minor metabolites were present in small quantities. No unchanged NC-00723 was detected in the faeces of dogs. Most of the administered radioactivity was excreted in the faeces as NC-00751, with a number of unidentified metabolites present in small quantities.

**Excretion**

The excretion of radioactivity is set out in the following table.

**Excretion of radioactivity expressed as percentage of dose.**

| | **Oral dosing 15 mg/kg bw** | | **Intravenous dosing 15 mg/kg bw** | | **Oral dosing 120 mg/kg bw** | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** |
| Urine | 12.7 | 14.3 | 39.7 | 42.5 | 18.8 | 20.1 |
| Cagewash | 0.2 | 0.3 | 0.5 | 0.4 | 0.8 | 0.9 |
| Faeces | 83.7 | 78.2 | 53.5 | 52.5 | 75.7 | 72.4 |

Total recovery ranged from 92.7% to 96.1% of the administered dose. After oral dosing at 15 mg/kg bw, peak urinary excretion occurred at 0–6 h in males and 6–12 h in females. Faecal excretion peaked in males at 6–12 h after dosing and in females at 12–24 h after dosing. After oral dosing at 120 mg/kg bw, maximum urinary excretion was at 0–6 h for both males and females. Maximum faecal excretion was at 6–12 h.

After IV dosing, urinary excretion peaked at 0–6 h after dosing, while faecal excretion peaked at 12–24 h in both males and females. The excretion of radioactivity in the faeces after IV dosing suggests either biliary or gastrointestinal secretion of NC-00723 or its metabolites.

**16. Hawkins DR, Kirkpatrick D, Aikens PJ & Saxton J (1996) $^{14}$C-NC-00723 and $^{14}$C-NC-00751. Storage stability in fortified human faeces. Laboratory Study No: MTO 45. Sponsor Study No: PCR-1117.**

Laboratory: Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England
Date of study: 26 October 1995–30 January 1996
Test chemical: NC-00723: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester.
Radiolabelled: Huntingdon Life Sciences Ltd. Batch no. MRH/NTS 31/37/2
19.97 Ci/mol purity 52.77 μCi/mg, purity: >98%
Non-labelled: Monsanto Company, Batch no. 95MP-028-3, purity >98%
Second chemical: NC-00751: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine
Radiolabelled: Huntingdon Life Sciences Ltd. Batch no. MRH/MTO 44/21
23.63 μCi/mg, 8.61 mCi/mmol purity: >98%
Non-labelled: Monsanto Company, Batch no. IP-1123-27-5, purity ≥99.2%

**Study design**

Faeces from non-dosed healthy volunteers was obtained and stored at -15$^{o}$C until required, when 5 g of faeces was used in each sample tube. Radiolabelled NC-00723 at 5 μg/g faeces or NC-00751 at 50 μg/g faeces was added. Levels were selected to be similar to those seen in human faeces after a single oral dose of $^{14}$C-NC-00723.

In the first trial, following addition of test compound, faeces were shaken for 2 min and kept at room temperature for around 30 min before extraction in the following protocols:

1. Extracted immediately
2. Stored at <-15$^{o}$C for 14 days then extracted
3. Stored at <-15$^{o}$C for 29 days then extracted
4. 1:1 (w/v) 1M citric acid added, extracted immediately
5. 1:1 (w/v) 1M citric acid added, Stored at <-15$^{o}$C for 14 days then extracted
6. 1:1 (w/v) 1M citric acid added; stored at <-15$^{o}$C for 29 days then extracted

The extracts were assessed for radioactivity and the metabolites determined.

In the second trial, human faeces were placed into centrifuge tubes and mixed with radiolabelled NC-00723 and 1M or 2M citric acid. The tubes were shaken for 2 min, stored at ambient temperatures for 30 min, then samples were extracted.

**Results**

The extractability of radioactivity was increased by the addition of citric acid. NC-00723 was rapidly converted to NC-00751 in faeces without citric acid. Degradation continued in frozen samples, although the rate of degradation was decreased with the addition of citric acid. No degradation of NC-00751 to other metabolites was seen at any time.

When citric acid was added to faeces before incubation at room temperature, there was little degradation of NC-00723 to NC-00751 at any time, including following frozen storage for 7 days. Thus it was recommended that, to prevent degradation of NC-00723 to NC-00751, faeces should be acidified with citric acid before frozen storage.

**17. Kirkpatrick D & Aikens PJ (1998) $^{14}$C-NC-00764. Stability in simulated gastric fluid and intestinal fluid. Laboratory Study No: MON 141. Sponsor Study No: PCR-1229.**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire England |
| Date of study: | 6 August 1998–29 September 1998 |
| Test chemical: | NC-00764: N-(3,3-dimethylbutyl)-beta L-aspartyl-L-phenylalanine methyl ester. |
| Radiolabelled: | Huntingdon Life Sciences Ltd. Batch no. MRH/NTS39/48 67.48 μCi/mg, 25.54 mCi/mmol purity: >98% |
| Non-labelled: | Monsanto Company, Batch no. 95MP115-25, purity >96.8% |
| Reference chem: | NC-00769: N-(3,3-dimethylbutyl)-beta-L-aspartyl-L-phenylalanine; Monsanto Company, Batch no. KVI-88, purity 96.6% $^{14}$C-NC-00754: N-(3,3-dimethylbutyl) -L- aspartic acid: Huntingdon Life Science Ltd, Batch No: MRH/NTS39/68, 93.61 μCi/mg, |
| GLP: | UK, EC, OECD |

**Study design**

Simulated gastric and intestinal fluids were prepared both with and without enzymes, resulting in four test solutions. Gastric fluid was prepared with 2 g NaCl and 3.2 g pepsin in 7 mL HCl and made up to 500mL with water. Similar gastric fluid without pepsin was also prepared. Intestinal fluid contained 6.8 g potassium phosphate in 125 mL water. Sodium hydroxide (0.2M, 50mL) and 10 g pancreatin were added. The pH was adjusted to 7.5, and the volume made up to 500 mL. A similar intestinal fluid, without pancreatin was prepared.

Test solutions and simulated gastric or intestinal fluids were warmed to 37$^{o}$C before mixing. Test solutions were prepared to contain 6 μg/mL $^{14}$C-NC-00764, and the mixture was incubated at 37$^{o}$C. Aliquots of 1 mL were removed at interval up to 2h after mixing. The reaction in each aliquot was stopped by mixing with 1 mL citric acid and 2 mL acetonitrile. All samples were analysed, and the amount of test article and degradants in each sample was calculated as a proportion of the total radioactivity.

The storage stability of NC-00764 was also analysed, with samples of each test fluid mixed with citric acid and acetonitrile. A sample was withdrawn immediately after mixing, with the remainder snap frozen and stored for 18 days before analysis.

**Results**

NC-00764 was stable in simulated gastric fluid both with and without pepsin for 2 h, with NC-00769 forming 2% of the content at this time. In simulated intestinal fluid without pancreatin NC-00764 was also stable, with NC-00769 forming 2.5% of the material after 2 h.. In simulated intestinal fluid with pancreatin, rapid hydrolysis to NC-00769 occurred, with hydrolysis essentially complete within 1 min.

The hydrolysis of NC-00764 was therefore enzyme catalysed, and would therefore be expected to occur *in vivo*. NC-00764 was stable stored frozen in each of the incubation mixtures.

## ACUTE STUDIES

### Parent compound

No studies were done using Neotame.

### Degradation products

**1. Bechtel CL (1998) Single Dose Gavage Study in Rats with NC-00764. Lab Project No. EHL 97173. Sponsor Monsanto Company, Deerfield Illinois. Sponsor Study No. PCR-1134**

| | |
|---|---|
| Laboratory: | Monsanto Company, Environmental Health Laboratory, St Louis, Missouri |
| Date of study: | 5 November 1997–20 November 1997 |
| GLP: | US FDA |
| Test chemical: | NC-00764, Lot no. 95MP115-25 |
| Test species: | Crl CD (SD) BR VAF/Plus rats: Charles River Laboratories Inc, Raleigh, NC |
| Dose: | 0, 0.6, 2.0 or 6.0 mg/kg bw in 0.5% aqueous methylcellulose, using 10/sex/group |

### Study design

Rats were housed individually in controlled conditions with free access to food and water, with exception of fasting before dosing. Rats were dosed by oral gavage at 0, 0.6, 2.0 or 6.0 mg/kg bw using 10/sex/group. The concentration of the test material for 2.0 and 6.0 mg/kg bw was verified. Rats were observed twice daily for mortality and morbidity. On the day of dosing, rats were examined for abnormal clinical signs at 1, 2.5 and 4 h after dosing, and daily thereafter until the end of the 14 day observation period. Bodyweights were recorded pre-fasting, immediately pre-dosing and on days 7 and 14 after dosing. Food consumption was measured on days 7 and 14 after dosing. All rats were examined macroscopically after the end of the study. The thoracic, abdominal and pelvic cavities were opened, and all organs and tissues were examined and any gross lesions preserved.

### Results

No deaths occurred during the study, and all rats appeared normal throughout the study. There were no statistically significant differences in bodyweight or food consumption. Bodyweight gain at 6.0 mg/kg bw in females on post-dosing day 14 was decreased 23% in comparison to controls ($p<0.05$). There were no treatment-related macroscopic post-mortem abnormalities detected. One male at 2.0 mg/kg bw/day had a pale yellow focus on the surface of the liver, but this was not considered treatment related.

**2. Bechtel CL (1998) Single Dose Gavage Study in Rats with NC-00777. Lab Project No. EHL 97176. Sponsor Monsanto Company, Deerfield Illinois. Sponsor Study No. PCR-1189**

| | |
|---|---|
| Laboratory: | Monsanto Company, Environmental Health Laboratory, St Louis, Missouri |
| Date of study: | 25 November 1997–10 December 1997 |
| GLP: | US FDA |
| Test chemical: | NC-00777, Lot no. KW-1307-272 |
| Test species: | Crl CD (SD) BR VAF/Plus rats: Charles River Laboratories Inc, Raleigh, NC |
| Dose: | 0, 0.6, 2.0 or 6.0 mg/kg bw in 0.5% aqueous methylcellulose, using 10/sex/group |

**Study design**

Rats were housed individually in controlled conditions with free access to food and water, with exception of fasting before dosing. Rats were dosed by oral gavage at 0, 0.6, 2.0 or 6.0 mg/kg bw using 10/sex/group. The concentration of the test material for 2.0 and 6.0 mg/kg bw was verified. Rats were observed twice daily for mortality and morbidity. On the day of dosing, rats were examined for abnormal clinical signs at 1, 2.5 and 4 h after dosing, and daily thereafter until the end of the 14 day observation period. Bodyweights were recorded prefasting, immediately pre-dosing and on days 7 and 14 after dosing. Food consumption was measured on days 7 and 14 after dosing. All rats were examined macroscopically after the end of the study. The thoracic, abdominal and pelvic cavities were opened, and all organs and tissues were examined and any gross lesions preserved.

**Results**

No unscheduled deaths occurred during the study. On the day of dosing, males in all treated groups and a female at 2.0 mg/kg bw had soft stools. All rats appeared normal throughout the rest of the study. This effect is not considered a toxicologically significant effect of NC-00777. There were no statistically significant differences in bodyweight or food consumption during the study. There were no treatment-related macroscopic post-mortem abnormalities detected. One control female and one female at 2.0 mg/kg bw/day had a distended uterus. This was considered normal for rats of this age, and was not related to treatment. There were therefore no effects detected following dosing with NC-00777 at up to 6.0 mg/kg bw.

**3. Bechtel CL (1998) Single Dose Gavage Study in Rats with NC-00779. Lab Project No. EHL 97177. Sponsor Monsanto Company, Deerfield Illinois. Sponsor Study No. PCR-1199**

| | |
|---|---|
| Laboratory: | Monsanto Company, Environmental Health Laboratory, St Louis, Missouri |
| Date of study: | 8 December 1997–23 December 1997 |
| GLP: | US FDA |
| Test chemical: | NC-00779, Lot no. DZG-1373-163 |
| Test species: | Crl CD (SD) BR VAF/Plus rats: Charles River Laboratories Inc, Raleigh, NC |
| Dose: | 0, 0.3, 1.0 or 3.0 mg/kg bw in 0.5% aqueous methylcellulose, using 10/sex/group |

**Study design**

Rats were housed individually in controlled conditions with free access to food and water, with exception of fasting before dosing. Rats were dosed by oral gavage at 0, 0.3, 1.0 or 3.0 mg/kg bw using 10/sex/group. The concentration of the test material for 1.0 and 3.0 mg/kg bw/day was verified. Rats were observed twice daily for mortality and morbidity. On the day of dosing, rats were examined for abnormal clinical signs at 1, 2.5 and 4 h after dosing, and daily thereafter until the end of the 14 day observation period. Bodyweights were recorded prefasting, immediately pre-dosing and on days 7 and 14 after dosing. Food consumption was measured on days 7 and 14 after dosing. All rats were examined macroscopically after the end of the study. The thoracic, abdominal and pelvic cavities were opened, and all organs and tissues were examined and any gross lesions preserved.

**Results**

No unscheduled deaths occurred during the study and all rats appeared normal throughout the study. There were no statistically significant differences in bodyweight or food consumption during the study. There were no treatment-related macroscopic post-mortem abnormalities detected. One female at 1.0 mg/kg bw/day had a distended uterus. This was considered normal for rats of this age, and was not related to treatment. There were no effects detected following dosing with NC-00779 at up to 3.0 mg/kg bw.

**SHORT-TERM STUDIES**

**1. Thomford P (1994) Two week Dietary Range-Finding Study of NC-00723 in Mice. Lab Project No. HWI 6211-285. Sponsor The NutraSweet Company, Deerfield Illinois. Sponsor Study No. PCR-0936**

| | |
|---|---|
| Laboratory: | Hazleton Wisconsin Inc., Madison Wisconsin |
| Date of study: | 2 December 1993–9 June 1994 |
| GLP: | US FDA |
| Test chemical: | NC-00723, Lot no. DEF-1037-249S-3: NutraSweet Company, purity: not specified |
| Test species: | Crl CD(ICR) BR VAF/Plus mice: Charles River Laboratories Inc, Portage, Michigan. |

**Study design**

Groups of mice (10 /sex/group) were fed NC-00723 in the diet at levels to achieve doses of 0, 10, 30, 100 or 300 mg/kg bw/day for 14 days. Mice were acclimatised for 15 days before treatment, and were housed individually in controlled conditions with free access to food and water. The diets were prepared weekly, with the concentrations adjusted based on bodyweight and food consumption. Animals were checked twice daily for mortality and moribundity during the study period. Mice were removed from their cages weekly and examined for poor health and abnormal clinical signs. The examination included, but was not limited to, the condition of the skin and fur, ears, eyes, mucous membranes, mouth and teeth, body and limb conformation and the genitalia. An evaluation of respiration, circulation, the CNS and general behaviour was done. The bodyweight was measured on the day of acquisition, 8 days and 1 day before dosing, and on study days 1, 3, 8 and 15. Food consumption was measured for days -8 to -1, days 1, 2, 3, days 4–8 and days 8–15.

Blood samples were taken from all mice on day 15 for haematological or biochemical parameters, using 5/sex/group for each examination. Haematologic parameters are detailed in Appendix B. A limited clinical chemistry examination, including glucose, urea nitrogen, AST, ALT and alkaline phosphatase levels was done. Post-mortem examinations were performed at the end of treatment. Macroscopic examination included an examination of all external surfaces of the body, all orifices, the cranial cavity, external surfaces of the brain and spinal cord, the nasal cavity and paranasal sinuses and the thoracic, abdominal and pelvic cavities and viscera. The weights of the adrenals, brain, heart, kidneys, liver with drained gallbladder and testes were measured. The organ to bodyweight and organ to brain weight ratios were calculated. A limited microscopic examination was performed, with the adrenals, brain, heart, kidney, liver, lungs, sternum and bone marrow, testes, thyroid with parathyroid and any lesions examined.

**Results**

**Mortality/clinical signs**

No animals died during this study and there were no treatment-related clinical signs. Incidental abnormalities included yellowed hair coat and an opaque left eye.

**Bodyweight and food consumption**

There were no significant changes in bodyweight in any treatment group at any time. Bodyweight gain was not affected by treatment. Food consumption was not affected by treatment.

**Clinical pathology**

No differences between groups on haematological examination were found. In males at 300 mg/kg bw/day, plasma glucose levels were decreased by 20% compared to controls. No differences between groups were seen for females.

**Gross pathology and organ weights**

There were incidental macroscopic abnormalities, including irregular pupils, opaque cornea, a fluid filled uterus and cystic ovary. One male at 300 mg/kg bw/day had light areas in the liver. There were no treatment-related effects on organ weight.

**Histopathology**

One high-dose male with grossly visible pale areas in the liver had hepatocellular necrosis visible on microscopic examination. Other incidental liver changes included chronic active inflammation. Incidental findings in the kidney included proteinaceous casts, cysts, pelvic dilatation, tubular epithelium hyperplasia and chronic inflammation. These were not considered to be treatment related.

**Conclusion**

In this study, there were no abnormal clinical signs or changes in bodyweight or food consumption related to treatment.

**2. Thomford P (1994) Two week Dietary Range-Finding Study of NC-00723 in Mice. Lab Project No. HWI 6211-297. Sponsor The NutraSweet Company, Deerfield Illinois. Sponsor Study No. PCR-0992**

| | |
|---|---|
| Laboratory: | Hazleton Wisconsin Inc., Madison Wisconsin |
| Date of study: | 17 May 1994–14 December 1994 |
| GLP: | US FDA |
| Test chemical: | NC-00723, Lot no 94UP8-1-7, NutraSweet Company, purity: not specified |
| Test species: | Crl CD(ICR) BR VAF/Plus mice: Charles River Laboratories Inc, Portage, Michigan. |

**Study design**

Groups of mice (10 /sex/group) were fed NC-00723 in the diet at levels to achieve doses of 0, 500, 1000, 2000, 4000 or 8000 mg/kg bw/day for 14 days. The diets were prepared weekly, with the concentrations adjusted based on bodyweight and food consumption. Animals were checked twice daily for mortality and moribundity during the study period. Mice were removed from their cages weekly and examined for poor health and abnormal clinical signs. The examination included, but was not limited to, the condition of the skin and fur, ears, eyes, mucous membranes, mouth and teeth, body and limb conformation and the genitalia. An evaluation of respiration, circulation, the CNS and general behaviour was also done. The bodyweight was determined 5 and 1 day before dosing, and on study days 1, 3, 8 and 15.. Food consumption was measured for days -5 to -3, on days -3, -2, -1, 1, 2, 3, days 4–8 and days 8–15.

Blood samples were taken from all mice on days 16 or 17 for haematological or biochemical parameters, using 5/sex/group for each examination, with haematologic parameters examined detailed in Appendix B. A limited clinical chemistry examination, including glucose, urea nitrogen, AST, ALT and alkaline phosphatase levels was done. Post-mortem examinations were performed at the end of treatment. Macroscopic examination included an examination of all external surfaces of the body, all orifices, the cranial cavity, external surfaces of the brain and spinal cord, the nasal cavity and paranasal sinuses and the thoracic, abdominal and pelvic cavities and viscera. The weights of the adrenals, brain, heart, kidneys, liver with drained gallbladder and testes were measured. The organ to bodyweight and organ to brain weight ratios were calculated. A limited range of tissues were preserved but not examined.

**Results**

**Mortality/clinical signs**

No animals died during this study and there were no treatment related clinical signs. Incidental findings included opaque eyes in both males and females, dilated pupils in males and red skin in females.

**Bodyweight and food consumption**

There were no significant changes in bodyweight in any treatment group at any time. Bodyweight gain was statistically significantly decreased at 8000 mg/kg bw/day in males from days 1 to 3 and days 1 to 8 (75 to 77% decrease), but was not significantly decreased at 4000 mg/kg bw/day (31% and 25% decrease for the same time periods).

In females, decreases in bodyweight gain of more than 20% were seen from 2000 mg/kg bw/day for days 1 to 8, while on days 8 to 15, decreases were more than 50% at 500 mg/kg bw/day. No clear dose-response relationship in the decreases in the bodyweight gain for females was seen.

Food consumption in males was decreased 15-19% compared to controls for selected time periods at 8000 mg/kg bw/day, before returning to control levels. However, day 1 observations were limited by extensive food spillage. In females, an 11% decrease in food consumption at 8000 mg/kg bw/day was seen for days 8 to 15; food consumption could not be measured on day 1 due to spillage.

**Clinical pathology**

There were no significant differences between groups for any clinical chemistry or haematological parameters.

**Gross pathology and organ weights**

There were no treatment-related gross pathological changes. Incidental findings included ovarian cysts and opaque corneas; however, this later finding was also observed in controls and is considered of no toxicological significance. In males, non-dose-related increased adrenal weights were seen in all treatment groups. The relationship to treatment is difficult to determine, as no histopathological observations were made.

**Conclusion**

In this study, bodyweight gain was decreased at high doses. No abnormal clinical signs or changes in clinical or gross pathology were noted.

**3. Thomford P (1994) Two week Dietary Toxicity Study of NC-00723 in Rats. Lab Project No. HWI 6211-291. Sponsor The NutraSweet Company, Deerfield Illinois. Sponsor Study No. PCR-0949**

| | |
|---|---|
| Laboratory: | Hazleton Wisconsin Inc., Madison Wisconsin |
| Date of study: | 29 December 1993–23 August 1994 |
| GLP: | US FDA |
| Test chemical: | NC-00723, Lot no. DEF-1037-272-2: NutraSweet Company, purity: not specified |
| Test species: | Crl CD(SD)BR VAF/Plus rats: Charles River Laboratories Inc, Portage, Michigan. |

**Study design**

Groups of rats (10 /sex group for toxicology and 5/sex/group for the satellite groups) were fed NC-00723 in the diet at levels to achieve doses of 0, 10, 30, 100 or 300 mg/kg bw/day for 14 days. Animals were checked twice daily for mortality and moribundity during the study period. On days 1, 2, 3, 8 and 15, rats were removed from their cages and examined for poor health and abnormal clinical signs. The examination included, but was not limited to, the condition of the skin and fur, ears, eyes, mucous membranes, mouth and teeth, body and limb conformation and the genitalia. An evaluation of respiration, circulation, the CNS and general behaviour was done.

The bodyweight was measured on the day of acquisition, 8 days and 1 day before dosing, and on study days 1, 3, 8 and 15. Food consumption was measured for days -8 to -1, days 1, 2, 3, days 4–8 and days 8–15.

Ophthalmological examinations were done on all rats before assignment to treatment groups and rats in the toxicology group on day 15. Blood samples were taken from rats in the toxicology groups on day 16 for haematological examination and biochemical parameters (see Appendix B, with the exception of reticulocyte count, creatinine phosphokinase, gamma-glutaryl transpeptidase, serum lactate dehydrogenase, ornithine decarboxylase, triglycerides and uric acid). Urine samples were also taken at this time for urinalysis (see Appendix B, with the exception of leucocytes, chloride, potassium, sodium, nitrite and osmolality). Post-mortem examinations were performed on the animals that died during the study and at the end of treatment all animals were killed for macroscopic and microscopic postmortem examination (see Appendix C). The weight of the lungs and seminal vesicles were not determined. Microscopic examination of the diaphragm, gall bladder, head, lacrimal gland, smooth muscle, oviduct, tonsils and trachea were not done. Macroscopic examination included an examination of all external surfaces of the body, all orifices, the cranial cavity, external surfaces of the brain and spinal cord, the nasal cavity and paranasal sinuses and the thoracic, abdominal and pelvic cavities and viscera.

Satellite rats had blood samples taken on day 8 and day 16, with the samples held for possible future analysis, however samples were not analysed for this report.

**Results**

**Mortality/clinical signs**

No animals died during this study. In females at the highest dose (300 mg/kg bw/day), excess salivation (1/10), sores (1/10) and scabs around the mouth (3/10) were seen (also seen in one female at 100 mg/kg bw). However, these findings are isolated and not considered to be treatment related.

**Bodyweight and food consumption**

There were no significant changes in bodyweight in any treatment group at any time. Bodyweight gain was reduced in males at 30 ($p \leq 0.01$, 16.5% decrease) and 300 mg/kg bw/day ($p \leq 0.01$, 17.4% decrease) from days 1 to 8, and in females from days 8 to 15 at 30 ($p \leq 0.01$, 27.4% decrease), 100 ($p \leq 0.05$, 19.5%) and 300 mg/kg bw/day ($p \leq 0.05$, 20.8% decrease).

Food consumption was markedly decreased in males on days 1 to 3 and in females on day 1 from 30 mg/kg bw/day. At 300 mg/kg bw/day on day 1, decreases in males were 29% ($p<0.001$) and in females were 37% ($p<0.001$). In males at 300 mg/kg bw/day, the food consumption over the 15 day dosing period was also significantly reduced ($p<0.05$, 10.4% decrease).

**Ophthalmoscopic examinations**

One male rat at 300 mg/kg bw/day had chronic dacryoadenitis in both eyes, and one female at 300 mg/kg bw/day had a vitreous haemorrhage in the right eye. These findings were not considered to be treatment related.

**Clinical pathology**

No differences between groups in relation to haematological parameters were found. There were no treatment-related changes in relation to clinical chemistry in males at any dose. In females, AST and ALT levels were increased in all treatment groups, with AST levels increased by 22%, 38% ($p \leq 0.05$), 17.7% and 31.9% at 10, 30, 100 and 300 mg/kg bw/day respectively. ALT levels were increased by 26%, 41% ($p \leq 0.05$), 11% and 33% at 10, 30, 100 or 300 mg/kg bw/day respectively. However, the increase in both these enzymes was only statistically significant at 30 mg/kg bw/day and lacked a dose-response relationship. There were no differences between groups for urinalysis parameters.

**Gross pathology and organ weights**

On macroscopic examination in males, dark foci were detected in the stomach in one animal in each group and in 3 in the high dose group. In females, there were no treatment-related macroscopic changes observed. The absolute and relative (to bodyweight or to brain weight) spleen weight was decreased at 300 mg/kg bw/day in males 16.6% ($p \leq 0.05$) and 14.2% ($p \leq 0.01$) or 18.2% ($p \leq 0.01$) respectively). No changes in organ weight were observed in females at any dose. In particular, liver weights were not affected by treatment in either sex.

**Histopathology**

In males, there was focal mucosal congestion in the stomach in one rat at 30 and at in one rats at 100 mg/kg bw/day. There was also erosion of the glandular mucosa of the stomach, with incidences of 1, 2, 2, 1 and 3 at 0, 10, 30, 100 and 300 mg/kg bw/day. No notable abnormal histopathological findings were found in females.

**Conclusion**

Few abnormal clinical signs were observed with the findings of increased salivation at the high dose was not clearly treatment-related. Neotame did not affect the bodyweight of treated animals over the short period of this study. Effects on bodyweight change were mainly related to early decreases in food consumption. Food consumption returned to normal during the 14-day study. Changes in liver enzyme levels in females were not supported by changes in organ weight or macroscopic or microscopic liver changes and lacked dose response relationship. These changes therefore, are not considered likely to be treatment-related. Increased spleen weight seen in males at 300 mg/kg bw/day was not correlated to any histopathological changes; given the short dosing period, its significance is unclear. Small increases in the incidence of erosion of the glandular mucosa of the stomach in males were also seen, however, these changes were not dose-related and the reporting pathologist stated that these are common observations in rats of this strain and sex.

**4. Thomford P (1994) Two week Range-Finding Dietary Study of NC-00723 in Rats. Lab Project No. HWI 6211-296. Sponsor: The NutraSweet Company, Deerfield Illinois. Sponsor Study No. PCR-0994**

| | |
|---|---|
| Laboratory: | Hazleton Wisconsin Inc., Madison Wisconsin |
| Date of study: | 15 July 1994–14 December 1994 |
| GLP: | US FDA |
| Test chemical: | NC-00723, Lot no. 94MP041-10: NutraSweet Company, purity: not specified |
| Test species: | Crl CD(SD)BR VAF/Plus rats: Charles River Laboratories Inc, Portage, Michigan. |

**Study design**

Groups of rats (5/sex/group) were fed NC-00723 in the diet at levels to achieve doses of 0, 200, 600, 2000, 4000 or 6000 mg/kg bw/day for 14 days (males) or 15 days (females). Food was freely available, except for the pre-sampling fasting period, and water was freely available at all times.

Animals were checked twice daily for mortality and moribundity during the study period. At least once weekly, rats were removed from their cages and examined for poor health and abnormal clinical signs. The examination included, but was not limited to, the condition of the skin and fur, ears, eyes, mucous membranes, mouth and teeth, body and limb conformation and the genitalia. An evaluation of respiration, circulation, the CNS, and general behaviour was done. The bodyweight was measured 7 and 3 days before dosing, and on study days 1, 3, 8 and 15. Food consumption was measured daily for days -7 to 7, and for days 8–15.

Blood samples were taken on day 16 (males) or day 17 (females) for haematological and biochemical parameters (see Appendix B, with the exception of reticulocyte count, creatinine phosphokinase, gamma-glutaryl transpeptidase, serum lactate dehydrogenase, ornithine decarboxylase, triglycerides and uric acid). Urine samples were taken at this time for urinalysis (see Appendix B, with the exception of leucocytes, chloride, potassium, sodium, nitrite and osmolality). Post-mortem examinations were performed on the animals that died during the study and at the end of treatment all animals were killed for macroscopic postmortem examination (see Appendix C). The weight of the lungs and seminal vesicles were not determined. Tissues were collected but no microscopic examination was done. Macroscopic examination included an examination of all external surfaces of the body, all orifices, the cranial cavity, external surfaces of the brain and spinal cord, the nasal cavity and paranasal sinuses and the thoracic, abdominal and pelvic cavities and viscera.

**Results**

**Mortality/clinical signs**

No rats died during the study. An increased incidence of red nasal discharge was seen in males at the 4000 and 6000 mg/kg bw/day dose levels (1,2 animals respectively) and in females at 2000, 4000 and 6000 mg/kg bw/day (1,2 and 2 animals respectively). An increased incidence of red colouration on the head was seen in females at 2000, 4000 or 6000 mg/kg bw/day (1, 2 and 5 animals respectively).

**Bodyweight and food consumption**

Both males and females showed a decreased food intake in the first three days of treatment, with decreases from 2000 mg/kg bw/day in males and from 600 mg/kg bw/day in females. In females, food consumption was decreased by 32% in comparison to controls at 600 mg/kg bw/day ($p<0.01$), increasing to a 92% decrease at 6000 mg/kg bw/day. By day three, decreases were only seen at 6000 mg/kg bw/day, being a 22% decrease in males ($p<0.05$) and a 9.5% decrease in females (not statistically significant) and no significant differences were seen in food consumption for the rest of the feeding period.

Bodyweight was lower than controls on day 3. In males at 4000 mg/kg bw/day, bodyweight was 9.9% lower ($p<0.05$), while at 6000 mg/kg bw/day it was 14.6% lower ($p<0.001$). In females on day 3, bodyweight at 6000 mg/kg bw/day was 10% lower than controls ($p\leq0.01$). Bodyweight was not significantly lower than controls for the rest of the study.

Bodyweight gain was decreased in rats on days 1 to 3 and days 1 to 8, and in males for the whole study. From days 1 to 3, bodyweight gains in males were 60% lower at 2000 mg/kg bw/day, 91% lower at 4000 mg/kg bw/day, while males at 6000 mg/kg bw/day lost weight. For females in this interval, bodyweight gain at 2000 mg/kg bw/day was 24% lower than controls, at 4000 mg/kg bw/day was 99% lower than controls, while females at 6000 mg/kg bw/day lost weight. For days 1 to 8, significant decreases in bodyweight gain were only seen at 6000 mg/kg bw/day, with decreases in males of 33% and in females of 43%. In males for days 1 to 15), bodyweight gain at 6000 mg/kg bw/day was 19.6% lower than controls, which was not statistically significant.

**Clinical pathology**

On haematological examination, males showed a statistically significant decrease in haemoglobin at 2000 mg/kg bw/day, and a statistically significant decrease in haematocrit at 2000 and 4000 mg/kg bw/day. The changes were not considered of biological significance and were not considered treatment-related. No changes were noted in females.

On clinical chemistry examination, there was a non-statistically significant increase in cholesterol in males, with increases of up to 27% at 6000 mg /kg bw/day. This was not considered of biological significance, nor was a statistically significant increase in potassium levels in females at 6000 mg/kg bw/day.

**Gross pathology and organ weights**

In males, the absolute spleen weight was increased 32% at 2000 mg/kg bw/day ($p<0.05$). As no effects were seen at the higher dose, this is not considered to be treatment-related. No other changes in absolute organ weight, or in organ weight:bodyweight or organ weight:brain weight were seen. There were no abnormalities noted on macroscopic examination. No histopathological examinations were performed in this study.

**5. Nicholls IM (1997) NC-00723 Dietary Preference Feasibility Study. Lab Project No. 96/NST 030/0603. Sponsor Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-1132**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study: | 14 February 1996 to 21 March 1996 |
| Test chemical: | NC-00723, Lot no. 95MP028-3, Monsanto Company, purity: not specified |
| Test species: | Crl CD(SD)BR VAF/Plus rats (Charles River (UK) Ltd, Margate, Kent UK) |

**Study design**

Rats were housed individually in cages provided with two food bowls, each containing 50 g of food. In the pre-treatment phase, the same diet was used in both jars, and the position of the jars was not altered. Any preference for one jar was noted. During the first preference phase, treated diets were provided in one jars and basal diet in the other (controls received only basal diet). During this phase, the position of the jars in the cage was altered on a daily basis. The groups (5/sex/group) were offered diets containing 0, 50, 150, 500, 1500, 5000 or 15000 ppm Neotame in RM1(E)SQC diet (equivalent to 0, 3,10,30,100 or 1000 mg/kg bw/day in male rats, or 0, 50 or 15000 ppm Neotame in LAD 2 SQC diet (equivalent to 0,3 or 1000 mg/kg bw/day in male rats).

The two diets were used to determine whether there would be an effect on preferences. The first preference phase continued for 3 days. Following this, rats were given the treated diet in both jars for 7 days in a treatment phase. This was followed by a second preference phase, to determine if acclimatisation to the test diet altered the initial preferences determined. A positive control diet containing quinine sulphate at 5000 ppm was also used.

Rats were observed twice daily during the study for any reaction to treatment or signs of ill health. Food consumption was determined daily throughout the study, and was reported as food consumption from each jar in the cage. Bodyweight was measured 7 days before treatment, and on days 0, 3, 10 and 13, being the beginning and end of each study phase. At the end of the study, rats were killed and discarded without post-mortem examination.

**Results**

*Preference for basal diet versus treated diet*

No preference for either jar was identified during the pre-treatment phase. During preference phase 1, a preference for the basal diet was seen from 150 ppm, with consumption of the treated diet only 40% of the total intake in males and 45% in females. At the highest concentration, virtually none of the treated diet was eaten (1% of the total intake). Consumption of quinine sulphate in the first preference phase was similar (1.2% in males, 0% in females).

*Food consumption*

During the treatment phase, food consumption was decreased at 15000 ppm by more than 10% in comparison to controls, while consumption of quinine sulphate was decreased to 63% of controls in males and 44.9% in females.

During the second preference phase, a similar consumption pattern was observed, although the preference for basal diet at 150 ppm was more marked, with the treated diet making up 30% of the total intake in males and 29% of the total intake in females.

*Affects on bodyweight*

There were only limited effects on bodyweight in this study. On day 10 (end of the treatment phase), the bodyweight of males at 15000 ppm was 11% lower than controls (not statistically significant). No significant differences were seen in females. Bodyweight gain was decreased at 5000 and 15000 ppm in rats from days 3 to 10, with bodyweight gain in males being 63% and 44% of controls and in females 55% and 60% of controls respectively.

*Conclusion*

It was determined that the study design used was suitable to evaluated dietary preferences in rats. No differences were found between the two diets tested on food preference, with further studies using RM1(E) SQC FG. The positive control was not considered to be necessary in future studies.

**6. Nicholls IM (1997) NC-00723 Dietary Preference Study. Lab Project No. 96/NST 031/0752. Sponsor Monsanto Company, Skokie Illinois.**
**Sponsor Study No. PCR-1150**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study: | 27 March 1996 to 3 May 1996 |
| Test chemical: | NC-00723, Lot no. 95MP028-3: Monsanto Company, purity: not specified |
| Test species: | Crl CD(SD)BR VAF/Plus rats. source: Charles River (UK) Ltd, Margate, Kent UK |

**Study design**

Rats were acclimatised for 13 days in controlled conditions. Rats were housed individually in cages provided with two food bowls, each containing 50 g of food. In the pre-treatment phase (5 days), the same diet was used in both jars, and the position of the jars was not altered. Any preference for one jar was noted. During the first preference phase, treated diets were provided in one jars and basal diet in the other (controls received only basal diet). During this phase the position of the jars in the cage was altered on a daily basis. Rats (14/sex/group) were offered diets containing 0, 50, 150, 500, 1500, 5000 or 15000 ppm NC-00723. Samples of the diet were analysed for homogeneity and compound concentration. The first preference phase continued for 5 days. Following this, rats were given the treated diet in both jars for 5 days in a treatment phase. This was followed by a second, 5-day preference phase, where a choice between treated and untreated diet was offered, to determine if acclimatisation to the test diet altered the initial preferences determined.

Rats were observed twice daily during the study for any reaction to treatment or signs of ill health. Food consumption was determined daily throughout the study, and was reported as food consumption from each jar in the cage. Bodyweight was measured 5 days before treatment, and on days 0, 5, 10 and 15, being the beginning and end of each study phase. At the end of the study, rats were killed and discarded without post-mortem examination.

**Results**

**Food consumption and bodyweight**

Total food consumption was decreased during the treatment phase in males and females from 5000 ppm. Decreases in males of 12% at 5000 ppm and 25% at 15000 ppm were seen, while in females, decreases of 7% and 16% were seen at 5000 and 15000 ppm. In both preference phases, a preference for the basal diet was seen from 150 ppm, with intakes of the treated diet making up from 23% to 32% of the total food intake.

No significant differences were seen in bodyweight at any time during the study. Bodyweight gain during the treatment phase was statistically significantly decreased in males at 5000 ppm (42%, $p<0.01$) and 15000 ppm (95%, $p<0.001$). In females at 15000, bodyweight gain was decreased 45%, but this was not statistically significant.

**Conclusion**

The results demonstrated Neotame administered in the diet to rats at concentrations in the diet as low as 150ppm decreases the palatability of rats for the treated diets.

**7. Willoughby CR (1996) NC-00723 Tolerance Study in the Rabbit. Lab Project No. 95/NST022/0345. Sponsor The Monsanto Company, Deerfield Illinois. Sponsor Study No. PCR-0998**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk, England |
| Date of study: | 12 April 1994 to 14 December 1994 |
| Test chemical: | NC-00723, Lot no. 94MP081-4 |
| Test species: | New Zealand White Rabbits, Froxfield Farms (UK) Limited, Hampshire England |
| GLP: | US FDA, OECD, UK |

**Study design**

Neotame in 0.5% aqueous methylcellulose was administered by gavage to two female rabbits. Dosing commenced at 50 mg/kg bw/day for two days. Rabbits were given a 2-day recovery period between each dosing period. Dosing was increased to 100, 250, 500 and 1000 mg/kg bw/day, with dosing to cease if a reaction to treatment was detected.

Rabbits were examined frequently each day for any reaction to treatment, and were removed from the cage and examined on days 2, 6, 10, 14 and 18 (second day of dosing on each occasion). Bodyweight and food consumption were determined daily. At the end of the study, a macroscopic examination of both rabbits was performed.

**Results**

No abnormal clinical signs were detected during the study. As no controls were used in this study, no comparison for bodyweight was possible, however the bodyweight of each rabbit increased over the study period, with minor fluctuations occurring. At 500 and 1000 mg/kg bw/day, the food consumption in either one or both rabbits was decreased in comparison to the intake recorded on the previous day.

At 500 mg/kg bw/day, the food consumption for one rabbit was 80% of the mean of the previous two untreated days intake, while at 1000 mg/kg bw/day, intakes were 73 or 64% of the mean intake on the previous two untreated days. No macroscopic abnormalities were detected on post-mortem examination.

**Conclusion**

The results demonstrated Neotame administered by gavage to rabbits at 500 mg/kg bw/day decreases food consumption in rabbits.

**8. Thomford P (1994) Two Week Dietary Toxicity Study of NC-00723 in Dogs. Lab Project No. HWI 6211-292. Sponsor The NutraSweet Company, Deerfield Illinois. Sponsor Study No. PCR-0952**

| | |
|---|---|
| Laboratory: | Hazleton Wisconsin Inc, Madison Wisconsin |
| Date of study: | 12 April 1994 to 14 December 1994 |
| Test chemical: | NC-00723, Lot no. 94UP8-1-7: The NutraSweet Company, purity: not specified |
| Test species: | Beagle dogs: Hazleton Research Products Inc, Kalamazoo, Michigan. |
| Dose: | 0, 10, 30, 100 or 300 mg/kg bw/day |
| Number: | 4/sex/group |
| GLP: | US FDA |

**Study design**

Dogs were housed individually in controlled conditions, with free access to water. Food was provided daily. The concentration of NC-00723 in the diet was based on bodyweight and food consumption to yield the intended doses of 0, 10, 30, 100 or 300 mg/kg bw/day. Dietary samples were retained for later analysis. The homogeneity of the lowest and highest dose diet was analysed before treatment, the concentration of NC-00723 in non-refrigerated, refrigerated and frozen samples was analysed, and the concentration of material in the test diet was assayed.

Dogs were observed twice daily for mortality or moribundity. Food consumption was determined daily from 10 days before treatment until the end of the study. Bodyweight was determined before food being offered on days -1, 1, 2, 3, 7, 8, 15 and on the day of post-mortem examination. A physical examination was done before food being offered and 3 to 5 h after feeding on days 7 and 14, and included heart rate, respiratory rate, rectal temperature and general body condition. An ECG was performed on each dog during the acclimation period and on days 7 and 14 at 3 to 5 h after feeding. An ophthalmological examination was done during acclimation and on day 13.

Blood was taken for haematology and clinical chemistry pre-feeding on days -7, 7 and 14 and 3 to 4 h after feeding on days 1, 7 and 14. Urine was collected overnight on days -7 and 14. Clinical pathology examinations performed on samples are detailed in Appendix B. Blood was taken before feeding and 3, 6, 9 and 24 h after feeding on days 1 and 14 and the plasma collected and frozen. Analysis results were not reported here.

At the end of the study, a macroscopic examination, including the external surface of the body, all orifices, the cranial cavity, the external surface of the brain and spinal cord, the nasal cavity and paranasal sinuses, and the thoracic, abdominal and pelvic cavities and their viscera was performed. Organs were weighed and samples taken for histopathological examination, as detailed in Appendix C.

**Results**

**Dietary analysis and test article consumption**

The homogeneity of the diet was acceptable, with all samples having concentrations within 6%. The concentration of NC-00723 in the diet was also acceptable, with samples within 12% of theoretical levels.

**Mortality/Clinical observations**

No dogs died during the study. There was a slight increase in vomiting seen in males, with 2 males each at 30, 100 and 300 mg/kg bw/day vomiting during the study. No other treatment-related clinical signs were seen. There were no treatment-related changes seen in the heart rate, respiratory rate, body temperature, ECG or ophthalmological examination.

**Bodyweight and food consumption**

Food consumption was decreased in males at 300 mg/kg bw/day on day 2 of the study. In females at this dose, food consumption was decreased 26% on day 1 and 12 % on day 3. None of the changes in food consumption seen were statistically significant.

There were no statistically or biologically significant changes in bodyweight seen during the study. Bodyweight gain was decreased in males for days 1 to 8 by 15% and 23% at 100 and 300 mg/kg bw/day respectively, and for the whole study by 33, 22 and 44% at 30, 100 and 300 mg/kg bw/day respectively. In females, bodyweight gain was increased by 60% during days 1 to 8 at 10 mg/kg bw/day ($p<0.01$), but was decreased by 50% ($p<0.05$) at 300 mg/kg bw/day. Over the study, bodyweight gain in females at 300 mg/kg bw/day was decreased by 31%.

**Clinical pathology**

On haematological examination, females at 3 h after feeding on day 1 showed a decrease in haemoglobin at 300 mg/kg bw/day ($p<0.05$) and a decrease in haematocrit at 100 and 300 mg/kg bw/day ($p<0.05$). On day 7, haemoglobin and haematocrit were both decreased, however the decrease was not statistically significant. Given the lack of consistency of these findings, and that they were only seen in females, they are unlikely to be treatment related.

On clinical chemistry examination, males showed a statistically significant ($p<0.05$) decrease in AST levels at 100 and 300 mg/kg bw/day on day 7, and at 300 mg/kg bw/day on day 14. The relevance of this finding is unclear.

There were no treatment-related changes on urinalysis.

**Gross pathology and organ weights**

The absolute weight of adrenals was increased in females by 10 to 29%, without a clear dose-response relationship. The weight of the thyroid with parathyroid was increased in treated males by up to 45%, also without a clear dose-response relationship.

There were no abnormal treatment-related findings on macroscopic or microscopic examination, and thus the observed organ weight changes were not considered toxicologically significant.

**Conclusion**

There were no deaths seen, and the only abnormal clinical sign observed was an increase in vomiting in some males. Minor changes in food consumption were seen at 300 mg/kg bw/day early in the study in both sexes. Bodyweight gain was decreased in males from 30 mg/kg bw/day and in females at 300 mg/kg bw/day over the study period; the changes seen in males were not clearly dose related. The minor haematological changes seen in females at 300 mg/kg bw/day and clinical chemistry changes seen in males at 100 or 300 mg/kg bw/day were not considered to be of toxicological significance. No abnormalities were found on gross or histopathological examination.

**9. Lemen JK (1998) Four week Dietary Study of NC-00764/NC-00777/NC-00779 Mixture in Rats. Lab Project No. MSE-N Study Number 97127 MSL Number 15754. Sponsor The Monsanto Company, Skokie Illinois: Sponsor Study No. PCR-1186**

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|---|---|
| Laboratory: | Monsanto Safety Evaluation, St Louis MO. |
| Date of study: | 17 November 1997–19 December 1997 |
| GLP: | OECD, US FDA |
| Test chemicals: | NC-00764/NC-00777/NC-00779 Mixture |
| | NC-00764: lot no. 95MP115-25 |
| | NC-00777: lot no. KW-1307-272 |
| | NC-00779: lot no. DZG-1373-163 |
| Test species: | Crl CD(SD)BR VAF/Plus rats: Charles River Laboratories Inc, Portage, Michigan. |

**Study design**

Groups of rats (5/sex/group) were fed a mixture of NC-00764, NC-00777 and NC-00779 in the diet at levels to achieve doses of 0, 0.2/0.2/0.1, 0.6/0.6/0.3, 2.0/2.0/1.0 and 6.0/6.0/3.0 mg/kg bw/day for 28 days using 15 rats/sex/group. These degradation products were studied as they may be formed in products containing NC-00723 and stored under conditions of high humidity or temperature.

Animals were observed twice daily for mortality and morbidity, as well as abnormal clinical signs. A detailed clinical examination was performed weekly. Bodyweight was determined on days -8, -1, 1, 2, 4, 8, 12, 15, 22 and 28. Food consumption was determined for days -8 to -1, 1 to 4, 4 to 8, 8 to 12, 12 to 15, 15 to 18, 18 to 22, 22 to 25 and 25 to 28. A detailed ophthalmoscopic examination was done on animals before the start of the study and in week 4. At the end of the study, blood and urine samples were collected for clinical chemistry evaluation as detailed in Appendix B, and a complete gross necropsy with organ weights (detailed in Appendix C) was performed.

Tissues (as specified in Appendix C) were examined histopathologically. The concentration of test article and homogeneity in the diet was determined in week 1 and 4.

**Results**

**Diet homogeneity and compound concentration**

The concentration of the test compounds in the diet was satisfactory, being with 16% of the intended concentration at all times. The homogeneity of the diet was satisfactory. The average test article consumption was 0.19/0.19/0.10, 0.57/0.57/0.3, 1.87/1.87/0.91 and 5.63/5.63/2.80 mg/kg bw/day in males and 0.2/0.2/0.1, 0.55/0.55/0.27, 1.87/1.87/0.94 and 5.70/5.70/2.87 mg/kg bw/day in females.

**Mortality/Clinical signs**

No rats died during the study. There were no treatment-related clinical signs observed during the study. No abnormal findings were detected on ophthalmoscopic examination.

**Bodyweight and food consumption**

There were no treatment-related effects on bodyweight, bodyweight gain or food consumption. Bodyweight gain was very slightly increased at higher doses (not statistically or biologically significant.

**Clinical pathology**

There were no treatment-related abnormalities on haematological, clinical chemistry or urinalysis examinations.

**Gross pathology and organ weights**

There were no treatment-related effects on organ weight (either absolute or relative). No treatment-related abnormalities were seen on gross or histopathological examination.

**Conclusions**

In this study, no treatment-related abnormalities were ween at the highest dose tested of the mixture of NC-00764/NC-00777/NC-00779 at doses of 5.63/5.62/2.80 mg/kg bw/day in males or 5.70, 4.70, 2.87 mg/kg bw/day in females.

**SUBCHRONIC STUDIES**

**1. Thomford PJ (1995) Thirteen Week Dietary Range-Finding Study of NC-00723 in Mice Lab Project No. HWI 6211-294. Sponsor The NutraSweet Company, Deerfield Illinois. Sponsor Study No. PCR-0989**

Laboratory: Hazleton Wisconsin Inc, Madison Wisconsin
Date of study: 5 August 1994 to 13 November 1994
Test chemical: NC-00723, Lot no. 94MP053-13
Test species: Crl CD-1 (ICR)BR VAF/Plus mice: Charles River Laboratories, Portage Michigan.

**Study design**

Mice were acclimatised for 15 days before treatment. They were individually housed in controlled conditions, with food and water freely available, with the exception of food withdrawal before routine blood sampling. NC-00723 was administered in the diet at concentrations to give doses of 0, 100, 1000, 4000 or 8000 mg/kg bw/day for 13 weeks, using 20/sex/group, with an additional 20/sex/group at each treatment level used for a satellite study. Dietary samples were taken in weeks 1, 3, 5, 9 and 13 for analysis of homogeneity and dietary concentration.

Mice were observed twice daily throughout the treatment period for mortality, moribundity and abnormal clinical signs or poor health. On a weekly basis, animals were removed from their cage, palpated and examined. Particular attention was paid to the condition of the skin and fur, the ears, eyes, mucous membranes, mouth and teeth, body and limb conformation and the genitalia. The respiratory, circulatory and CNS systems and general behaviour were also evaluated. Food consumption was measured during the week before dosing, daily for days 1, 2 and 3, then for days 4 to 8, and weekly thereafter. Bodyweight was determined on arrival, 8, 3, 2 and 1 days before treatment, on days 1, 3 and 8, then weekly during the study.

Blood was obtained from 10/sex/group at week 14 for haematology and from 10/sex/group for clinical chemistry examinations. Haematology parameters were those listed in Appendix B, while a limited clinical chemistry examination included glucose, urea nitrogen, albumin, globulin, AST, ALT, alkaline phosphatase, total protein and total bilirubin. No urinalysis was done. Blood was also taken on days 2, 4, 31 and 95 from satellite animals, the plasma extracted, frozen, and analysed for plasma levels.

At the end of the study period, all animals were killed for postmortem examination of gross pathology, organ weights and histopathology. The macroscopic examination included close examination of all external features and orifices, and cranial, thoracic, abdominal and pelvic cavities and their viscera, the brain and spinal cord, and the nasal cavity and paranasal sinuses. The following organs were weighed and the organ:bodyweight and organ:brain weight ratios calculated: adrenals brain, heart, lungs, ovaries, pituitaries, kidneys, liver with gallbladder, spleen, testes, thyroids and parathyroids. Samples of all tissues were fixed (see Appendix C); however, the tissues from all control and high dose (8,000mg/kg bw/day) animals were examined microscopically. At all other doses the adrenals, brain (medullary, cerebellar and cortical sections), heart, kidneys, lesions, liver, lungs, pancreas, testes and thyroid with parathyroid were examined. Bone marrow smears were prepared, but were held for possible future examination.

**Results**

**Homogeneity and stability of test material**

The homogeneity of the diet was acceptable, and all samples analysed were within 10% of the proposed concentration. The achieved average daily intake over the course of the study was 100.2, 1003, 3997 and 8016 mg/kg bw/day in males and 101.7, 1009, 4327 and 8109 mg/kg bw/day in females.

**Mortality/clinical signs**

No treatment related deaths were seen during the study. There was a slight increase in the incidence of yellow stained hair coat in males. The number of animals with opaque eyes (either one or both) was also slightly increased at higher doses (males: 2, 2, 2, 0, 3; females: 1, 2, 6, 5, 5 at 0, 100, 1000, 4000 or 8000 mg/kg bw/day respectively). Given the relatively low incidence of these findings and the lack of clear dose relationship, these are considered isolated findings of no clinical significance.

**Bodyweight and food consumption**

Food consumption was decreased in males on day 1 from 1000 mg/kg bw/day, with food consumption unable to be assessed at 4000 mg/kg bw/day due to excessive spillage. At 8000 mg/kg bw/day, mean food consumption was decreased (not significant) by 17% in males and 19% in females on day 1, with no notable decrease in intake for the rest of the study.

There were no significant differences in bodyweights at any dose in either male or female mice. Bodyweight gain was decreased in males from 1000 mg/kg bw/day over the first 3 days (up to 140%, $p<0.001$), and during the study at 8000 mg/kg bw/day ($p<0.01$), with decreases of 16 to 30% in comparison to controls seen. At 4000 mg/kg bw/day in males, decreases of 10 to 13% ($p<0.05$) were seen intermittently throughout the study. In females, decreased bodyweight gain was only seen during days 1 to 3, with decreases seen from 1000 mg/kg bw/day.

**Clinical pathology**

No treatment-related changes in haematological parameters were seen in males. In females, a slight, statistically significant decrease in MCV was seen from 4000 mg/kg bw/day; however, this finding was not considered to be toxicologically significant as the values were within historical control ranges and no other parameters were affected. No treatment-related changes in clinical chemistry were seen during the study.

**Analyte analysis**

Although NC-00723 levels were initially determined, the levels of NC-00751 in the plasma interfered with the accuracy of the readings. There was also evidence that NC-00723 was unstable in the frozen samples, and thus the data was not considered reliable due to this technical difficulty. The mean levels of NC-00751 over the course of the study in males were 0.52, 5.1, 15 and 34 μg/mL and in females were 0.57, 6.0, 17 and 29 μg/mL. There was therefore no consistent difference in plasma levels between sexes. When plasma levels were adjusted for dose, there was no clear dose relationship, and a large amount of variability was seen. There was no evidence of accumulation throughout the study, as plasma levels did not increase at observations later in the study.

**Gross pathology and organ weights**

There were slight increases in the absolute and relative liver weights in high dose mice. The absolute liver weight at 8000 mg/kg bw/day was increased 9% in males and 12% in females ($p<0.05$).

The liver weight relative to bodyweight was increased 15 or 16% ($p<0.001$) at 8000 mg/kg bw/day and at 4000 mg/kg bw/day was increased 7% ($p<0.05$) in males and 9% ($p<0.01$) in females. In females the liver weight relative to brain weight was increased 11% at 4000 mg/kg bw/day ($p<0.05$) and 14% ($p<0.01$) at 8000 mg/kg bw/day.

However, these were not accompanied by other histopathological findings. There were no other notable treatment-related changes in organ weights.

On macroscopic examination, one high dose male had a dark focal area in the stomach. No other significant abnormalities were notes.

**Histopathology**

On histopathological examination, there was a slight increase in chronic inflammation in the kidney (4, 3, 4, 6, 8) at 0, 100, 1000, 4000 and 8000 mg/kg bw/day respectively in both sexes and the pancreas (0, 0, 0, 0, 1) in males. However, there was no dose-response.

**Conclusions**

No mortality or significant treatment-related clinical signs were seen during the study. Food consumption was decreased in both sexes at 8000 mg/kg bw/day, and in males at 1000 mg/kg bw/day. The food consumption in males at 4000 mg/kg bw/day could not be assessed due to excessive spillage; the decrease at 1000 mg/kg bw/day is therefore considered treatment related (specifically related to reduced diet palatability). Bodyweight gain was also decreased from 1000 mg/kg bw/day in both sexes during the first few days of the study. There were no significant treatment-related changes in clinical chemistry or haematology. Treatment-related increases in liver weights were seen at 8000 mg/kg bw/day and increases in relative liver weight were seen at 400 mg/kg bw/day. These changes were not associated with any histopathological findings or enzyme changes. The NOEL based on liver weight changes, was 1000 mg/kg bw/day.

**2. Mitchell DJ (1995) NC-00723: Toxicity Study by dietary administration to CD rats for 13 weeks followed by a 4 week reversibility study Lab Project No. 95/NST 017/0211. Sponsor The NutraSweet Company, Deerfield Illinois. Sponsor Study No.PCR-0988**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study: | 17 August 1994 to 28 December 1994 |
| GLP: | OECD, UK, Japan, USFDA |
| Test chemical: | NC-00723, Lot no. 94MP081-3 |
| Test species: | Crl CD(SD)BR VAF/Plus rats: Charles River (UK) Ltd, Margate, Kent UK |

**Study design**

Neotame was administered in the diet at concentrations to give doses of 0, 100, 300, 1000 or 3000 mg/kg bw/day for 13 weeks, using 25/sex for 0, 1000 and 3000 mg/kg bw/day and 20/sex for 100 and 300 mg/kg bw/day. 5 rats/sex at 0, 1000 and 3000 were selected during week 12 of dosing to continue in a 4-week recovery phase. In a satellite study, 24/sex/group were given NC-00723 at 100, 300, 1000 or 3000 mg/kg bw/day; no control group was used.

Neotame was incorporated into the diet weekly to provide the required concentration to achieve mg/kg bw/day. Samples were taken of each batch for analysis of homogeneity. Confirmation that the proposed dietary concentration had been achieved was done in weeks 1, 3, 5, 9 and 13.

The rats were observed twice daily throughout the treatment period for mortality. On a weekly basis, animals were removed from their cage, palpated and examined. Particular attention was paid to behaviour, body temperature, buccal cavity, build, coat, secretions, excreta, eyelids, muscle reactions, posture, reflexes, respiratory system, skin and teeth. Close observations for signs relating to neurotoxicity (including convulsions, tremors, gait abnormalities and behaviour changes) were also made. Food consumption was measured daily during the week before dosing and the first week of dosing, then weekly. Bodyweight was determined 7 days before treatment, on days 0, 1, 2 and 7, then weekly during the treatment and reversibility stages. Water consumption was measured during weeks 1, 5 and 11 of treatment, then in week 3 of reversibility study. Ophthalmological examination was carried out pretest and at week 12 of treatment and at the end of the reversibility study. Particular attention was paid to the palpebrae and adjacent structures, conjunctiva, cornea and sclera, anterior chamber and iris, lens and vitreous and the ocular fundus.

A bone marrow sample was obtained from the femur at postmortem examination. Smears were made and either stained or left unstained. The cellularity and composition of the marrow was assessed.

Blood was obtained from 10 rats/sex/group at week 6, and from all surviving rats at week 13 for haematology and clinical chemistry examinations (see Appendix B). Urine was collected during week 12, and urinalysis done (see Appendix B). Blood was also taken during weeks 1, 5, and 12 for toxicokinetic sampling. At the end of the study period, all animals were killed for postmortem examination of gross pathology, organ weights and histopathology (see Appendix C). The macroscopic examination included close examination of all external features and orifices, neck and associated tissues, and cranial, thoracic, abdominal and pelvic cavities and their viscera. The external and cut surfaces of all organs were examined, with abnormalities noted and preserved. Postmortem examinations were also carried out on rats that died during the study. The kidneys from females in the reversibility study were examined microscopically; however, no other histopathological examinations of these animals were performed. Samples of kidney and liver from 5 rats/sex at 0, 1000 and 3000 mg/kg bw/day were prepared for possible electron microscopy examination; although they were not examined.

**Results**

**Homogeneity and stability of test material**

The homogeneity of the diet was acceptable, and all samples analysed were within 10% of the proposed concentration.

**Mortality/clinical signs**

No treatment related deaths were seen during the study. At 3000 mg/kg bw/day, a number of female rats were noted to be thin. There was a slight increase in brown staining of the nose and muzzle at 3000 mg/kg bw/day. Other findings, considered incidental, included hair loss, skin reddening, a hunched posture and bloody nasal discharge.

**Bodyweight and food consumption**

Food consumption was decreased in males on day 1 at 1000 mg/kg bw/day (33%, $p<0.001$) and during the first week at 3000 mg/kg bw/day (decreases up to 70%, $p<0.001$, with an average decrease for the week of 11.2%, $p<0.01$). In males at 3000 mg/kg bw/day, food consumption was also decreased from week 7 until the end of the study, with decreases of 8 to 12 %, $p<0.01$ or $p<0.001$. In females, food consumption was decreased on the first day in all treatment groups (from 20% at 100 mg/kg bw/day, $p<0.05$ to 80% at 3000 mg/kg bw/day, $p<0.001$). At 3000 mg/kg bw/day on day 2, food consumption was decreased 26% ($p<0.001$). Decreased food consumption was also seen in week 6 in females from 300 mg/kg bw/day, but was not otherwise seen during the study. No differences were seen between groups during the reversibility study. Water consumption was decreased on day 1 in treated groups, probably related to the decreased food intake. No other differences were seen during the study.

Bodyweight was lower than controls in males at 3000 mg/kg bw/day throughout the study, with bodyweights from 10 to 14% lower than controls ($p<0.001$). In females, bodyweight at 3000 mg/kg bw/day was lower than controls until day 14, although the difference was less than 10% on days 7 and 14. The overall bodyweight gain in males at 3000 mg/kg bw/day was 21% lower than controls for the 13 week study, while in females at 3000 mg/kg bw/day it was 8% lower than controls. During the reversibility phase, the differences between control and 3000 mg/kg bw/day males decreased, but was still 11% after 4 weeks of control diet.

**Ophthalmoscopic examinations**

No treatment-related ophthalmological changes were seen.

**Clinical pathology**

In males, a decrease in MCV at 3000 mg/kg bw/day (53 versus 56 in controls, $p<0.01$) after week 5 was seen. After week 12, the MCV values in males were 54, 52, 53, 52 and 52 for 0, 100, 300, 1000 and 3000 mg/kg bw/day. The values at 100, 1000 and 3000 mg/kg bw/day were statistically significantly lower than controls ($p<0.05$). No changes were seen in females at any dose, and no differences between groups were seen at the end of the reversibility phase. The changes to MCV in males are not considered to be of biological significance, as they are relatively small, and do not demonstrate a dose-response.

On blood chemistry examination, both males and females showed a decrease in total cholesterol.

The decreases are presented in the table below.

**Cholesterol levels in plasma**

| Dose (mg/kg bw/day) | 0 | 100 | 300 | 1000 | 3000 |
|---|---|---|---|---|---|
| Week 6 males | 88 | 83 | 89 | 77 | 70* |
| Week 6 females | 92 | 83 | 86 | 76** | 69*** |
| Week 13 males | 78 | 74 | 69 | 63** | 61*** |
| Week 13 females | 82 | 76 | 77 | 70** | 65*** |

*$p<0.05$, ** $p<0.01$, *** $p<0.001$

The decreases were considered to be treatment related, given the persistence throughout the study and may be related to the decreased food consumption and bodyweight gains. Slight decreases in globulin levels and a consequent increase in the albumin/globulin ratio were seen throughout the study at 3000 mg/kg bw/day in males and at week 13 in females. Alkaline phosphatase levels were increased in both males and females at week 13 at 3000 mg/kg bw/day and at 1000 mg/kg bw/day in males. After the reversibility phase, the alkaline phosphatase had returned to normal in males, but was still elevated in females, although not statistically significant. These findings were not accompanied by histopathological changes in the liver, bone or GI tract.

No treatment-related abnormalities were seen on urinalysis.

**Gross pathology and organ weights**

In males at 3000 mg/kg bw/day, the absolute and relative weight of a number of organs were decreased.

The changes in organ weight at this dose are presented in the table below.

**Decreases in organ weight (% absolute and relative to brain or bodyweight) in males at 3000 mg/kg bw/day.**

| Organ | Absolute | Relative to bodyweight | Relative to brain weight |
|---|---|---|---|
| Adrenals | 14.6** | 1.9 | 13.6** |
| Heart | 13** | -1 | 11.9** |
| Kidney | 12.7** | -0.5 | 11.5** |
| Liver | 15.9** | 2.2 | 14.4* |
| Prostate | 17.2*** | 20 | 30.1*** |
| Spleen | 27.9*** | 16.8** | 26.7*** |
| Thymus | 23.7* | 10.9 | 22* |

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

No statistically significant changes in organ weights were seen in females at any dose, or in males at doses of 1000 mg/kg bw/day or less. No abnormalities were noted on macroscopic post-mortem examination. However, following the 4-week reversibility period all the organ weights of treated animals were not statistically significant to those of controls with the exception of the prostate.

**Histopathology**

The only findings on histopathological examination were an increase in cortico-medullary mineralisation in the kidneys of females, at higher doses with an incidence of 4/20, 2/20, 2/20, 10/20 and 13/20 (significant at $p<0.01$) at 0, 100, 300, 1000 or 3000 mg/kg bw/day respectively. These findings are related to sexual maturity and are not significant considering that effects were reversible and no other renal parameters were affected.

A low incidence of focal necrosis or leucocyte infiltration in the liver was detected; however, as this occurred only in one or two rats it was not considered to be treatment related.

There were no histopathological findings correlating with the decreased organ weights observed in males at 3000 mg/kg bw/day, and the biological significance of these findings is therefore unclear.

**Conclusions**

The observed intermittent decreases in food consumption at low doses are considered to be related to palatability of the diet. The only findings of significance throughout the study were decreases in bodyweight and bodyweight gain at the high dose, decreases in plasma cholesterol from 1000 mg/kg bw/day, and decreases in a range of organ weights at 3000 mg/kg bw/day. The NOEL, based on the decrease in plasma cholesterol levels was 300 mg/kg bw/day.

**3. Thomford PJ (1995) 13-Week Dietary Toxicity Study of NC-00723 in Dogs followed by 4-week Reversibility Period. Lab Project No. HWI 6211-295. Sponsor The NutraSweet Company, Deerfield Illinois. Sponsor Study No. PCR-0990**

Laboratory: Hazleton Wisconsin, Madison, Wisconsin.
Date of study: 24 August 1994 to 21 December 1994
GLP: USFDA
Test chemical: NC-00723, Lot no. 94MP081-4
Test species: Beagle dogs: HRP Inc, Kalamazoo Michigan.

**Study design**

Dogs were acclimatised for 30 days before treatment, and a thorough examination was performed during this time. They were housed individually in controlled conditions. Water was freely available, and food was offered for a 3-h period once daily. The concentration of test material in the diet was adjusted to give the required dose based on food consumption and bodyweight. Dietary samples were retained, and the homogeneity and concentration of test material in the diet was assessed. The intended doses were 0, 60, 200, 600 or 2000 mg/kg bw/day, using 6/sex/group at 0, 600 and 2000 mg/kg bw/day and 4/sex/group at 60 and 200 mg/kg bw/day. At 0, 600 and 2000 mg/kg bw/day, 2/sex/group were retained for a four-week reversibility study immediately after the cessation of dosing. At week 3 of the study, the highest dose was decreased to 1200 mg/kg bw/day due to significant decreases in food consumption and bodyweight loss at this dose. The top dose is therefore referred to as 1200/2000 mg/kg bw/day throughout the study.

All dogs were observed twice daily for mortality and moribundity. Examinations were done at the start and end of the feeding period, and included (but were not limited to) the condition of the skin and fur, the ears, eyes, mucous membranes, mouth and teeth, body and limb conformation and genitalia. Food consumption was measured daily for 2 weeks during the pre-treatment period and for weeks 1 to 4 of treatment. Subsequently food consumption was measured weekly. Bodyweight was determined weekly pre-feeding throughout the study. A detailed examination of all dogs was done 3 to 5 h after feeding in weeks 2, 6, 13 and at the end of the reversibility phase. The physical examination included the heart rate, respiratory rate, rectal temperature, respiratory and circulatory system and general behaviour. Neurological assessments were also performed at these times, including the pupillary, patellar, righting, hopping and blinking reflexes. An ECG was also done at these times.

An ophthalmological examination was done pre-treatment and in weeks 6, 12 and 17. Clinical pathology tests were done pre-treatment and in weeks 2, 6, 13 and 18. Blood was collected pre-feeding and urine was collected overnight. The haematological, clinical chemistry and urinalysis parameters examined are detailed in Appendix B. Blood was also collected before feeding and 1.5, 3, 6, 12 and 24 h after feeding on day 1 and during weeks 6 and 12, the plasma separated and frozen and NC-00723 and NC-00751 levels were analysed.

After 13 weeks, 4 dogs/sex/group were killed and examined. The remaining dogs were examined after a 4-week recovery phase. A macroscopic examination was done, including the external surface, all orifices, cranial cavity, external surface of the brain, excised portion of the spinal cord, the nasal cavity and paranasal sinuses, and the thoracic, abdominal and pelvic cavities and viscera. Organs (detailed in Appendix C) were weighed and the organ to bodyweight and organ to brain weight ratios calculated. Tissues were preserved (detailed in Appendix C) and examined microscopically. Bone marrow smears were prepared for examination if required. Samples of the liver, kidney and spleen were fixed for electron microscopy examination if required. The serum and gut associated lymphoid tissue was collected and stored for potential immunotoxicity studies.

**Results**

**Homogeneity and stability of test material**

The homogeneity of the diet was acceptable, and all samples analysed were within 5 to 6% of the proposed concentration, with the exception of the 60 mg/kg bw/day diet in week 3, which could not be verified analytically. The achieved test article intake for males over the course of the study was 59.7, 195.4, 596.7 and 1152.8 mg/kg bw/day and for females was 60.4, 203.8, 602.3 and 1127.3 mg/kg bw/day at 60, 200, 600 and 1200 mg/kg bw/day.

**Mortality/clinical signs**

There were no deaths during the study. All dogs at the two highest doses of 600 and 1200/2000 mg/kg bw/day had white and grey discoloured faeces. Three high dose males were noted to be thin, while for females 0, 0, 1, 1 and 4 dogs at 0, 60, 200, 600 and 1200/2000 mg/kg bw/day respectively were thin. These signs were not observed after the reversibility period.

**Bodyweight and food consumption**

Food consumption was statistically significantly decreased at 1200/2000 mg/kg bw/day in both sexes for weeks 1 and 2, with food intake decreased more than 10% in comparison to controls until week 10. At 600 mg/kg bw/day, females had decreased food consumption during the first 2 weeks although only week 1 was significant ($p<0.05$, 20% and 11% for week 1 and 2 respectively). No other notable decreases in food consumption were seen throughout the study.

Bodyweight was statistically significantly decreased ($p<0.05$) in males on 1200/2000 mg/kg bw/day in week two (15% in comparison to controls) and from week 5 until the end of the study, with decreases of up to 19% in comparison to control. During the reversibility phase, the males had returned to the same bodyweight as control dogs by the end of the second week.

In females at 1200/2000 mg/kg bw/day, bodyweight was statistically significantly ($p<0.05$) decreased by 17% in week 3. For the rest of the study, the decreases were between 15 and 18% in comparison to controls. No statistically significant decreases, or decreases greater than 10% were seen in any other groups. The cumulative bodyweight gain was statistically significantly lower than controls at 1200/2000 mg/kg bw/day, with decreased weight gains in males of 57% in comparison to controls and in females of 75% in comparison to controls. Statistically significant decreases were also seen in females at 600 mg/kg bw/day, with bodyweight gains of less than half those seen in controls up to week 5. The cumulative bodyweight gain in females at 600 mg/kg bw/day was decreased 24% in comparison to controls throughout the study, and in males was decreased more than 15% in comparison to controls throughout the study.

**Physical parameters**

In males at 1200 mg/kg bw/day, mean heart rates was decreased at week 2 (72. 64. 74. 73, 51 beats per half minute, $p<0.001$); however, this finding was isolated to males and was not observed at later intervals. No changes were seen in females at any stage, or in males at any other times. There were no treatment-related changes seen in heart or respiratory rates, body temperatures, neurological effects, ECG or ophthalmological examinations.

**Clinical pathology**

On haematological examination, at week 2, activated partial thromboplastin time was decreased ($p\leq0.01$) in males at 600 mg/kg bw/day and increased ($p\leq0.05$) in females at 1200/2000 mg/kg bw/day. At week 13, the erythrocyte count was decreased in both sexes at 1200/2000 mg/kg bw/day ($p<0.01$). Haemoglobin and haematocrit were also statistically significantly decreased, and in females MCV and MCH were increased. The changes seen at week 13 at the highest dose were considered to be related to treatment.

On clinical chemistry examination in week 2, males had a statistically significant increase in triglyceride levels ($p<0.01$) at 1200/2000 mg/kg bw/day. Cholesterol and alkaline phosphatase levels were increased in a dose-related manner, and glucose levels were decreased in a dose-related manner. In females in week 2, AST and ALT levels were statistically significantly decreased at 1200/2000 mg/kg bw/day, and ALT levels were also statistically significantly decreased at 600 mg/kg bw/day. Glucose levels were slightly decreased, and cholesterol and triglyceride levels were slightly increased. In week 6, glucose levels were statistically significantly decreased and triglyceride and alkaline phosphatase levels were statistically significantly increased in males at 1200/2000 mg/kg bw/day ($p<0.05$). No dose-related changes in serum enzymes were seen in females in week 6. In week 13, alkaline phosphatase levels were statistically significantly increased in males at 1200/2000 mg/kg bw/day, and in females from 200 mg/kg bw/day. ALT levels were statistically significantly decreased in females from 600 mg/kg bw/day. The changes in AST, ALT and alkaline phosphatase levels were considered treatment-related. The changes in glucose levels were probably related to decreased food consumption.

No changes were seen in urinalysis parameters at any time during the study.

**Gross pathology and organ weights**

The absolute weight of the liver was increased in males in week 12 at 1200/2000 mg/kg bw/day (18%). In females, liver weight was increased at 600 mg/kg bw/day from 12 to 23%. The relative liver weight (to bodyweight) was statistically significantly increased at 1200/2000 mg/kg bw/day in males (49% more than controls) and in females from 600 mg/kg bw/day (27 to 35%). Absolute spleen weight was decreased in all male treatment groups (15% to 43%), although none of the decreases were statistically significant, and spleen weights were increased in females at 200 and 1200/2000 mg/kg bw/day only (not statistically significant). There were no significant abnormalities found on macroscopic examination.

**Histopathology**

Mineralisation was seen in the kidneys at all doses, with a slight increase in vacuolisation in the kidney (males 0, 0, 2, 1,2; females 1, 2, 4, 2, 3) at 0, 60, 200, 600 or 1200/2000 mg/kg bw/day, respectively. In the liver, lymphohistiocytic infiltrate was seen at all doses, with increased hepatocellular glycogen from 600 mg/kg bw/day (males 0, 0, 2, 1, 2; females 1, 2, 4, 2, 3, although no dose response). There was a high incidence of immature prostates, with 2, 3, 4, 4 and 4 at 0, 60, 200, 600 and 1200/2000 mg/kg bw/day respectively. Splenic congestion, thymic haemorrhage and uterine hypertrophy were seen as incidental findings.

**Conclusions**

Food consumption was decreased from 600 mg/kg bw/day in females, with decreases in cumulative bodyweight gain also seen. Increases in alkaline phosphatase levels were seen from 200 mg/kg bw/day in females (although this was a marginal increase) and at 1200/2000 mg/kg bw/day in males. Absolute liver weight was increased in high dose males and in females from 600 mg/kg bw/day. Microscopically, lymphohistiocytic infiltration was seen in the liver cells from 600 mg/kg bw/day, although this lacked a dose-response. However, there was no histopathological evidence of liver toxicity, cholestasis or lesions in the bone or GI tract which would account for the elevations in liver enzymes.

The NOEL, based on changes on clinical pathology parameters and on liver weight changes, was 200 mg/kg bw/day.

**CHRONIC TOXICITY/ONCOGENICITY STUDIES**

**1. Thomford PJ (1997) 104-Week Dietary Carcinogenicity Study with NC-00723 in CD-1 Mice. Lab Project No. Covance 6211-303. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-1014**

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|---|---|
| Laboratory: | Covance Laboratories Inc, Madison Wisconsin |
| Date of study: | 21 April 1995 to 12 December 1997 |
| GLP: | US FDA |
| Test chemical: | NC-00723, Lot no. 95MP028-3 |
| Test species: | Crl CD1(ICR) VAF/Plus mice; source Charles River Laboratories, Portage Michigan. |

**Study design**

Mice were acclimatised for 17 days before commencement of treatment. They were housed individually in controlled conditions, with free access to food and water. Diets were prepared to give the required doses, and were analysed for achieved concentration in weeks 1, 13, 26, 39, 52, 65, 78, 91 and 104. Homogeneity was established pre-test and week 23. The stability of the test compound in the diet had previously been established. In the main toxicity study, mice received 0, 50, 400, 2000 or 4000 mg/kg bw/day, using 140/sex for control and 70/sex/dose for the treated mice. Satellite groups of 35/sex/group were also established at 50, 400, 2000 or 4000 mg/kg bw/day. Doses were selected based on the findings in the 2-week and 13-week toxicity study.

Mice were observed twice daily for mortality, moribundity, signs of poor health and abnormal behaviour. Mice were removed from their cages and examined at least once monthly for the first 52 weeks, and at least fortnightly for the rest of the study. The examination included, but was not limited to, the condition of the skin and fur, the ears, eyes, mucous membranes, mouth and teeth, body and limb conformation, genitalia, evaluation of respiratory, circulatory and CNS systems and the general behaviour of the animals. Particular attention was paid to mass development, including the date of onset, location, size, appearance and progression of grossly visible or palpable masses.

Food consumption was determined for one week during acclimation, the on days 1 to 4 and 4 to 8, weekly until week 26, and then at least every 2 weeks until the end of the study. All mice were weighed on arrival, 10 and 3 days before treatment, on days 1, 4 and 8, then weekly until week 26 and at least fortnightly until the end of the study. Any mice killed at an unscheduled time had a blood film taken, which was stained and held for examination if required. Blood was taken from all mice at the terminal sacrifice, and an erythrocyte count and total and differential white cell count done. Blood was taken from the satellite animals between 6 and 7:30 am in weeks 1, 13, 26 and 52, and between 7:30 and 9 am in week 14. The plasma was harvested and stored frozen until analysis. Satellite animals were discarded without further examination.

All animals that died during the study, and all mice at the end of the study had a macroscopic post-mortem examination. This included an examination of the external surface of the body, all orifices, the cranial cavity, the brain and spinal cord and the thoracic, abdominal and pelvic cavities and their viscera. Organs were weighed and tissues examined microscopically as detailed in Appendix C.

**Results**

**Homogeneity and stability of test articles**

The homogeneity and concentration of the prepared diets was suitable at all times. Achieved doses over the course of the study were 0, 50, 400, 2000 and 4004 mg/kg bw/day for males and 0, 50, 402, 2008 and 4016 mg/kg bw/day for females.

**Mortality and clinical signs**

There were no treatment-related increases in mortality during the study. In males, incidences of masses (including small and large movable and stationary masses) was 57, 31, 50, 33 and 35 at 0, 50, 400, 2000 and 4000 mg/kg bw/day, respectively, and thus no dose-related increase in mass presence was observed.

In females, the number of animals with decreased activity was slightly increased with treatment (34, 15, 15, 18 and 20) at 0, 50, 400, 2000 and 4000 mg/kg bw/day. There was no increase in the number of masses detected, with 23, 9, 6, 10 and 9 found at 0, 50, 400, 2000 and 4000 mg/kg bw/day, respectively.

**Bodyweight and food consumption**

In males, food consumption was statistically significantly decreased from 400 mg/kg bw/day intermittently throughout the study, with decreases of up to 9% ($p<0.01$). In treated females, sporadic statistically significant decreases in food consumption were seen in all treatment groups, with decreases of up to 7%. No decreases in food consumption of more than 10% were seen at any time, and the decreases, although statistically significant, were not considered of biological significance, being small and probably related to decreased palatability.

Bodyweight in males was consistently decreased from 400 mg/kg bw/day, with decreases of up to 7% ($p<0.001$). Decreases of a similar magnitude were seen at higher doses. In females, decreases of up to 10% were seen at 2000 mg/kg bw/day, with decreases of up to 8% ($p\leq0.001$) seen at 400 mg/kg bw/day. Cumulative bodyweight gain was decreased in males from 400 mg/kg bw/day, with decreases of 18-20% at 400 mg/kg bw/day for the period of weeks 1 to 77. In females, decreases in cumulative bodyweight gain of 15% were seen at 50 mg/kg bw/day for weeks 1 to 77, with decreases of 20% seen at 400 mg/kg bw/day for the same period.

**Clinical pathology**

No differences between groups were seen in relation to haematological parameters. No clinical chemistry or urinalysis examination was conducted.

**Organ weight and gross pathology**

There were no treatment-related effects on absolute or relative organ weight. On gross post-mortem examination, changes were found in all groups, consistent with findings expected in aging mice. No treatment-related increases in any abnormalities were identifies.

**Histopathology**

In males at high dose (4000 mg/kg bw/day) the incidence of hepatocellular adenomas was slightly increased although not statistically significant (13/140, 13/70, 11/70. 10/70, 14/70) at doses of 0, 50, 400, 2000 and 4000 mg/kg bw/day, respectively.

However, no increase in the incidence of carcinomas was seen and the incidence of adenomas was within the historical control ranges observed in this strain of mice. There was no increase in the incidence of pre-neoplastic lesions at 2000 or 4000 mg/kg bw/day. There were no treatment-related non-neoplastic histopathological findings.

In the absence of statistical significance, a dose-response relationship or any increase in the incidence of carcinomas or pre-neoplastic lesions the incidence of adenomas in male rats is not considered treatment related.

**Conclusions**

The observed decreases in bodyweight and bodyweight gain are considered to be related to the reduced palatability of the diet containing high levels of Neotame. No treatment-related changes were seen on clinical pathology or post-mortem examination.

In conclusion, this long-term study in mice showed no evidence of carcinogenic potential in mice up to a period of 104 weeks. The NOEL, based on the absence of adverse effects at the highest dose tested, was 4000 mg/kg bw/day.

**2. Mitchell DJ (1997) NC-00723: 52-WeekToxicity Study by dietary administration to CD rats with exposure *In Utero* followed by a 4 week reversibility study Lab Project No. NST/029/972374. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-1011**

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|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study: | 19 April 1996 to 23 May 1997 |
| GLP: | OECD, UK, EC |
| Test chemical: | NC-00723, Lot no. 96NK002-6 |
| Test species: | Crl CBR VAF/Plus Sprague Dawley rats, selected from *in utero* phase of NST/029/972354 at 21 days) |

**Study design**

Rats were selected from the *in utero* exposure assessment (NST/029/972354) at 21 days of age. The toxicity phase of this study commenced when rats were 26 to 28 days old. Rats were housed individually in controlled conditions with free access to food and water, except during urine collection periods and for overnight fasting before blood sampling. Rats were fed diets with NC-00723 at levels to produce doses of 0, 10, 30, 100, 300 or 1000 mg/kg bw/day, with 20 rats/sex/group for the toxicity study, an additional 10 rats/sex/group at 0, 100, 300 and 1000 mg/kg bw/day for a reversibility study, 6/sex /group at 0, 10 or 30 and 24/sex/group at 100, 300 or 1000 mg/kg bw/day in a satellite phase. During the treatment phase, results from the toxicity and reversibility animals were pooled, resulting in 30/sex/group at 0, 100, 300 or 1000 mg/kg bw/day. The satellite rats were dosed but used only for plasma level sampling. Clinical signs, bodyweight and food consumption were recorded for these animals but were not reported.

Diets were prepared, and sampled for homogeneity pre-test and in week 12. Diets were sampled for achieved concentrations in weeks 1, 6, 13, 26, 39 and 52. Dietary concentrations were adjusted weekly for the first 26 weeks, then every two weeks thereafter to give the required doses.

Animals were observed twice daily for mortality, morbidity and abnormal clinical signs. Animals found *in extremis* were killed, and a complete post-mortem examination was done on all decedents. During the reversibility phase, rats were examined at least once daily. Once weekly, rats were removed from their cages and a more thorough clinical examination performed. This included, but was not limited to, behaviour changes, body temperature, buccal cavity, build, coat, abnormal secretions, excreta, eyelids, muscle reaction, posture, reflexes, respiration, skin and teeth. Signs considered indicative of neurotoxicity (convulsions, tremors, gait abnormalities and behavioural changes) were particularly noted.

Rats were weighed on days 0, 3 and 7, weekly for weeks 2 to 26, then every second week until the end of the study. Food consumption was determined for days 1, 2, 3, 4 to 7, weekly for weeks 2 to 26, then for one week in each two-week period until the end of the study. A complete ophthalmologic examination was done on all rats pre-test, in weeks 12, 26, 38 and 51 of the toxicity phase and week 4 of the reversibility phase. The examination in week 51 was repeated by an independent toxicologist.

Blood samples were taken in weeks 13, 26, 39 and 52 of toxicity phase from 10/sex/group. Samples were taken at the end of the reversibility phase from all animals. Haematology and clinical chemistry examinations (see Appendix B) were performed. A bone marrow smear from the femur was prepared at necropsy. Slides were prepared for examination and retained in the archives for examination if required. Samples for urinalysis were taken in weeks 12, 25, 38 and 51 of the toxicity study and week 3 of the reversibility study. Parameters examined are detailed in Appendix B. Blood was taken from satellite animals in weeks 1, 13, 26 and 53. In weeks 1 and 13, all groups were sampled at 8 am, with additional samples taken from 100, 300 and 1000 mg/kg bw/day groups at 2 pm, 8 pm and 2 am. The plasma was separated and citric acid added before freezing. Samples were later analysed for test article levels.

A detailed post-mortem examination was carried out on all animals. This included an inspection of the external features and orifices, neck and associated tissues, the cranial, thoracic, abdominal and pelvic cavities and their viscera inspected. The required organs were weighed (see Appendix C), and examined. Any abnormalities were noted and samples preserved as required.

**Results**

**Homogeneity and stability of test articles**

The homogeneity and stability of the prepared diets was suitable at all times. The concentrations were within 10% of those needed to achieve the required doses. Achieved doses over the course of the study were 0, 10.1, 30.2, 100.2, 302.0 and 1006.4 mg/kg bw/day for males and 0, 10.1, 30.3, 100.5, 301.9 and 1006.3 mg/kg bw/day for females.

**Mortality and clinical signs**

There was no treatment-related increase in the incidence of mortalities. There was a slight increase in the incidence of aggressiveness in males, with the incidence being 0/30, 0/20, 2/20, 0/30, 2/30 and 4/30 at 0, 10, 30, 100, 300 or 1000 mg/kg bw/day. An increase in irritability was also seen in males, the incidence being 4/30, 3/20, 5/20, 4/30, 9/30 and 9/30 at 0, 10, 30, 100, 300 or 1000 mg/kg bw/day. These increases were not seen in females.

These effects were not considered to be treatment-related, as there is no dose-response relationship, irritability is a common finding in older single housed male rats and was not observed in females.

The incidence of brown staining on the head was increased in males with occurrences of 6, 6, 6, 12, 13, and 12 at doses of 0, 10, 30, 100 and 300 mg/kg bw/day. This may be related to the brown diet adhering to the muzzle and decreased grooming associated with the decreased palatability of the diet. Other incidental clinical signs observed included piloerection, decreased grooming of the body, ataxia, abnormal gait and hair loss. These were seen at low incidences in all groups, and were not considered treatment related. There were no abnormal clinical signs noted during the reversibility phase.

**Bodyweight and food consumption**

In the rats obtained from the *in utero* phase, males at 100 mg/kg bw/day commenced dosing with a bodyweight 13% lower than controls, while females at 100 mg/kg bw/day were 15% lower than controls. In males, there were no other significant differences in bodyweight for any treatment group. In females at 100 mg/kg bw/day, the difference from control had decreased to 7% by the end of week 2 of dosing.

From weeks 28 in males and 24 in females to week 52, bodyweight in rats at 100 mg/kg bw/day was statistically significantly lower than controls. This difference was less than 10% until week 40, increasing to an 11.5% difference by week 50. At 300 mg/kg bw/day, bodyweight was statistically significantly lower than controls from weeks 24 to 30, however the difference was less than 10%. From weeks 34 to 52, females at 300 mg/kg bw/day had statistically significantly lower bodyweight than controls, with the difference 12.7% by week 52. At 1000 mg/kg bw/day females had statistically significantly lower bodyweights from weeks 48 to 52, with the differences ranging from 10.5% to 13.4%. No significant differences in bodyweight were observed in any groups during the reversibility phase.

Cumulative bodyweight gain was decreased in females at 300 mg/kg bw/day for weeks 0 to 26 (8.4%). For weeks 0 to 52, females at 300 mg/kg bw/day had a 14.3% lower cumulative bodyweight gain than controls and at 1000 mg/kg bw/day had a 15.7% lower cumulative bodyweight gain than controls.

Food consumption in males at 1000 mg/kg bw/day was decreased in weeks 10 and 11. In females, there were intermittent decreases throughout the study from 100 mg/kg bw/day. In week 28 decreases were 11.7%, 11.1% and 10.6% at 100, 300 and 1000 mg/kg bw/day respectively. Although there was not a clear dose relationship, the decrease in food consumption in females from 100 mg/kg bw/day was considered to be treatment related.

**Ophthalmoscopic examination**

At week 26, there were reports of small, light grey, translucent spherical bodies in the centre of the lens. This finding, which was particularly apparent in male rats, was termed nuclear sclerosis (see table below). However, there was no real dose-response, incidences were comparable to controls in weeks 38 and 51 and the effect was reversible (in week 4 reversibility phase). The reporting ophthalmologist did not consider this finding of any clinical significance or to be comparable with the clinical lesion of nuclear sclerosis reported for dogs or in humans.

A second ophthalmologist conducted a separate examination during week 51 and concluded that there were no compound-related ocular effects.

**Incidence of bilateral nuclear sclerosis and faint nuclear sclerosis.**

| | Week 12 | | Week 26 | | Week 38 | | Week 51 | |
|---|---|---|---|---|---|---|---|---|
| | male | Female | male | Female | male | Female | male | female |
| 0 mg/kg bw/day | 0 | 0 | 0 | 1 | 5 | 6 | 9 | 8 |
| 10 mg/kg bw/day | 0 | 0 | 2 | 1 | 4 | 1 | 4 | 2 |
| 30 mg/kg bw/day | 0 | 0 | 2 | 0 | 4 | 2 | 5 | 4 |
| 100 mg/kg bw/day | 0 | 0 | 2 | 0 | 4 | 2 | 5 | 5 |
| 300 mg/kg bw/day | 0 | 0 | 4 | 3 | 9 | 6 | 14 | 8 |
| 1000 mg/kg bw/day | 1 | 0 | 6 | 2 | 8 | 5 | 11 | 6 |

**Clinical pathology examination**

On haematological examination, there were increases in MCH in males at 300 mg/kg bw/day in weeks 26, 39 and 52, and also at 100 mg/kg bw/day in week 52. The prothrombin time was increased at 300 mg/kg bw/day in males at weeks 39 and 52 of the toxicity phase, and at the end of the reversibility phase. These changes were all relatively small, but are considered treatment related due to the consistency of the findings. There were no consistent significant changes in haematological parameters in females at any dose.

On clinical chemistry examinations, changes in sodium and chloride levels were the only consistent findings throughout the study, and although the changes were statistically significant, they were small and not considered of biological significance. Changes in other parameters, including glucose, creatinine, bilirubin and globulin levels were seen intermittently, but were not considered treatment related. There were no changes in plasma cholesterol levels throughout the study.

There were no significant treatment-related changes in urinalysis. Changes in sodium and chloride levels were consistent with variations in plasma levels, and the osmolality of the urine was increased at higher dose levels, which probably reflects slightly decreased water consumption associated with decreased food consumption.

**Analyte examination**

The plasma concentration of NC-00723 was variable throughout the study, however it was clear that levels were not increasing with increasing time spent on study. Plasma NC-00723 was below the level of detection for females at 10 mg/kg bw/day. Plasma levels of NC-00751 were much higher, and showed a dose relationship, which is consistent with the rapid metabolism of NC-00723 to NC-00751 seen in rats in other studies. No accumulation of NC-00723 or NC-00751 was seen during the study.

**Organ weights**

The absolute heart weight was decreased in females from 100 mg/kg bw/day, with decreases of 12.5% to 13.8%. This decrease was also seen in heart weight relative to brain weight, with decreases of 10 to 13%, but was not seen for heart weight relative to bodyweight.

The absolute weight of the pituitary was decreased in females from 30 mg/kg bw/day, however the changes were not dose related (0, 0, 20%, 25%, 10% and 15% at 0, 10, 30, 100, 300 and 1000 mg/kg bw/day). The thymus weight was also decreased in a non-dose related manner.

**Gross and histopathology**

In females, the mammary glands were thickened (2/17, 1/20, 2/20, 1/20, 0/20 and 2/19), appeared active (0, 3, 2, 2, 0, 1) and had masses present (0, 1, 1, 1, 1, 2) at doses of 0, 10, 30, 100, 300 or 1000 mg/kg bw/day, respectively. Pituitary masses were also detected in females (2, 2, 4, 2, 4, 3), and were present at a low incidence in males (0, 0, 1, 1, 0, 2) at doses of 0, 10, 30, 100, 300 or 1000 mg/kg bw/day, respectively. There was a slight increase in the incidence of thickened stomach wall in males (1, 1, 1, 1, 2, 2) at doses of 0, 10, 30, 100, 300 or 1000 mg/kg bw/day, respectively.

On histopathological examination, there was a range of findings at a low incidence in the adrenal cortex, with no clear dose relationship. Pituitary adenomas were seen in both males (0, 0, 2, 2, 2, 2) and females (1, 2, 7, 1, 4, 3), with pituitary haemorrhage also seen in females (1, 0, 0, 1, 1, 1) at doses of 0, 10, 30, 100, 300 or 1000 mg/kg bw/day, respectively. In the mammary glands of females, fibroadenomas were seen at 0, 0, 1, 0, 1 and 0. Secretory activity and ductular dilation was noted in females (2, 2, 2, 3, 1, 2).

**Conclusions**

Bodyweights were lower than controls from 100 mg/kg bw/day. Although these rats had commenced the study at a lower bodyweight, the differences between groups had stabilised in the early stages of the study, before further decreased bodyweight gain leading to lower bodyweights later in the study. Food consumption was also decreased in females from 100 mg/kg bw/day. The observed decreases in bodyweight and bodyweight gain are considered to be related to the reduced palatability of the diet containing high levels of Neotame. No significant treatment-related findings were seen in clinical pathology, organ weight or gross or histopathological examination.

The NOEL, based on the absence of adverse effects at the highest dose tested, was 1000 mg/kg bw/day.

**3. Mitchell DJ (1997) NC-00723: Oncogenicity Study by dietary administration to CD rats with exposure *In Utero.* Lab Project No. NST020/972379. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-1000**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study: | 7 May 1995 to 19 May 1997 |
| GLP: | OECD, UK, EC |
| Test chemical: | NC-00723, Lot no. 95MP028-3 |
| Test species: | CDBR VAF/Plus Sprague Dawley rats, selected from *in utero* phase of NST/020/970389 at 21 days) |

**Study design**

Rats were selected from the *in utero* exposure assessment (NST/020/970389) at 21 days of age. The toxicity phase of this study commenced when rats were 25 to 29 days old. Rats were housed individually in controlled conditions with free access to food and water, except during urine collection periods and for overnight fasting before blood sampling. Rats were fed diets with NC-00723 at levels to produce doses of 0, 50, 500 or 1000 mg/kg bw/day. In the oncogenicity study, there were 147 rats/sex in control, 74 males and 75 females at 50 mg/kg bw/day, 75 males and 73 females at 500 mg/kg bw/day and 75/sex at 1000 mg/kg bw/day. An additional 12 rats/sex/group were used in a satellite phase. These rats were used for water consumption, clinical pathology measurements and plasma levels sampling, and were killed in week 82.

Neotame was incorporated into the diet at 40000 ppm as a premix. This concentrated diet was used to formulate diets for dose groups. The diets were sampled for homogeneity in week 1. Samples were taken to monitor the achieved concentrations in weeks 1, 13, 26, 39, 52, 65, 78, 91 and 104. Dietary concentrations were adjusted weekly for the first 26 weeks, then every two weeks thereafter to give the required dosages in mg/kg bw/day.

Animals were observed twice daily for abnormal clinical signs and for dead or moribund animals. Animals found *in extremis* were killed, and a complete post-mortem examination was done on all decedents. During the reversibility phase, rats were examined at least once daily. Once weekly, rats were removed from their cages and a more thorough clinical examination performed. This included, but was not limited to, behaviour changes, body temperature, buccal cavity, build, coat, abnormal secretions, excreta, eyelids, muscle reaction, posture, reflexes, respiration, skin and teeth. Signs considered indicative of neurotoxicity (convulsions, tremors, gait abnormalities and behavioural changes) were particularly noted. The presence of any masses, including their location, size, consistency, time of first observation and subsequent history was noted.

Rats were weighed at pre-treatment, weekly for 26 weeks, then every second week until the end of the study. Food consumption was determined for days 1 to 3 and 4 to 7, weekly for 26 weeks, then for one week in each two-week period until the end of the study. Water consumption was determined for rats in the satellite group in weeks 27, 54 and 79.

Blood samples were taken in weeks 26, 52 and 78 from the satellite animals. In week 104, samples were taken from 10/sex/group of the oncogenicity animals. Haematology and clinical chemistry examinations (see Appendix B) were performed. Samples for urinalysis were taken in weeks 21, 51, 77 and 103. Parameters examined are detailed in Appendix B. Blood was also taken from satellite animals for analyte analysis in weeks 13, 30, 54 and 82, the plasma separated and citric acid added before freezing. Samples were later analysed for test article levels.

A detailed post-mortem examination was carried out on all animals. This included an inspection of the external features and orifices, neck and associated tissues, the cranial, thoracic, abdominal and pelvic cavities and their viscera inspected. The required organs were weighed (see Appendix C), and examined. Any abnormalities were noted and samples preserved as required.

## Results

### Homogeneity and stability of test articles

The homogeneity and stability of the prepared diets was suitable at all times. The concentrations were within 10% of those needed to achieve the required doses. The achieved dosages were 0, 50.7, 508.5 and 1016.4 for males and 0, 50.8, 507.3 and 1013.8 for females.

### Mortality and clinical signs

There was no treatment-related increase in the incidence of mortalities. Aggressiveness was noted in males, with the incidence being 38/147, 15/74, 24/75 and 24/75 at 0, 50, 500, or 1000 mg/kg bw/day and limited aggressiveness observed in females the incidence being 1/147, 3/75, 2/73 and 4/75 at 0,50,500 or 1000 mg/kg bw/day. Irritability was also seen in males, with 78/147, 35/75, 39/75 and 39/75 rats affected; in females the incidences were 41/147, 12/75, 15/73 and 25/75 respectively. These effects were not considered to be treatment related as controls showed higher incidences and there was no dose-response.

The incidence of brown staining on the head was slightly increased in both males and females. This may be related to decreased grooming associated with the decreased palatability of the diet. Other incidental clinical signs observed included piloerection and hunched posture. These were seen at low incidences in all groups, and were not considered treatment related. There was no treatment-related increase in the incidence of palpable masses, either for the number of animals with masses or the number of masses detected in each group. There were no significant differences in the mean time of onset for the detection of masses for any treatment groups.

### Bodyweight and food consumption

In females, bodyweight was statistically significantly lower than controls at all doses in week 18. Female bodyweights were consistently lower than controls at 500 or 1000 mg/kg bw/day from week 11, and at 50 mg/kg bw/day from week 18 until the end of the study, with bodyweights up to 17% lower than controls during this period. In males at 500 mg/kg bw/day, bodyweights were statistically significantly lower than controls from week 5, with all doses lower than controls from week 22. During these weeks, the bodyweights in the treated groups were more than 10% lower than control. Thus treatment-related changes in bodyweight were seen in all treatment groups in both sexes. Statistically significant decreases ($p<0.05$) in the cumulative bodyweight gain were seen in all treatment groups. The decreases were greater than 10% for weeks 0 to 78 in all treatment groups, and for weeks 0 to 104 for all female treatment groups. Food consumption was decreased in females during week 50, but no other change in food consumption was seen during the study. There were no significant differences in water consumption between treatment groups at any stage during the study.

### Clinical pathology

There were few differences between groups on haematological examination. In week 52 in males, the MCHC was slight decreased at 50 and 500 mg/kg bw/day; as this was not seen at the high dose or at other time periods it was not considered treatment related. On clinical chemistry examination, there were very small inconsistent increases or decreases in the sodium and chloride levels in the plasma in both sexes.

In week 52, ALT, AST and glucose levels were decreased in males at the high dose. These effects were not seen in females, or in males at any other time period and are not considered treatment related. There were no other notable differences between groups. On urinalysis, males at 500 or 1000 mg/kg bw/day in week 77 had slightly decreases urine specific gravity. This was not considered to be treatment related, and no other urinary changes were observed at any time.

**Organ weights**

In males, at a dose of 1000 mg/kg bw/day the absolute adrenal weight was decreased by 27% in comparison to controls ($p<0.01$). The heart weight was decreased in all male treatment groups from 12% (50 mg/kg bw/day, $p<0.05$) to 17% (1000 mg/kg bw/day, $p<0.001$). No other changes in absolute organ weight were seen in males. In females, the absolute weight of the uterus was increased in all treatment groups in comparison to controls, with increases of 14% at 50 and 500 mg/kg bw/day, and of 34% at 1000 mg/kg bw/day. The absolute weight of the thyroids with parathyroid was decreased from 500 mg/kg bw/day. None of these increases were statistically significant, and the relationship to treatment is not established. In males, adrenal weight relative to brain weight was significantly decreased ($p<0.01$, 27%), and in females the uterus weight relative to bodyweight was increased at 500 and 1000 mg/kg bw/day (38 to 46%, $p<0.05$).

**Gross and histopathology**

There were no treatment-related findings on gross post-mortem. There was an apparent increase in the incidence of activated mammary glands in females at 1000 mg/kg bw/day. When this was consolidated with the findings for thickened mammary glands (without activation), there were no longer significant differences between groups, and thus this is an incidental finding.

On histopathological examination, there were no treatment-related increases in the incidence or onset of neoplasm. The incidence of renal adenoma was increased in males at 50 mg/kg bw/day, but as this was not dose related, and there was no increase in pre-neoplastic or non-neoplastic findings at any dose, this is not considered to be related to treatment. A range of tumours were seen, consistent with those expected in Sprague Dawley rats of this age.

There were no treatment-related non-neoplastic findings on histopathological examination. The incidence of female rats with corticomedullary mineralisation in the kidney was increased in all treatment groups (79/147, 46/75, 54/73 and 47/75 at 0, 50, 500 and 1000 mg/kg bw/day respectively). This is a relatively common finding, and the increase was only statistically significant at the middle dose of 500 mg/kg bw/day. The incidence of progressive nephropathy in treated rats was decreased in comparison to controls, as was the incidence of mineralisation of the pelvic epithelium and the incidence of transitional cell hyperplasia. In males, the incidence of focal cystic degeneration of the liver was decreased in all treatment groups (significantly at 500 and 1000 mg/kg bw/day). Other incidental findings were seen on histopathological examination, including papillary cysts in the kidney, necrosis of the femoral marrow, thymic haemorrhage, erosion of the glandular stomach and cystic follicular hyperplasia of the thyroid in females. Microscopic examination of the brain did not reveal any changes, particularly evidence of neuronal degeneration or necrosis which could be attributed to treatment.

**Conclusions**

No significant treatment-related findings were seen in clinical pathology, organ weight or gross or histopathological examination. Under the conditions of the study Neotame was not considered carcinogenic in rats up to a period of 104 weeks.

The NOEL, based on the absence of adverse effects at the highest dose tested, was 1000 mg/kg bw/day.

**4. Thomford PJ (1997) 52-Week Dietary Toxicity Study of NC-00723 in Dogs Followed by a 4-week Reversibility Period. Lab Project No. Covance 6211-304. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-1017**

| | |
|---|---|
| Laboratory: | Covance Laboratories Inc, Madison Wisconsin |
| Date of study: | 24 March 1996 to 14 November 1997 |
| GLP: | US FDA |
| Test chemical: | NC-00723, Lot no. 96NK 005-2 |
| Test species: | Beagle dogs source: Covance Research Product Inc., Kalamazoo, Michigan |

**Study design**

Dogs were acclimatised for 36 days before treatment, with a full physical and neurological examination, haematological and clinical chemistry tests performed during this period. Dogs were housed individually in controlled conditions, with water freely available, and food available for a limited period each day. The diet was prepared based on bodyweight and food consumption to give doses of 0, 20, 60, 200 or 800 mg/kg bw/day using 4/sex/group, with an additional 2/sex/group at 0, 200 and 800 mg/kg bw/day for a reversibility study.

All dogs were observed twice daily for mortality and moribundity, with observations made both before and after feeding. Observations included but were not limited to the conditions of the skin and fur, the ears, eyes, mucous membranes, mouth and teeth, body and limb conformation and the genitalia. A detailed physical examination was done pre-test and in weeks 13, 26, 39 and 52 weeks, and in week 56 for the reversibility phase dogs. The examination included measuring the heart rate, rectal body temperature and respiratory rate.

Bodyweight was determined pre-treatment, on day 1 and weekly throughout the test. Food consumption was determined daily for two weeks before treatment and during weeks 1, 2, 52 and 53, as well as weekly for weeks 3 to 51 and 54 to 56. A neurological examination was done pre-treatment and in weeks 13, 26, 39, 52 and 56. The pupillary light, patellar, righting, hopping and palpebral reflexes were assessed, as well as general observations including assessment of gait. An ECG was done pre-treatment and in weeks 13, 26, 39, 52 and 56, and an ophthalmological examination was also done at these times, including assessment of the anterior portion of the eye and the ocular fundus.

Blood was collected 1.5, 3, 6, 12 and 24 h after feeding on day 6 and in weeks 13, 26 and 52. Samples were placed into sodium heparin and paraoxon esterase, the plasma separated and frozen. Blood and urine were collected pre-treatment and in weeks 13, 26, 39, 52 and 55/56 for clinical pathology parameters, as detailed in Appendix B.

At the end of the study, all animals were examined for any gross abnormalities. The examination included the external surface, all orifices, cranial cavity, brain and spinal cord, nasal cavity, paranasal sinuses and the thoracic, abdominal and pelvic cavities and viscera. Organ weights, organ to bodyweight ratio and organ to brain weight rations were determined, and samples preserved for microscopic examination as detailed in Appendix C. Samples of the liver, spleen and kidney were preserved for electron microscopy, but were not examined, as were bone marrow smears.

**Results**

**Homogeneity and stability of test articles**

Homogeneity and concentration of the treated diets were considered acceptable with most analyses within 5% of intended levels. The achieved test article consumption was 20.1, 59.9, 200.6 and 802.1 mg/kg bw/day in males and 19.9, 59.7, 198.7 and 796 mg/kg bw/day in females.

**Mortality and clinical signs**

There were no deaths during the study. The only notable clinical signs observed were discoloured faeces at 800 mg/kg bw/day. There were no treatment related changes in heart rate, respiratory rate or body temperature. There were no abnormal findings on the neurological examination, ophthalmological examination or ECGs.

**Bodyweight and food consumption**

In males, bodyweight at 800 mg/kg bw/day was more than 10% lower than controls from week 3 until the end of the study. In females at 800 mg/kg bw/day in week 6 the bodyweight was 11% lower than controls. Bodyweight was not more than 10% lower than controls at any other dose and any point in the study, and none of the lower bodyweights were statistically significantly different to controls. Cumulative bodyweight gain was statistically significantly lower than controls in males at 800 mg/kg bw/day up to week 8. Decreased cumulative weight gains of more than 10% (not statistically significant) were seen at all doses in males. No statistically significant differences in cumulative bodyweight gain were seen in females at any time during the study. Food consumption in males was statistically significantly decreased in males at 800 mg/kg bw/day, at day14 and 19% lower than controls for weeks 1 and 2, respectively ($p<0.05$). In females at 800 mg/kg bw/day food consumption was 12 to 23% lower than controls for weeks 1 to 3 (not statistically significant).

**Clinical pathology**

There were no treatment-related changes on haematological examination. In both sexes, alkaline phosphatase activity was increased throughout the study at 800 mg/kg bw/day ($p\leq0.001$). These increases were not seen at lower doses, and had resolved at the end of the reversal phase. Isoenzyme analysis indicated that this was of hepatic origin rather than other sources of alkaline phosphatase. There was a non-statistically significant increase in cholesterol levels seen; however this may be due to normal variation. No abnormalities were seen in urinalysis throughout the study.

**Gross pathology and organ weights**

No abnormalities were detected on macroscopic examination. There were no statistically significant treatment-related changes in absolute or relative organ weights, with the exception of increased liver weight relative to brain weight in females at 200 and 800 mg/kg bw/day ($p \leq 0.05$). However, absolute liver weight and liver weight relative to bodyweight was unchanged at 200 and 800 mg/kg bw/day. In the absence of other clinical or histopathological changes, the increased liver weight relative to brain weight in female dogs is not considered toxicologically significant.

**Histopathology**

On histopathological examination, hepatocellular vacuolation was observed in both male and female controls and animals receiving doses of 800mg/kg bw/day. This is an incidental finding of no clinical significance, as there were no significant differences in incidence between controls and high-dose animals, no dose-response relationship and at no other doses was this observed.

There was a relatively high incidence of tubular mineralisation in the kidney in all groups of females, without any dose relationship. Other incidental findings included chronic inflammation and mineralisation of the thyroid, atrophy or degeneration of the testes in one high-dose male, mineralisation of the ovaries in one female at 200 mg/kg bw/day and uterus dilatation in one female at 60 mg/kg bw/day.

**Conclusions**

There were no significant treatment related changes in clinical pathology, organ weights or gross or histopathology. The NOEL, based on the significant increases in alkaline phosphatase activity was 200 mg/kg bw/day.

**REPRODUCTION STUDIES**

**1. Mitchell DJ (1997) NC-00723: 52-Week Toxicity Study by dietary administration to CD rats with exposure *In Utero* and followed by a Reversibility Phase. Lab Project No. NST029/972354. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-1011**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study: | 20 December 1995 to 24 April 1996 |
| GLP: | UK, US FDA |
| Test chemical: | NC-00723, Lot no. 96NK002-6 |
| Test species: | Crl: CDBR VAF/Plus Sprague Dawley rats, Charles River UK, Margate, Kent |

**Study design**

Rats were housed individually except during pairing in controlled conditions with food and water available *ad libitum*. NC-00723 was included in the diet at levels to give doses of 0, 10, 30, 100, 300 or 1000 mg/kg bw/day using 25/sex/group in the treatment groups. The top dose for dams was reduced to 300 mg/kg bw/day from day 14 of lactation to minimize differences in offspring bodyweight.

The dietary concentration of NC-00723 was calculated weekly using bodyweight and food consumption data, although males during pairing received diets calculated on the intake by females. Diets were sampled for homogeneity and achieved concentration in week 1 and 4, gestation week 2 (females) and lactation week 2. Adult male and female rats were maintained on the treated diets for 29 days before pairing, during mating, gestation and lactation. $F_1$ rats were fed at the appropriate dose from weaning until selection for the toxicity phase at approximately 26 to 28 days of age.

Females were paired on a one-to-one basis with males from the same treatment group. They were checked daily for evidence of mating, including a vaginal smear for the presence of spermatozoa. The day that mating was detected was determined to be the first day of gestation, and the rats were separated. Pairs were allowed a maximum of one week to mate.

Rats were observed twice daily for signs of reaction to treatment and mortality. Bodyweight was determined for males at the start of treatment, on days 3 and 7 and weekly until pairing. Females were weighed at the start of treatment, on days 3 and 7 then weekly until mating, on days 0, 6, 13 and 20 of gestation and days 1, 7, 14, 18 and 21 of lactation. Food consumption was determined on the first 3 days, then weekly until pairing, and weekly in females through gestation and lactation. The time from the detection of mating until the commencement of parturition was recorded. All females were inspected twice daily from gestation day 20 for onset, progress and completion of parturition.

On day 1 of lactation, the number of stillborn and live pups was recorded. Pups were counted on days 4, 7, 14 and 21 of lactation, and the litters were culled to 8 pups (4 males, 4 females) on day 4 of lactation. Litters were examined daily for signs of ill health or reaction to treatment. Bodyweight was recorded on a litter basis on days 1, 4, 7 and 14, and on an individual pup bases on day 21. On day 21, one male and one female were selected from each litter to go on to the oncogenicity phase. For some doses, pup numbers were reduced due to insufficient survivors or insufficient litters. All adults and weanlings not used in the toxicity phase, as well as the culled day 4 offspring were killed and discarded without post-mortem examination.

**Results**

**Achieved doses**

Doses were generally within 10% of target dosages, although males during the first week of treatment had intake in the range of 81 to 88% of target, due to decreased food intake.

**Mortality and clinical signs**

There were no treatment-related clinical signs, and no deaths during the study.

**Bodyweight and food consumption**

Food consumption was decreased in both sexes from 300 mg/kg bw/day during week one, particularly in males. There were no significant effects on food consumption seen during gestation or lactation.

Bodyweight was decreased in males during the first three days of treatment. Cumulative bodyweight gain was significantly decreased from 100 mg/kg bw/day in males at the end of the pre-pairing period. Over this period, the bodyweight gain was 79 to 82% of that seen in controls. In females, decreased weight gain or bodyweight loss was seen during the first 3 days of treatment. Overall bodyweight gain before pairing was reduced by 12 to 17% in comparison to controls from 100 mg/kg bw/day. During gestation and lactation, the bodyweight and bodyweight gain of treated females was similar to controls.

**Reproductive performance**

There were no treatment-related effects on the percentage of rats mating, the conception rate or fertility index. Litter sizes and sex ratio were not affected by treatment. The early bodyweights of the offspring were not affected by treatment, but the cumulative bodyweight gain at 300 and 1000 mg/kg bw/day was decreased for days 14 to 21 (up to 18%). At weaning, the offspring in these groups had bodyweights around 8 to 11% lower than controls.

**Conclusion**

Neotame did not affect reproductive capacity in rats up to a dose of 1000 mg/kg bw/day for 52-weeks.

**2. Mitchell DJ (1997) NC-00723: Oncogenicity Study by dietary administration to CD rats with exposure *In Utero. In Utero Phase.* Lab Project No. NST020/970389. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-1000**

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|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study: | 15 March 1995 to 15 June 1995 |
| GLP: | UK, US FDA |
| Test chemical: | NC-00723, Lot no. 95MP028-3 |
| Test species: | Crl: CDBR VAF/Plus Sprague Dawley rats, Charles River UK, Margate, Kent |

**Study design**

Rats were housed individually except during pairing in controlled conditions with food and water available *ad libitum*. NC-00723 was included in the diet at levels to give doses of 0, 50, 500 or 1000 mg/kg bw/day using 85/sex/group in the treatment groups and 170/sex/group for controls. The two top doses for dams were both reduced to 300 mg/kg bw/day from day 14 of lactation to minimise differences in offspring bodyweight. The dietary concentration of NC-00723 was calculated weekly using bodyweight and food consumption data, although males during pairing received diets calculated on female intake. Diets were sampled for homogeneity and achieved concentration in week 1, gestation week 2 (females), lactation week 2 and lactation week 3 (500 and 1000 mg/kg bw/day groups only). Adult male and female rats were maintained on the treated diets for 29 days before pairing, during mating, gestation and lactation. $F_1$ rats were fed at the appropriate dose from weaning until selection for the oncogenicity phase at approximately 25 to 29 days of age.

Females were paired on a one-to-one basis with males from the same treatment group. They were checked daily for evidence of mating, including a vaginal smear for the presence of spermatozoa. The day that mating was detected was determined to be the first day of gestation, and the rats were separated. Pairs were allowed a maximum of one week to mate.

Rats were observed twice daily for signs of reaction to treatment and mortality. Bodyweight was determined for males at the start of treatment and weekly until pairing. Females were weighed weekly until mating, on days 0, 6, 13 and 20 of gestation and days 1, 7, 14 and 21 of lactation. Food consumption was determined weekly until pairing, and weekly in females through gestation and lactation. The time from the detection of mating until the commencement of parturition was recorded. All females were inspected twice daily from gestation day 20 for onset, progress and completion of parturition.

On day 1 of lactation, the number of stillborn and live pups was recorded. Pups were counted on days 4, 7, 14 and 21 of lactation, and the litters were culled to 8 pups (4 males, 4 females) on day 4 of lactation. Litters were examined daily for signs of ill health or reaction to treatment. Bodyweight was recorded on a litter basis on days 1, 4, 7 and 14, and on an individual pup bases on day 21. On day 21, one male and one female were selected from each litter to go on to the oncogenicity phase. For some doses, pup numbers were reduced due to insufficient survivors or insufficient litters. All adults and weanlings not used in the oncogenicity phase, as well as the culled day 4 offspring were killed and discarded without post-mortem examination.

**Results**

**Achieved doses**

The average doses before pairing were 50.2, 494, and 985 mg/kg bw/day for males and 50.7, 501 and 988 mg/kg bw/day for females. During gestation average doses for females were 51.3, 508 and 1018 mg/kg bw/day and during lactation (up to day 13) were 54, 564 and 1173 mg/kg bw/day.

**Mortality and clinical signs**

There were no treatment-related clinical signs, and no deaths during the study.

**Bodyweight and food consumption**

Food consumption was decreased in both sexes from 500 mg/kg bw/day during week 1 (6–8%, $p<0.001$). Food consumption was also decreased in males in all treatment groups during week 4 (3%, $p<0.05$). During gestation, food consumption was decreased 3% in females from 500 mg/kg bw/day for days 7 to 13, 14 to 19 and the whole of gestation. There were no changes in food consumption seen during lactation.

Bodyweight was statistically significantly decreased in rats from 500 mg/kg bw/day for the four weeks before pairing, with decreases of 3 to 4% in comparison to controls. Cumulative bodyweight gain was also decreased at these doses. During gestation and lactation, females had statistically significantly lower bodyweights from 500 mg/kg bw/day ($p<0.01$), with decreases of 3 to 4%.

**Reproductive performance**

There were no treatment-related effects on the percentage of rats mating, the conception rate or fertility index. Litter sizes and sex ratio were not affected by treatment. The early bodyweights of the offspring were not affected by treatment, but the cumulative bodyweight gain was decreased for days 14 to 21 (9 to 10%, $p<0.001$) from 500 mg/kg bw/day.

In the offspring selected for the oncogenicity phase, all mean bodyweights were within 5% of the control rats.

**Conclusion**

Neotame did not affect reproductive capacity in rats up to a dose of 1000 mg/kg bw/day for 104-weeks.

**3. Willoughby CR (1996) Reproductive performance range-finding dietary study of NC-00723 in rats. Lab Project No. 94/NST015/1137. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-0987**

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|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study: | 1 June 1994–11 September 1994 |
| GLP: | UK, US FDA |
| Test chemical: | NC-00723, Lot no. 94UP8-3-4 |
| Test species: | Crl: CDBR VAF/Plus Sprague Dawley rats, Charles River UK, Margate, Kent |

**Study design**

Rats were acclimatised for 5 days before commencement of dosing. They were housed in controlled conditions in groups during the acclimatisation phase, then individually for the rest of the study except during pairing. Food and water were freely available. NC-00723 was included in the diet at concentrations to give doses of 0, 10, 30, 100, 300 or 1000 mg/kg bw/day, using 8 rats/sex/group. Females were fed treated diet for 29 days before pairing until termination of the study when offspring were 25 days old. Males were fed treated diet for 29 days before pairing until after successful littering of the females. During pairing, males received diets prepared to the females' specifications. No dietary analysis was conducted during this study.

Rats were paired on a one-to-one basis within treatment groups for a maximum of 3 weeks. Vaginal smears were done on females for 10 days before pairing to determine the regularity of the oestrus cycle. The cage was checked daily for any copulation plugs, and a vaginal smear to detect spermatozoa was performed daily. The day on which evidence of mating was detected was designated day 0 of gestation, and the rats were separated. The pre-coital interval was recorded.

Rats were observed twice daily for mortality and morbidity, and daily for evidence of reaction to treatment. Bodyweight of males was determined on days 7 and 3 before treatment, daily for the first week of treatment, then twice weekly until the end of the study. Bodyweight of females was determined 7 and 3 days before treatment, daily for the first week of treatment, twice weekly until pairing, on days 0, 3, 7, 10, 14, 17 and 20 of gestation and days 1, 4, 7, 11, 14, 18, 21 and 25 of lactation. Food consumption was determined daily for one week pre-treatment, and for the first week of treatment and weekly thereafter until pairing. Following conclusion of pairing, food consumption was determined weekly for males. For females, food consumption was determined twice weekly during gestation and lactation.

Females were inspected two or three times daily for the onset, progress and completion of parturition. Gestation length was recorded. All offspring were examined approximately 24 h after birth. The number of offspring born (both alive and dead), the litter weight of live offspring, the sex ratio and any individual observations were determined.

Pups were examined daily for signs of reaction to treatment and mortality. On day 4, the litter size was reduced to 8 pups (4/sex where possible). Culled offspring, and any pups found dead were examined internally and externally for any abnormalities. The bodyweight of the litter was determined on days 1, 4, 7, 11, 14, 18, 21 and 25. The sex ratio was determined on days 1, 4 and 25.

At the end of the study, females and their litters were killed and examined internally and externally for any macroscopic abnormalities. The number of implantation sites was recorded. Females not littering by day 25 after mating were killed and examined, including determination of any implantation sites. Male adults were also examined macroscopically.

**Results**

One female at 300 mg/kg bw/day developed mastitis and was killed for humane reasons. This was not considered to be related to treatment. No other abnormal clinical signs were noted. Food consumption in males was decreased in comparison to controls during the first week and week 4 from 100 mg/kg bw/day. At 100 mg/kg bw/day, week 1, decreases were between 14 and 29% ($p<0.01$, $p<0.001$). Similar decreases in males were seen at 300 and 1000 mg/kg bw/day. In females, food consumption was decreased in comparison to controls by 12.7% ($p<0.01$) at 1000 mg/kg bw/day during week one. No other statistically significant decreases in food consumption in females were seen, although at the highest dose, food consumption was more than 10% lower than controls from day 18 of lactation. Based on food consumption and bodyweight, compound intake was generally within 10% of the theoretical values.

Bodyweight in males was statistically significantly lower than controls from 30 mg/kg bw/day on days 1 to 4, with decreases of around 5% ($p<0.05$) at this dose. The differences in bodyweight increased with increasing dose and are considered to be related to treatment. Bodyweight in males at 30 mg/kg bw/day were also lower than controls (8.6%, $p<0.05$) on day 25. Consistent lower bodyweights were seen in males at 1000 mg/kg bw/day. In females, the only significantly lower bodyweights were seen at 1000 mg/kg bw/day on days 1 and 4, with decreases of 4.7 and 1%.

All females were cycling during the study, with a small number of rats having irregular cycles. These were not treatment related. One female at 100 mg/kg bw/day had a pre-coital interval of 13 to 16 days; all other rats mated within 4 days of pairing. There were no treatment-related effects on the number of animals mating, the conception rate, or the mean gestation length. Litter size and survival were not affected by treatment, and there were no treatment-related effects on sex ratio. Reproductive information is presented in the following table:

**Reproductive parameters for Sprague-Dawley rats.**

| **Dose (mg/kg bw/day)** | **0** | **10** | **30** | **100** | **300** | **1000** |
|---|---|---|---|---|---|---|
| % mating | 100 | 100 | 100 | 100 | 100 | 100 |
| Conception rate % | 88 | 100 | 100 | 100 | 100 | 100 |
| Mean gestation length (days | 22.4 | 22.3 | 22.8 | 22.6 | 22.4 | 22.4 |
| Implantation sites | 17.9 | 16.1 | 17.3 | 17.9 | 17.3 | 16.5 |
| Total pups (day 1) mean | 16.6 | 15.0 | 15.9 | 16.1 | 15.1 | 15.3 |
| Post implantation survival index (%) | 93 | 93 | 92 | 90 | 88 | 92 |
| Live birth index (%) | 97 | 99 | 98 | 99 | 100 | 96 |
| Viability index day 4 (%) | 97 | 99 | 100 | 97 | 98 | 98 |
| Pups at day 25 (mean) | 8 | 8 | 8 | 7.9 | 7.9 | 8 |

The litter bodyweight was decreased on days 21 and 25 of lactation at 1000 mg/kg bw/day by 12% ($p<0.05$). Bodyweight gain was also decreased for days 1 to 25 at 1000 mg/kg bw/day and for days 18 to 21 of lactation at 300 mg/kg bw/day (26%, $p<0.01$).
On post-mortem examination, no treatment-related findings were seen in males or pups. In females, the liver lobes were thickened and swollen in two animals (one each at 100 and 1000 mg/kg bw/day); however, the significance of this finding is unclear.

**Conclusion**

Neotame did not affect reproductive capacity in rats up to a dose of 1000 mg/kg bw/day in a range finding study in rats.

**4. Willoughby CR (1996) Reproductive performance range-finding dietary study of NC-00723 in rats. Lab Project No. 94/NST019/0533. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-1007**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study: | 21 September 1994–2 January 1995 |
| GLP: | OECD, UK, US FDA |
| Test chemical: | NC-00723, Lot no. 94MP081-4 |
| Test species: | Crl: CDBR VAF/Plus Sprague Dawley rats, Charles River UK, Margate, Kent |

**Study design**

Rats were acclimatised for 5 days before commencement of dosing. They were housed in groups during the acclimatisation phase, then individually for the rest of the study except during pairing. Food and water were freely available. NC-00723 was included in the diet at concentrations to give doses of 0, 500, 1000, 1500, 2000, 2500 or 3000 mg/kg bw/day, using 8 rats/sex/group. Females were fed treated diet for 29 days before pairing until lactation day 21. Males were fed treated diet for 29 days before pairing until after successful littering of the females. Selected $F_1$ pups were fed test diet for 14 days after weaning. During pairing, males received diets prepared to the females' specifications. No dietary analysis was conducted during this study.

Rats were paired on a one-to-one basis within treatment groups for a maximum of 3 weeks. Vaginal smears were done on females for 10 days before pairing to determine the regularity of the oestrus cycle. The cage was checked daily for any copulation plugs, and a vaginal smear to detect spermatozoa was performed daily. The day on which evidence of mating was detected was designated day 0 of gestation, and the rats were separated. The pre-coital interval was recorded.

Rats were observed twice daily for mortality and morbidity, and daily for evidence of reaction to treatment. Bodyweight of males was determined on days 7 and 3 before treatment, daily for the first four days of treatment, then twice weekly until the end of the study. Bodyweight of females was determined 7 and 3 days before treatment, daily for the first four days of treatment, twice weekly until pairing, on days 0, 3, 7, 10, 14, 17 and 20 of gestation and days 1, 4, 7, 11, 14, 18, and 21 of lactation. Food consumption was determined daily for one week pre-treatment and for the first week of treatment, then weekly thereafter until pairing. Following conclusion of pairing, food consumption was determined weekly for males. For females, food consumption was determined twice weekly during gestation and lactation.

Females were inspected three times daily for the onset, progress and completion of parturition. Gestation length was recorded. All offspring were examined approximately 24 h after birth. The number of offspring born (both alive and dead), the litter weight of live offspring, the sex ratio and any individual observations were determined. Pups were examined daily for signs of reaction to treatment and mortality. On day 4, the litter size was reduced to 8 pups (4/sex where possible). Culled offspring, and any pups found dead were examined internally and externally for any abnormalities. The bodyweight of the litter was determined on days 1, 4, 7, 11, 14, 18, and 21. The sex ratio was determined on days 1, 4 and 21. On day 21, 2/sex/litter were selected and maintained on treated diet, with 1/sex/litter receiving the same diet formulation as the parents (LAD 2) and the others receiving RM1 diet. Pups were monitored daily, with bodyweights recorded daily from day 21 to 28 and days 31 and 35. Food consumption was determined daily for days 21 to 27, 28–30 and 31–34.

At the end of the study, females and their litters were killed and examined internally and externally for any macroscopic abnormalities. The number of implantation sites was recorded. Females not littering by day 25 after mating were killed and examined, including determination of any implantation sites. Male adults were also examined macroscopically.

**Results**

One male at 2500 mg/kg bw/day was killed for humane reasons following damage to the buccal cavity. This was not considered to be related to treatment. Brown staining of the coat, an ungroomed appearance, thin rats and some tremors on handling were noted at a slightly increased incidence from 2500 mg/kg bw/day. Food consumption in males was decreased in comparison to controls during the first week in all treatment groups, with consumption of the first day at 500 mg/kg bw/day only 25% of control food intake. Over the first week, food intake at 500 mg/kg bw/day in males was decreased 14% ($p<0.05$) in comparison to controls. Similar decreases were seen during the first week in females, with no difference in food consumption between groups in females during gestation or lactation. Compound intake was generally within 10% of intended levels.

Bodyweight in males was statistically significantly lower than controls from 500 mg/kg bw/day on days 1 and 2, although at this dose decreases were only around 6% ($p<0.01$ or $p<0.05$). The differences in bodyweight increased with increasing dose and are considered to be related to treatment. In females, significantly lower bodyweights were seen from 500 mg/kg bw/day on days 1, 2 and 10, with decreases of 6 to 8%. During gestation, bodyweights were significantly lower than controls from 1500 mg/kg bw/day (9%, $p<0.05$). There were no statistically significant lower bodyweights during lactation, however on day 1, females from 1500 mg/kg bw/day had bodyweights around 12% lower than controls.

All females were cycling during the study, with one rat having irregular cycles. This was not treatment related. No treatment-related changes were seen in the pre-coital interval. There were no treatment-related effects on the number of animals mating, the conception rate, or the mean gestation length. Litter size and survival were not affected by treatment, and there were no treatment-related effects on sex ratio. Reproductive information is presented in the following table:

**Reproductive parameters for Sprague-Dawley rats.**

| Dose (mg/kg bw/day) | 0 | 500 | 1000 | 1500 | 2000 | 2500 | 3000 |
|---|---|---|---|---|---|---|---|
| % mating | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Conception rate % | 88 | 88 | 100 | 100 | 100 | 100 | 100 |
| Mean gestation length (days | 22.4 | 22.4 | 22.4 | 22.4 | 22.3 | 22.4 | 22.5 |
| Implantation sites | 18.0 | 16.6 | 18.5 | 16.9 | 15.6 | 16.9 | 17.3 |
| Total pups (day 1) | 16.0 | 15.1 | 16.0 | 15.8 | 15.0 | 15.0 | 15.5 |
| Post implantation survival index (%) | 89 | 92 | 87 | 93 | 95 | 89 | 90 |
| Live birth index (%) | 97 | 98 | 99 | 99 | 99 | 99 | 100 |
| Viability index day4 (%) | 99 | 98 | 99 | 100 | 100 | 99 | 99 |

The litter bodyweight was decreased on days 21 of lactation from 1500 mg/kg bw/day by 10% ($p<0.05$) or more. Bodyweight gain was also decreased for days 1 to 21 from 1500 mg/kg bw/day and for days 18 to 21 of lactation from 500 mg/kg bw/day (30%, $p<0.001$).

Food consumption in pups maintained on treated diet until day 38 showed differences between the two diets. In pups fed the adult treated diet, females from 2000 mg/kg bw/day had statistically significantly decreased food consumption on the second day after weaning (d22), with decreases of around 31%. Other groups, including rats from 1000 mg/kg bw/day had decreases in food consumption of more than 10% in comparison to controls, however these were not statistically significant. Weanling rats offered the RMI diet had lower food consumption, with statistically significant decreases seen in males from 1000 mg/kg bw/day and in females from 1500 mg/kg bw/day (45 to 55%). Compound intakes in treated weanlings were higher than intended over the whole of the 2-week period; this was probably due to historical control information being used to calculate the required dietary concentration. In males on LAD diet, bodyweights were lower than controls from 1500 mg/kg bw/day for days 22 to 24 (15%, $p<0.05$ or 0.01) while in females on this diet, they were lower from 1000 mg/kg bw/day only on day 22 (12%, $p<0.05$). On the RMI diet, males at 1000 mg/kg bw/day had lower bodyweights on days 22 and 24 (8 to 10%), with consistently lower bodyweights seen from 2000 mg/kg bw/day throughout the observation period. in females, consistent lower bodyweights were seen from 1500 mg/kg bw/day throughout the observation period.

On post-mortem examination, adults had brown staining on the head, and one high dose male had a pale raised area on the surface of the spleen. No other treatment-related findings were seen in adults or pups.

**Conclusion**

Neotame did not affect reproductive capacity in rats up to a dose of 3000 mg/kg bw/day.

**5. Willoughby CR (1997) NC-00723: Two Generation Reproductive Study by Dietary Administration to CD Rats . Lab Project No. 96/NST024/0643. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-1001**

Laboratory: Huntingdon Life Sciences Ltd, Suffolk England
Date of study: 26 April 1995–18 January 1996
GLP: OECD, UK, US FDA

Test chemical: NC-00723, Lot no. 95MP028-3
Test species: Crl: CDBR VAF/Plus Sprague Dawley rats, Charles River UK, Margate, Kent

**Study design**

Rats were acclimatised for 10 days before commencement of dosing. They were housed individually during the acclimatisation phase and for the rest of the study except during pairing. Food and water were freely available. NC-00723 was included in the diet at concentrations to give doses of 0, 100, 300 or 1000 mg/kg bw/day, using 28 rats/sex/group. Females were fed treated diet for 29 days before pairing, while males were fed treated diet for 71 days before pairing. Treatment continued through pairing and until females were killed at weaning of the pups. During pairing, males received diets prepared to the females' specifications. The $F_1$ pups were treated at the appropriate concentration from weaning until selection for the next generation, and for at least 10 weeks after selection before pairing. Treatment was continued until weaning of the $F_2$ generation. Diet homogeneity was determined pre-study, and the achieved concentrations were determined at interval throughout the study.

Rats were paired on a one-to-one basis within treatment groups for a maximum of 3 weeks. Vaginal smears were done on females for 10 days before pairing to determine the regularity of the oestrus cycle. The cage was checked daily for any copulation plugs, and a vaginal smear to detect spermatozoa was performed daily. The day on which evidence of mating was detected was designated day 0 of gestation, and the rats were separated. The pre-coital interval was recorded. The same procedure was followed for both generations, however care was taken with $F_1$ pairings to avoid sibling matings.

Rats were observed daily for mortality and morbidity, and for evidence of reaction to treatment. Rats dying during the study were examined macroscopically for abnormalities. Bodyweight of males were determined at the start of the study, and weekly until the end of the study. Bodyweight of females was determined at the start of the study, weekly until pairing, on days 0, 6, 13, and 20 of gestation and days 1, 4, 7, 14, and 21 of lactation. Food consumption was determined weekly except during pairing.

In both generations, females were inspected two to three times daily for the onset, progress and completion of parturition. Gestation length was recorded. All offspring were examined approximately 24 h after birth. The number of offspring born (both alive and dead), the litter weight of live offspring, the sex ratio and any individual observations were determined. Pups were examined daily for signs of reaction to treatment and mortality. On day 4, the litter size was reduced to 8 pups (4/sex where possible). Culled offspring, and any pups found dead were examined internally and externally for any abnormalities. The bodyweight of the litters were determined on days 1, 4 (before and after culling), 7, 14, and 18. Individual pup bodyweights were determined on day 21. The sex ratio was determined on days 1, 4 and 21. Pups were monitored for signs of physical development, including pinna unfolding, hair growth, tooth eruption and eye opening. $F_1$ pups selected to produce the next generation were individually assessed for the time of vaginal opening or balanopreputial separation. The $F_1$ pups were also tested for activity level, using infrared cameras overnight, their ability to learn to swim a Y-maze, and their response to auditory and visual stimulation. Pups were selected for the next generation within a limited age range, and for a representational bodyweight. If it was not possible to select pups from different litters, additional pups were selected from available litters to ensure the correct number of rats in the second generation.

All adults ($F_0$ and $F_1$) were killed after weaning of the pups and examined internally and externally for any macroscopic abnormalities. Females failing to mate or give birth were killed on day 25 after last mating opportunity, or on day 25 post-coitum. Unselected $F_1$ pups and all $F_2$ pups were also examined for macroscopic abnormalities. The examination included a detailed examination of the cranial, thoracic, pelvic and abdominal cavities and their viscera. The external and cut surfaces of organs and tissues were examined, and the brain, epididymides, ovaries, prostate, seminal vesicles, spleen, testes, thymus and uterus with cervix were weighed. The number of implantation sites was also recorded. No histopathological examination was performed as all tissues were examined in a 13-week rat toxicology study (PCR 0988) up to dosages of 3000 mg/kg bw/day.

**Results**

There were no treatment-related clinical signs or deaths in either generation. In the $F_1$ generation, one male at 300 mg/kg bw/day was euthanased due to damage to the buccal cavity, and one female at 1000 mg/kg bw/day was euthanased after developing mastitis. These were not considered treatment related.

Food consumption in males in the first generation was decreased in comparison to controls throughout the study from 100 mg/kg bw/day. At 100 mg/kg bw/day decreases were between 4 and 9% ($p<0.05$). At 1000 mg/kg bw/day, decreases were between 5 and 9% ($p<0.05$). In females, food consumption was decreased in comparison to controls by 9% ($p<0.001$) at 1000 mg/kg bw/day during week one. No other statistically significant decreases in food consumption in females in the first generation were seen. In the second generation, food consumption was consistently decreased from week 4 in males at 1000 mg/kg bw/day (7–10%, $p<0.01$). No statistically significant decreases in food consumption was observed in females. Based on food consumption and bodyweight, compound intake was within 10% of the theoretical values at all times during the study.

In the first generation, bodyweight was statistically significantly lower than controls at 1000 mg/kg bw/day throughout the study, with decreases of 5 ($p<0.05$) to 9% ($p<0.001$). In $F_0$ males, statistically significantly lower bodyweights were also seen at 100 mg/kg bw/day throughout the study 4 ($p<0.05$) to 7% ($p<0.01$). Given the consistency of this finding, it is considered to be treatment related. In adults in the $F_1$ generation, consistent statistically significantly lower bodyweights were seen at 1000 mg/kg bw/day, with decreases in males from 8 to 12% ($p<0.01$, $p<0.001$) and in females from 7 to 10% ($p<0.01$).

All females in both generations had normal oestrus cycles during the study. There were no treatment-related differences in pre-coital intervals in either generation. There were no treatment-related effects on the number of animals mating, the conception rate, or the mean gestation length. Litter size and survival were not affected by treatment, and there were no treatment-related effects on sex ratio. Reproductive information is presented in the following tables for both generations:

**Reproductive parameters for Sprague-Dawley rats–$F_0$ generation.**

| Dose (mg/kg bw/day) | 0 | 100 | 300 | 1000 |
|---|---|---|---|---|
| % mating | 100 | 100 | 100 | 100 |
| Conception rate % | 96 | 100 | 96 | 96 |
| Mean gestation length (days) | 22.7 | 22.6 | 22.8 | 22.7 |
| Implantation sites | 16.5 | 15.6 | 15.6 | 16.5 |
| Total pups (day 1) | 14.9 | 14.1 | 14.3 | 15.2 |
| Viability index day 4 | 92 | 96 | 97 | 90 |
| Lactation index day 21 | 97 | 99 | 98 | 99 |

**Reproductive parameters for Sprague-Dawley rats–$F_1$ generation.**

| Dose (mg/kg bw/day) | 0 | 100 | 300 | 1000 |
|---|---|---|---|---|
| % mating | 100 | 96 | 96 | 100 |
| Conception rate % | 93 | 100 | 85 | 100 |
| Mean gestation length (days) | 22.8 | 22.6 | 22.7 | 22.8 |
| Implantation sites | 15.0 | 15.1 | 14.7 | 15.0 |
| Total pups (day 1) | 14.3 | 14.5 | 13.6 | 14.5 |
| Viability index day4 | 95 | 95 | 93 | 90 |
| Lactation index day 21 | 93 | 91 | 98 | 94 |

The litter bodyweight was decreased on days 1 and 21 of lactation at 300 and 1000 mg/kg bw/day for males in the F1 generation by 7 to 13% ($p<0.05$ to $p<0.001$). In female pups, bodyweight was reduced at both these times at 1000 mg/kg bw/day, and on day 21 at 300 mg/kg bw/day. In the second generation, pup weight at 1000 mg/kg bw/day was decreased on day 21 by around 15% in comparison to controls ($p<0.01$).

In $F_1$ pups, there were no treatment-related effects on physical development or on auditory or visual tests. There was a slight decrease in activity in males at 1000 mg/kg bw/day, and these rats also took longer in the swimming test (15 to 32% more time than controls). This may be related to the reduced bodyweight, although no similar effects were seen in females and these observations were within background control ranges. No treatment-related differences in sexual development (vaginal opening or balanopreputial separation) were seen.

On post-mortem examination, no treatment-related findings were seen in $F_1$ pups dying before day 21. In $F_0$ males and females a thickened stomach wall was seen; the increase in incidence was small, and is considered an incidental finding. In the $F_2$ pups, there was a slight increase in the incidence of renal pelvic cavitation; the significance of this was unclear. The treatment-related findings were noted in $F_1$ adults at any dose. The weight of the spleen and the thymus were slightly decreased in some groups of treated rats in both generations. There was no clear dose relationship, and the magnitude of the changes was small; this is therefore considered not treatment related.

As no effects were seen on any reproductive parameters, the NOEL for reproductive effects is 1000 mg/kg bw/day.

**Conclusion**

Neotame did not affect reproductive capacity in rats throughout 2 generations up to a dose of 1000 mg/kg bw/day.

**DEVELOPMENTAL STUDIES**

**1. Willoughby CR (1996) NC-00723: Dietary Teratology Study in the Rat. Lab Project No. 95/NST025/1381. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-0999**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study | 14 June 1995–8 December 1995 |
| GLP: | OECD, UK, US FDA |
| Test chemical: | NC-00723, Lot no. 94MP081-4 |
| Test species: | Crl: CDBR VAF/Plus Sprague Dawley rats, Charles River UK, Margate, Kent |

**Study design**

Female rats were acclimatised for 13 days and examined daily. Rats were housed individually except during pairing, in controlled conditions with free access to food and water. Doses of 0, 100, 300 or 1000 mg/kg bw/day, using 24/group were used, with dietary concentrations adjusted to give required doses. Dietary concentrations were assessed in week 1 of treatment and weeks 2 and 3 of gestation. The concentration was found to be acceptable at these times. Females were dosed for 28 days before pairing and until day 20 after mating. Vaginal smears were taken to determine the stage of the oestrus cycle, and females were checked for evidence of mating after pairing.

Rats were assessed twice daily for mortality and morbidity. Rats were observed once daily for abnormal clinical signs. Bodyweight was determined before treatment, on days 0, 1, 2, 3, 7, 10, 14, 21, and 28 of treatment before pairing and on days 0, 3, 7, 10, 14, 17 and 20 of gestation. Food consumption was assessed daily for the first week, weekly for the next 3 weeks and for days 0–2, 3–6, 7–9, 10–13, 14–16 and 17–19 of gestation. Following euthanasia, females were examined macroscopically for signs of disease or reaction to treatment. The number of corpora lutea, implantation sites (including checking apparently non-pregnant rats by staining the uterus), resorption sites and the number and distribution of foetuses in each uterine horn were determined.

Foetuses were weighed, sexed and examined. Half of each litter had the neck, thoracic and abdominal cavities dissected and examined. The foetuses were eviscerated, processed and stained with Alizarin red for skeletal examination. The remaining foetuses were fixed in Bouin's fixative and sectioned freehand. No histopathological examination was done.

**Results**

There were no treatment-related clinical signs, change in the general condition of the animals or mortality. Food consumption was decreased during the first week of treatment. On day 1, food consumption was decreased by 32% in comparison to controls at 300 mg/kg bw/day and 63% at 1000 mg/kg bw/day ($p<0.001$). However, by day 2 there was a reversal of the food consumption at 300 mg/kg bw/day (105%) and 1000 mg/kg bw/day (85%) of control values.

The food consumption during the whole of the first week was decreased at 1000 mg/kg bw/day by 9% ($p<0.05$). Test article consumption was within 5% of intended levels at all stages of treatment. Bodyweights of rats at 1000 mg/kg bw/day were statistically significantly lower during the first week (up to 7%) but were not significantly different to control for the duration of the study.

Two females at 100 and one at 300 mg/kg bw/day were not pregnant. This was not considered to be treatment related. The number of corpora, implantations, viable young, sex ratios, pre and post implantations, and foetal and placental weights were not affected by treatment.

On post-mortem examination, there were no treatment-related maternal findings. In the foetuses, no increase in visceral abnormalities were seen. There were incidences of incomplete skeletal ossification, with some slight elevation at high dose; however, these were not statistically significant, fell within historical control ranges and had no dose-response relationship.

**Conclusion**

Neotame is not teratogenic when administered in the diet at up to 1000 mg/kg bw/day.

**2. Willoughby CR (1996) NC-00723: Maternal Toxicity Range Finding Study Administered by Gavage to the Rabbit. Lab Project No. 95/NST023/0748. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-1038**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study: | 19 March 1995–28 April 1995 |
| GLP: | OECD, UK, US FDA |
| Test chemical: | NC-00723, Lot no. 94MP081-4 |
| Test species: | NZW rabbits, Froxfield Farms (UK) Limited, Froxfield, Hampshire England, 18–26 weeks |

**Study design**

Oestrus was synchronised in females by injection of luteinising hormone. Rabbits were acclimatised to laboratory conditions before treatment. They were housed individually in controlled conditions with free access to food and water. Females were naturally mated with stock males from the same source, and were given an injection of luteinising hormone after mating to ensure ovulation. NC-00723 in aqueous methylcellulose was given daily by oral gavage on gestation day 6 to 19, at 0, 30, 100, 300 or 1000 mg/kg bw/day, using 6/group. Rabbits were examined daily for abnormal clinical signs. A detailed examination was done on days 6, 12 and 19. Rabbits were removed from their cages and the behaviour, temperature, condition of the buccal cavity, build, coat, abnormal secretions, excreta, eyelids, eyes, muscle reaction, posture, reflexes, respiratory system, skin and teeth were assessed. Mortality was assessed twice daily. Bodyweight, food and water consumption were assessed daily.

On day 29 after mating, females were euthanased and examined macroscopically for any abnormalities. The reproductive tract was assessed, and the number of corpora lutea, implantation sites and resorption sites recorded. The number and distribution of foetuses in each uterine horn were recorded. Foetuses were killed and discarded without examination.

**Results**

One rabbit at 1000 mg/kg bw/day was found dead; no treatment-related abnormalities were found on post-mortem examination. One female at 1000 mg/kg bw/day also had a total litter loss. Two control females were not pregnant. The only treatment-related clinical signs were pale faeces at 1000 mg/kg bw/day. There were no significant differences in bodyweight at any time during gestation. Food consumption was decreased during gestation days 13 to 19 from 100 mg/kg bw/day, with intakes less than 77% of control rabbits.

There were no treatment-related effects on corpora lutea, implantations, viable young, early or late resorptions or pre or post implantation losses. No treatment-related macroscopic abnormalities were found on post-mortem examination.

**Conclusion**

Neotame is not teratogenic in rabbits when administered by gavage at up to 1000 mg/kg bw/day.

**3. Willoughby CR (1996) NC-00723: Teratology Study in the Rabbit by Gavage. Lab Project No. 96/NST026/0085. Sponsor The Monsanto Company, Skokie Illinois. Sponsor Study No. PCR-1023**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Suffolk England |
| Date of study: | 3 July 1995–30 April 1996 |
| GLP: | OECD, UK, US FDA |
| Test chemical: | NC-00723, Lot no. 94MP081-4 |
| Test species: | NZW rabbits, Froxfield Farms (UK) Limited, Froxfield, Hampshire England |

**Study design**

Oestrus was synchronised in females by injection of luteinising hormone. Rabbits were acclimatised to laboratory conditions for one week before treatment. They were housed individually in controlled conditions with free access to food and water. Females were naturally mated with stock males from the same source, and were given an injection of luteinising hormone after mating to ensure ovulation. NC-00723 in aqueous 0.5% w/v methylcellulose was given daily by oral gavage on gestation day 6 to 19, at 0, 50, 150, or 500 mg/kg bw/day, using 20/group. Where there were insufficient pregnant animals in the group (at 0 and 500 mg/kg bw/day), and five additional animals/group were added to the study later.

Rabbits were examined twice daily for abnormal clinical signs. A detailed examination was done on days 6, 12 and 19. Rabbits were removed from their cages and the behaviour, temperature, condition of the buccal cavity, build, coat, abnormal secretions, excreta, eyelids, eyes, muscle reaction, posture, reflexes, respiratory system, skin and teeth were assessed. Mortality was assessed twice daily. Bodyweight and food consumption were assessed daily, although food consumption was reported for days 1 to 5, 6 to 12, 13 to 19, 20 to 23 and 24 to 28 of gestation.

A pharmacokinetic phase was also conducted as part of the study, using 5/treatment group. Rabbits were treated in a similar fashion to the main study animals, although no detailed clinical examination was done. On days 6, 13 and 19, at 0.5, 1, 4, 8 and 24 h after dosing, blood samples were taken, plasma extracted and frozen and later analysed.

Rabbits were killed on day 20, and discarded without examination, other than to determine that they were pregnant.

Animals aborting during the study were killed and examined. The number of corpora lutea, and implantation sites were determined. Where possible, the foetuses were examined. On day 29 after mating, females were euthanased and examined macroscopically. The reproductive tract was assessed, and the number of corpora lutea, implantation sites and resorption sites recorded. The number and distribution of foetuses in each uterine horn were recorded. The weight of each foetus and placenta, and any external abnormalities were recorded. The neck, thoracic and abdominal cavity of each foetus was dissected, the contents examined and the sex recorded. Foetuses were prepared either for skeletal or visceral examination.

**Results**

One rabbit at 150 and one at 500 mg/kg bw/day were found dead. The rabbit at 150 mg/kg bw/day died as the result of a dosing injury. The cause of death in the rabbit at 500 mg/kg bw/day was not clear was attributed to a marked and persistent decrease in food consumption and a large decrease in bodyweight and therefore a treatment related affect cannot be definitely concluded. Two females at 500 mg/kg bw/day aborted late in pregnancy. Before abortion, they had decreased food consumption and lost bodyweight. There were no significant differences in bodyweight for any group during gestation. Food consumption was variable in treated rabbits during gestation, with intakes for days 13 to 19 decreased from 150 mg/kg bw/day.

NC-00723 Cmax levels ranged from 250 to 2600 ng/mL, while Cmax for NC-00751 was 21,100 to 121,300 ng/mL. The Cmax and AUC for NC-00723 increased with dose; however increases were not proportional to the dose. NC-00751 AUC values increased in a dose-related fashion. There was no evidence for increasing plasma levels with an increased duration of dosing. These results indicate that NC-00723 is absorbed following oral administration in the rabbit, and is extensively metabolised to NC-00751, as occurs in other species.

There were no treatment-related effects on corpora lutea, implantations, viable young, early or late resorptions or pre or post implantation losses. No treatment-related macroscopic abnormalities in the dams or foetuses were found on post-mortem examination.

**Conclusion**

Neotame is not teratogenic in rabbits when administered by gavage at up to 500 mg/kg bw/day.

## GENOTOXICITY STUDIES

*Gene mutation assays — NC-00723*

**1. Riccio ES & Stewart KR (1994) Salmonella-Escherichia coli / microsome plate incorporation assay of NC-00723. Laboratory Study No SRI 6160-A200-94 Sponsor The NutraSweet Company. Sponsor Study No: PCR-0963**

Laboratory: The NutraSweet Company, Preclinical Research, USA
Date of study: 25/07/94–02/12/94
GLP: US FDA
Guidelines: US-FDA 21 CFR Part 58
Test chemical: NC-00723 Lot No. 94UP8-3-4

**Study design**

NC-00723 in DMSO was tested for its ability to induce reverse mutations in *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100 and *Escherichia coli* strain WP2 (*uvr*A), with or without S9 microsomal activation mixture. Positive controls used were 9-aminoacridine hydrochloride, 2-anthramine, *N*-ethyl- *N* '-nitro- *N* -nitrosoguanidine, 2-nitrofluorene and sodium azide. The negative control was DMSO.

**Results**

**Preliminary range-finding and cytotoxicity test**

In a range-finding study using strain TA-100, concentrations up to 10,000 μg/plate were used, with or without activation by S9. There was no cytotoxicity at any concentration and levels of NC-00723 up to 10,000 μg/plate were therefore used in the mutagenicity tests.

**Mutagenicity tests**

Each series of tests was repeated three times using 312–10,000 μg NC-00723/plate, in the presence or absence of S9. In each of the tests, no positive response was observed with any of the tester strains, with or without microsomal activation with S9. Significantly increased mutation rates were seen with each of the positive controls.

**Conclusions**

Under the conditions of this study, NC-00723 was not mutagenic in *Salmonella typhimurium* or *Escherichia coli* in the presence or absence of S9.

**2. Rudd CJ & Mirsalis JC (1994) L5178Y mouse lymphoma (MOLY) cell *tk*$^{+/-}$ → *tk*$^{-/-}$ gene mutation assay with NC-00723. Laboratory Study No: SRI 6160-G001. Sponsor The NutraSweet Company. Sponsor Study No: PCR-0965**

Laboratory: The NutraSweet Company, Preclinical Research, USA
Date of study: 22/7/94–26/10/94
GLP: US FDA
Guidelines: US-FDA 21 CFR Part 58 Good laboratory practice for nonclinical laboratory studies
Test chemical: NC-00723 Lot No. 94UP8-3-4

**Study design**

**Preliminary range-finding and cytotoxicity test**

The cytotoxicity of NC-00723 in L5178Y mouse lymphoma cells was initially tested in the presence and absence of S9, using concentrations up to 3800 μg/mL, the highest dose being equivalent to a concentration of approximately 10 mM. Cell growth was inhibited in a concentration-dependent manner after treatment for 4 h with NC-00723 at 500–3800 μg/mL without S9, and at 250–3800 μg/mL with S9. In the absence of S9, the cell survival rate was 87% at 500 μg/mL and 17% at 1000 μg/mL. In the presence of S9, cell survival was 79% at 250 μg/mL and 65% at 500 μg/mL.

**Mutagenicity test**

Based on the cytotoxicity data, replicate mutagenesis experiments were performed with and without S9 using NC-00723 (in 99.9% DMSO) at concentrations of 100, 200, 400, 600, 800 and 1000 μg/mL. Positive controls were ethyl methanesulfonate (without S9) and 3-methylcholanthrene (with S9). The negative control was DMSO. Cells were grown in the presence of trifluorothymidine at 5 μg/mL.

**Results**

Cultures treated with 1000 μg/mL in the presence of S9 could not be evaluated for mutant frequency because of the cytotoxicity of this level of NC-00723. Thus, the highest concentration for which a result was obtained with S9 was 800 μg/mL with S9. No increase in the frequency of mutant (trifluorothymidine-resistant) cells was observed. The average frequency of mutant cells in cultures without S9 was 16–49 mutants/$10^6$ cells (0.6–1.4 times the solvent control values) and the frequency with S9 was 29–40 mutants/$10^6$ cells (0.8–1.1 times the solvent control values). The mutant frequencies of the positive control cultures, treated with ethyl methanesulfonate or 3-methylcholanthrene, were increased on average 9.1 and 7.4 times, respectively.

**Conclusion**

Under the conditions of this study, NC-00723 was not mutagenic in L5178Y mouse lymphoma cells in the presence or absence of an exogenous metabolic activation system (S9).

*DNA damage and other genotoxic effects — NC-00723*

**3. Suing KD & Winegar RA (1994) Measurement of chromosomal damage in Chinese hamster ovary (CHO) cells treated with NC-00723. Laboratory Study No: SRI 6160-C300-94. Sponsor The NutraSweet Company Sponsor Study No: PCR 0964**

Laboratory: The NutraSweet Company, Preclinical Research, USA
Date of study: 22/07/94–02/12/94
GLP: US FDA
Guidelines: US-FDA 21 CFR Part 58
Test chemical: NC-00723 Lot No. 94UP8-3-4

**Study design**

NC-00723 in DMSO was tested for its ability to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells in the presence or absence of S9 microsomal activation mixture. Positive controls were cyclophosphamide and methyl methanesulfonate for the tests with and without S9, respectively. The negative control was DMSO.

**Range-finding study**

In an initial range-finding study, CHO cells were treated with the test material at concentration levels of 0–3800 µg/mL, in the presence or absence of S9 (the highest dose being equivalent to a concentration of 10 mM NC-00723). In a second study, to determine the concentration-dependent nature of the cytotoxicity, CHO cells were incubated with NC-00723 at concentrations of 0–500 µg/mL and 0–2000 µg/mL, in the absence and presence of S9, respectively. In the absence of S9, cells were incubated for 21 h with the test material. In the presence of S9, cells were incubated with the test material for 3 h, followed by washing and incubation for 18 h. Colchicine was added to all cells 2.5 h before the end of the incubation period.

**Chromosomal aberration study**

The concentration ranges chosen for the first test were 62.5, 125, 250 and 500 µg/mL without S9 and 250, 500 and 1000 µg/mL with S9; and, replicate study was done at 0, 62.5, 125 and 250µg/mL without S9 and 0, 250, 500 and 1000 µg/mL with S9. The same method was used as described for the dose range-finding study. At the end of the incubation, the degree of confluence relative to controls and the general health of the cells were assessed. Cells containing metaphase chromosomes were examined for chromosomal aberrations including chromatid and chromosome gaps, deletions and exchanges.

**Results**

Cells treated with 500 µg/mL NC-00723 in the absence of S9 could not be scored due to poor cell morphology and low number of cells in metaphase. No significant increase in cells with structural aberrations was observed at any dose, with or without S9. The positive controls, cyclophosphamide and methyl methanesulfonate, produced significant increases in the number of cells with structural chromosomal aberrations.

**Conclusion**

Under the conditions of this assay, NC-00723 did not demonstrate any evidence of clastogenicity in Chinese hamster ovary cells in the presence or absence of an exogenous metabolic activation system (S9).

**4. Garrett SL, Kier LD, Carbone LA and McAdams JG (1997) Mouse bone marrow micronucleus assay of NC-00723. Laboratory Study No: MSL-15204, Sponsor The Monsanto Company. Sponsor Study No: PCR-1026**

Laboratory: Environmental Health Laboratory, Monsanto Chemical Company, USA
Date of study: 14/10/96–13/12/96
GLP: US FDA

Guidelines: OECD Guideline No. 474, US FDA and ICH
Test chemical: NC-00723 95MP028-3
Test species: CD-1(ICR)BR mice; Charles River Laboratories Inc

**Study design**

NC-00723 was tested for its ability to induce micronuclei in polychromatic erythrocytes (PCEs) of mice. Groups of mice (5/sex/time point/dose level) were administered a single oral gavage dose volume of 10mL/kg of NC-00723 suspended in 0.5% methylcellulose (w/v)/0.1% Tween 80 (v/v) at dose levels of 500, 1000 or 2000 mg/kg bw. Bone marrow was collected 24 and 48 h after treatment. Cells were examined for the presence of micronucleated PCEs and the ratio of PCE/total erythrocytes was calculated. A positive control group was administered cyclophosphamide in 0.5% methylcellulose (w/v)/0.1% Tween 80 (v/v) and bone marrow collected at 24 h only. A negative control group was administered 5% methylcellulose (w/v)/0.1% Tween 80 (v/v).

The dose levels were based on two range finding studies, one in which no toxicity was observed at dosages of 1000 or 2000 mg/kg bw and a further study in which 2000 mg/kg bw was confirmed as the maximum tolerated dose.

**Results**

There were no deaths or clinical abnormalities and no changes in bodyweights, frequency of micronucleated PCEs or PCE/total erythrocyte ratios in any NC-00723-treated groups compared to the test groups. In the cyclophosphamide-treated group, there was a significant increase in the incidence of micronucleated PCEs in both male and female mice. In addition, bodyweights were decreased for the female positive control group.

**Conclusion**

Under the conditions of this assay, NC-00723 did not induce an increase in micronuclei in the polychromatic erythrocytes of mice.

*Gene mutation assays — derivative NC-00751*

**1. Curtiss SW McAdams JG & Kier LD (1997) Ames/*Salmonella* assay of NC-00751. Laboratory Study No: MSL-15167. Sponsor: Monsanto Company Sponsor Study No. PCR 1137**

Laboratory: Ceregen, Monsanto, Environmental Health Laboratory, USA
Date of study: 03/12/96–13/01/97
GLP: US-FDA
Guidelines: OECD Guideline No. 471, US FDA and ICH
Test chemical: NC-00751, Lot No. IP-1281-72-4

**Study design**

NC-00751 in DMSO was tested for its ability to induce reverse mutations at various *his* loci in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537, with or without S9 microsomal activation mixture.

Positive controls used were 2-acetylaminofluorene, 9-aminoacridine, 2-anthramine, benzo[a]pyrene, cumene hydroperoxide, danthron, 4-nitroquinoline-*N*-oxide and sodium nitrite. The negative control was DMSO.

**Results**

**Preliminary range-finding and cytotoxicity test**

In a range-finding study using strain TA-100, concentration levels up to 5 mg/plate were used, with or without activation by S9. No cytotoxicity or precipitation of the test substance was seen at any concentration and levels of NC-00751 up to 5 mg/plate were therefore used in the mutagenicity tests.

**Mutagenicity tests**

Each series of tests was repeated three times using 0–5.0 mg/plate, in the presence or absence of S9. In each of the tests, no positive response was observed with any of the tester strains, with or without the presence of microsomal enzymes. Significantly increased mutation rates were seen with each of the positive controls.

**Conclusions**

Under the conditions of this study, NC-00751 was not mutagenic in *Salmonella typhimurium* in the presence or absence of an exogenous metabolic activation system (S9).

**2. Cabonce MA, Asbury KJ, McAdams JG, Wagner CA & Kier LD (1997) AS52/XPRT gene mutation assay of NC-00751 Laboratory Study No: EHL 96184, Sponsor: Monsanto Company. Sponsor Study No: PCR-1138**

Laboratory: Monsanto, Environmental Health Laboratory, USA
Date of study: 17/12/96–08/04/97
GLP: OECD, US-FDA
Guidelines: OECD Guideline No. 476, US FDA and ICH
Test chemical: NC-00751, Lot No. IP-1281-72-4

**Study design**

**Preliminary range-finding, cytotoxicity test**

Solubility of NC-00751 and cytotoxicity to AS52/XPRT Chinese hamster ovary (CHO) cells were initially tested using NC-00751 at 0–5000 µg/mL in DMSO and S9 concentrations of 0, 1, 5 and 10% (representing the percentage of S9 (v/v) in the S9/cofactor mixture). Precipitation of the 5000 and 2500 µg/mL doses was observed. No significant toxicity was observed at any dose level, with or without S9, and there were no significant test article-related increases in mean mutant frequency or dose response.

**Mutagenicity test**

Based on the cytotoxicity data, triplicate mutagenesis experiments were performed, with and without 5% S9, using NC-00751 at 313, 625, 1250, 2500 and 5000 µg/mL. A concentration of 5% S9 was selected as being the most appropriate in the absence of any indication of mutagenicity in initial experiments.

Positive controls were benzo[a]pyrene (with S9) and actinomycin D (without S9). The negative control was DMSO. Cells were grown in the presence of 10 μM 6-thioguanine.

**Results**

No significant increase in the frequency of mutant (6-thioguanine-resistant) cells was observed, with or without S9. The average frequency of mutant cells in cultures without S9 was 16–28 mutants/$10^6$ cells (0.57–1.01 times the solvent control value) and the frequency with S9 was 23–31 mutants/$10^6$ cells (1.12–1.53 times the solvent control value). The mutant frequencies of the positive control cultures, treated with benzo[a]pyrene and actinomycin D, were increased 27.1 and 2.5 times, respectively.

**Conclusions**

Under the conditions of this study, NC-00751 was not mutagenic in AS52 Chinese hamster ovary cells in the presence or absence of an exogenous metabolic activation system (S9).

*Gene mutations assays — derivative NC-00764*

**1. Curtiss SW, McAdams JG & Kier LD (1998) Ames/*Salmonella* assay of NC-00764. Laboratory Study No: EHL 96168. Sponsor: Monsanto Company. Sponsor Study No: PCR-1086**

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|---|---|
| Laboratory: | Monsanto, Environmental Health Laboratory, USA |
| Date of study: | 03/12/96–20/01/97 |
| GLP: | OECD, US-FDA |
| Guidelines: | OECD Guideline No. 471, US FDA and ICH |
| Test chemical: | NC00764 Lot No. IP-1123-264-1 |

**Study design**

NC-00764 in DMSO was tested for its ability to induce reverse mutations at various *his* loci in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537, with or without S9 microsomal activation mixture. Positive controls used were 2-acetylaminofluorene, 9-aminoacridine, 2-anthramine, benzo[a]pyrene, cumene hydroperoxide, danthron, 4-nitroquinoline-*N*-oxide and sodium nitrite. The negative control was DMSO.

**Results**

**Preliminary range-finding and cytotoxicity test**

In a range-finding study using strain TA-100, concentration levels up to 5 mg/plate were used, with or without activation by S9. No cytotoxicity or precipitation of the test substance was seen at any dose and levels of NC-00764 up to 5 mg/plate were therefore used in the mutagenicity tests.

**Mutagenicity tests**

Each series of tests was repeated three times using 0–5.0 mg/plate, in the presence or absence of S9. In each of the tests, no positive response was observed with any of the tester strains, with or without the presence of microsomal enzymes. Significantly increased mutation rates were seen with each of the positive controls.

**Conclusions**

Under the conditions of this study, NC-00764 was not mutagenic in *Salmonella typhimurium* in the presence or absence of an exogenous metabolic activation system (S9).

**2. Cabonce M, Asbury K, McAdams JG, Wagner CA & Kier LD (1998) AS52/XPRT gene mutation assay with NC-00764 Laboratory Study No: EHL 96183. Sponsor Monsanto Chemical Co. Sponsor Study No: PCR-1087,**

Laboratory: Monsanto, Environmental Health Laboratory, USA
Date of study: 26/11/96–28/4/97
GLP: US-FDA
Guidelines: OECD Guideline No. 476, US FDA and ICH
Test chemical: NC-00764 Lot No. IP-1123-264-1

**Study design**

**Preliminary range-finding and cytotoxicity test**

The solubility of NC-00764 and its cytotoxicity to AS52/XPRT Chinese hamster ovary cells were initially tested using NC-00764 at 0–5000 µg/mL in DMSO, and S9 concentrations of 0, 1, 5 and 10% (representing the percentage of S9 (v/v) in the S9/cofactor mixture). No significant cytotoxicity was observed at any dose level; precipitation of NC-00764 was seen at the 5000 µg/mL level in some experiments.

**Mutagenicity test**

Based on the cytotoxicity data, triplicate mutagenesis experiments were performed, with and without 5% S9, using NC-00764 at 313, 625, 1250, 2500 and 5000 µg/mL. A concentration of 5% S9 was selected as being the most appropriate in the absence of an indication of mutagenicity in initial experiments. Positive controls were benzo[a]pyrene (with S9) and actinomycin D (without S9). The negative control was DMSO. Cells were grown in the presence of 10 µM 6-thioguanine.

**Results**

No significant increase in the frequency of mutant (6-thioguanine-resistant) cells was observed, with or without S9. The average frequency of mutant cells in cultures without S9 was 23–52 mutants/$10^6$ cells (0.48–1.11 times the solvent control value) and the frequency with 5% S9 was 31—50 mutants/$10^6$ cells (1.11–1.78 times the solvent control value). The mutant frequencies of the positive control cultures, treated with benzo[a]pyrene and actinomycin D, were increased 6.2 and 2.7 times, respectively.

**Conclusion**

Under the conditions of this study, NC-00764 was not mutagenic in AS52 Chinese hamster ovary cells in the presence or absence of an exogenous metabolic activation system (S9).

**3. Garrett SL, Kier LD, Carbone LA & McAdams JG (1998) Mouse bone marrow micronucleus assay of NC-00764. Laboratory Study No: EHS 96164. Sponsor: Monsanto Company. Sponsor Study No: PCR–1090**

| | |
|---|---|
| Laboratory: | Environmental Health Laboratory, Monsanto Chemical Company, USA |
| Date of study: | 02/12/96–30/01/97. |
| GLP: | US FDA, OECD |
| Guidelines: | OECD Guideline No. 474 US FDA and ICH |
| Test chemical: | NC-00764 Lot No. IP-1123-264-1 |
| Test species: | CD-1(ICR)BR mice; Charles River Laboratories Inc |

**Study design**

NC-00764 was tested for its ability to induce micronuclei in polychromatic erythrocytes (PCEs) of mice. Groups of mice (5/sex/time point/dose level) were administered a single oral gavage dose volume of 10mL/kg of NC-00764 suspended in 0.5% methylcellulose (w/v)/0.1% Tween 80 (v/v) at dose levels of 500, 1000 and 2000 mg/kg bw. Bone marrow was collected 24 and 48 h after treatment. Cells were examined for the presence of micronucleated PCEs and the ratio of PCE/total erythrocytes was calculated. A positive control group was administered cyclophosphamide in 0.5% methylcellulose (w/v)/0.1% Tween 80 (v/v) and bone marrow collected at 24 h only. A negative control group was administered 5% methylcellulose (w/v)/0.1% Tween 80 (v/v).

Due to a procedural error, only four bone marrow samples from the female 48 h vehicle control group could be analysed. A supplemental micronucleus study was performed at a dosage of 0 and 2000 mg/kg, with bone marrow collected at 24 and 48 h after treatment, and a cyclophosphamide positive control sample at the 24 h time point only.

The dose levels were based on two range finding studies; one in which no toxicity was observed at dosages of 1000 or 2000 mg/kg bw and a further study in which 2000 mg/kg was confirmed as the maximum tolerated dose.

**Results**

There were no deaths or clinical abnormalities and no changes in frequency of micronucleated PCEs or PCE/total erythrocyte ratios in any NC-00764-treated groups compared to the test groups. There were no test article related changes in bodyweights. In the cyclophosphamide-treated group, there was a significant increase in the incidence of micronucleated PCEs in both male and female mice.

**Conclusion**

Under the conditions of this assay, NC-00764 did not induce an increase in micronuclei in the polychromatic erythrocytes of mice.

*Gene mutations assays — derivative NC-00777*

**1. Balwierz PS & Bunch RT (1998) Evaluation of the mutagenic potential of NC-00777 (E-3091) in the Ames/*Salmonella* microsome assay. Laboratory Study No: SA4692. Sponsor: Monsanto Company. Sponsor Study No: PCR 1191**

Laboratory: Monsanto, Environmental Health Laboratory, USA
Date of study: 23/09/97–22/10/97
GLP: US-FDA
Guidelines: Part 58 of Title 21 of the Code of Federal Regulations
Test chemical: NC-00777, Lot No. KW-1307-272 (SP1456)

**Study design**

NC-00777 in DMSO was tested for its ability to induce reverse mutations at various *his* loci in *Salmonella typhimurium* strains TA 97a, TA98, TA100, TA102 and TA1535, with or without S9 microsomal activation mixture. Positive controls used were ICR-191 acridine, sodium azide, 2-aminoanthracene, cumen hydroperoxide, 2-nitrofluorene and danthron. The negative control was DMSO.

**Results**

Two mutation assays were carried out, each with three replicates, using 0–5.0 mg/plate, in the presence or absence of S9. In both assays a precipitate was observed at the 1 and 5 mg/plate concentrations in the top agar before it was poured onto the plates, but was not seen in the plates when they were evaluated microscopically. In each of the tests, no increases in the mean number of revertants and no cytotoxicity were observed with any of the tester strains, with or without the presence of microsomal enzymes. Significantly increased mutation rates were seen with each of the positive controls.

**Conclusions**

Under the conditions of this study, NC-00777 was not mutagenic in *Salmonella typhimurium* in the presence or absence of an exogenous metabolic activation system (S9).

**2. Cabonce M, Asbury K, McAdams JG, Wagner CA & Kier LD (1998) Amended report for AS52/XPRT gene mutation assay with NC-00777. Laboratory Study Number EHL 97143. Sponsor: Monsanto Chemical Company. Sponsor Study No: PCR-1192**

Laboratory: Monsanto, USA
Date of study: 26/08/97–25/02/98
GLP: US-FDA
Guidelines: OECD Guideline No. 476 US FDA and ICH
Test chemical: NC-00777, Lot No. KW-1307-272

**Study design**

**Preliminary range-finding, cytotoxicity and mutagenicity tests**

Solubility of NC-00777 and cytotoxicity to AS52/XPRT CHO cells were initially tested in three experiments using NC-00777 at 0–5000 μg/mL in DMSO and S9 concentrations of 0, 1, 5 and 10% (representing the percentage of S9 (v/v) in the S9/cofactor mixture).

Precipitation of the test substance was seen at concentrations ≥ 1250 μg/mL. In the first experiment, using concentrations up to 5000 μg/mL, significant toxicity was seen at 0, 1, 5 and 10% S9 at concentrations of ≥ 156, 313, 313 and 625 μg/mL, respectively. In the second experiment, using concentrations up to 625 μg/mL, significant toxicity was seen at 0, 1, 5 and 10% S9 at concentrations of ≥ 115, 260, 350 and 425 μg/mL, respectively. In a third range-finding experiment, using concentrations up to 800 μg/mL, significant toxicity was seen at 0, 1, 5 and 10% S9 at concentrations of ≥ 300, 300, 300 and 400 μg/mL, respectively.

Three further experiments were carried out to determine potential mutagenicity and the concentration of S9 to be used in subsequent mutagenicity tests. NC-00777 concentrations were 0–410 μg/mL, with 0–10% S9.

**Mutagenicity tests**

Based on the cytotoxicity and mutagenicity data, triplicate mutagenesis experiments were performed, with and without 5% S9, using NC-00777 at 0–390 μg/mL. A concentration of 5% S9 was selected as being the most appropriate in the absence of any indication of mutagenicity in initial experiments. Positive controls were benzo[a]pyrene (with S9) and actinomycin D (without S9), the negative control was DMSO. Cells were grown in the presence of 10 μM 6-thioguanine.

**Results**

Significant cytotoxicity (≤ 50% relative survival) was seen at ≥ 280 and ≥ 350 μg/mL, in the absence and presence of S9, respectively. Relative survival of cells was 11% at 300 μg/mL in the absence of S9 and 26% in the presence of S9. No significant increase in the frequency of mutant (6-thioguanine-resistant) cells was observed, with or without S9. The average frequency of mutant cells in cultures without S9 was 11–36 mutants/$10^6$ cells (0.54–1.86 times the solvent control value) and the frequency with S9 was 17–33 mutants/$10^6$ cells (1.20–2.24 times the solvent control value). The mutant frequencies of the positive control cultures, treated with benzo[a]pyrene and actinomycin D, were increased 8.5 and 4.42 times, respectively.

**Conclusions**

Under the conditions of this study, NC-00777 was not mutagenic in AS52 CHO cells in the presence or absence of an exogenous metabolic activation system (S9).

*DNA damage and other genotoxic effects — derivative NC-00777*

**1. Soelter SG, Bunch RT & Nicolette J (1998) An evaluation of the potential of NC-00777 (E-3091) to induce micronucleated polychromatic erythrocytes in the bone marrow of mice (micronucleus test) (SA4714) Sponsor: Monsanto Company. Sponsor Study No: PCR-1196**

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| Laboratory: | Monsanto Safety Evaluation, GD Searle and Co., USA |
| Date of study: | 11/11/97–20/12/97 and 12/05/98–26/05/98 |
| GLP: | US FDA |
| Guidelines: | OECD 474 and ICH |
| Test chemical: | NC-00777, Lot No. KW-1307-272 |
| Test species: | CD-1(ICR)BR mice; Charles River Laboratories Inc |

**Study design**

NC-00777 was tested for its ability to induce micronuclei in polychromatic erythrocytes (PCEs) of mice. Groups of mice (5/sex/time point/dose level) were administered a single oral gavage dose volume of 10mL/kg of NC-00777 suspended in 0.5% methylcellulose (w/v)/0.1% Tween 80 (v/v) at dose levels of 0, 500, 1000 and 2000 mg/kg bw. Bone marrow was collected at 24 and 48 h after treatment. Cells were examined for the presence of micronucleated PCEs and the ratio of PCE/total erythrocytes was calculated. A positive control group was administered cyclophosphamide in 0.5% methylcellulose (w/v)/0.1% Tween 80 (v/v) and bone marrow collected at 24 h only. A negative control group was administered 5% methylcellulose (w/v)/0.1% Tween 80 (v/v).

The dose levels were based on a previous range-finding study (Monsanto study number PCR-1195, EHL study number 96212), in which no deaths were seen in mice at a dose level of 2000 mg/kg.

**Results**

There were no deaths or clinical abnormalities and no changes in bodyweights, frequency of micronucleated PCEs or PCE/total erythrocyte ratios in any NC-00777-treated groups compared to the test groups. In the cyclophosphamide-treated group, there was a significant increase in the incidence of micronucleated PCEs in both male and female mice. In addition, bodyweights were decreased for the female positive control group.

**Conclusion**

Under the conditions of this assay, NC-00777 did not induce an increase in micronuclei in the polychromatic erythrocytes of mice.

*Gene mutation assays — derivative NC—00779*

**1. Balwierz PS & Bunch RT (1998) Evaluation of the mutagenic potential of NC-00779 (E-3090) in the Ames Salmonella/microsome assay (SA4712). Laboratory Study No: SA4712. Sponsor: Monsanto Chemical Co. Sponsor Study No: PCR-1201**

Laboratory: GD Searle and Co, Monsanto, USA
Date of study: 24/09/97–16/10/97
GLP: US FDA
Guidelines: US-FDA 21 CFR Part 58
Test chemical: NC-00779, Lot No. DZG-1373-163 (SP1457)

**Study design**

NC-00779 in DMSO was tested for its ability to induce reverse mutations in *Salmonella typhimurium* strains TA1535, TA100, TA102, TA98 and TA97a, with or without S9 microsomal activation mixture. Positive controls used were ICR-191 acridine, sodium azide, 2-aminoanthracene, cumen hydroperoxide, 2-nitrofluorene and danthron. The negative control was DMSO.

**Results**

Initial and confirmatory mutagenicity tests carried out in triplicate using NC-00779 at concentrations ranging from 10–5000 μg/plate, in the presence or absence of a metabolic activation system (S9). In both mutation assays, a precipitate was seen at the 5000 μg/plate concentration in the top agar before it was poured onto the plates, but was not seen when the plates were examined by microscope. In each of the tests, no increases in the mean number of revertants and no cytotoxicity was seen with any of the tester strains, with or without S9. Significantly increased mutation rates were seen with each of the positive controls.

**Conclusions**

Under the conditions of this study, NC-00779 was not mutagenic in *Salmonella typhimurium* in the presence or absence of an exogenous metabolic activation system (S9).

**2. Cabonce M, Asbury K, McAdams JG & Wagner CA (1998) AS52/XPRT gene mutation assay with NC-00779. Laboratory Study No: EHL 97144. Sponsor: Monsanto Company. Sponsor Study No: PCR-1202**

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| Laboratory: | Monsanto, USA |
| Date of study: | 18/08/97–25/11/97 |
| GLP: | US-FDA |
| Guidelines: | OECD Guideline No. 476 US FDA and ICH guideline on specific aspects of regulatory genotoxicity tests) |
| Test chemical: | NC-00779, Lot No. DZG-1373-163 |

**Study design**

**Preliminary range-finding, cytotoxicity and initial mutagenicity tests**

Solubility of NC-00779 and cytotoxicity to AS52/XPRT Chinese hamster ovary cells were initially tested using NC-00779 at 0–5000 μg/mL in DMSO and S9 concentrations of 0, 1, 5 and 10% (representing the percentage of S9 (v/v) in the S9/cofactor mixture). Precipitation was seen at 2500 and 5000 μg/mL. No significant cytotoxicity (≤ 50% relative survival) was seen at any dose level, with or without S9.

An initial experiment to determine the mutagenicity potential of the test substance used NC-00779 at 0–5000 μg/mL in DMSO and S9 concentrations of 0, 1, 5 and 10%. No test article-related increase in mean mutant frequency or dose responses were seen, and positive controls (benzo[a]pyrene and actinomycin D) gave the expected positive responses.

**Confirmatory mutagenicity tests**

Based on the cytotoxicity and mutagenicity data, triplicate mutagenesis experiments were performed, with and without 5% S9, using NC-00779 at 0–5000 μg/mL. A concentration of 5% S9 was selected as being the most appropriate in the absence of any indication of mutagenicity in initial experiments. Positive controls were benzo[a]pyrene (with S9) and actinomycin D (without S9), the negative control was DMSO. Cells were grown in the presence of 10 μM 6-thioguanine.

**Results**

No significant increase in the frequency of mutant (6-thioguanine-resistant) cells was observed, with or without S9. The average frequency of mutant cells in cultures without S9 was 22–39 mutants/$10^6$ cells (0.93–1.67 times the solvent control value) and the frequency with S9 was 33–49 mutants/$10^6$ cells (1.23–1.84 times the solvent control value). The mutant frequencies of the positive control cultures, treated with benzo[a]pyrene and actinomycin D, were increased 10.4 and 3.1 times, respectively.

**Conclusions**

Under the conditions of this study, NC-00779 was not mutagenic in AS52 Chinese hamster ovary cells in the presence or absence of an exogenous metabolic activation system (S9).

*DNA damage and other genotoxic effects — derivative NC—00779*

**1. Nicolette, JJ & Bunch RT )1998) An evaluation of the potential of NC-00779 (E-3090) to induce micronucleated polychromatic erythrocytes in the bone marrow of mice (micronucleus test) SA4713). Sponsor: Monsanto Chemical Co. Sponsor Study No: PCR-1206**

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| Laboratory: | GD Searle & Co., Monsanto Chemical Company, USA |
| Date of study: | 02/12/97–11/02/98 |
| GLP: | US FDA |
| Guidelines: | US-FDA 21 CFR Part 58, OECD Guideline No. 474 and ICH |
| Test chemical: | NC-00779 Lot No. DZG-1373-163 (SP1457) |
| Test species: | CD-1(ICR)BR mice; Charles River Laboratories Inc |

**Study design**

NC-00779 was tested for its ability to induce micronuclei in polychromatic erythrocytes (PCEs) of mice. Groups of mice (5/sex/time point/dose level) were administered a single oral gavage dose volume of 10mL/kg of NC-00779 suspended in 0.5% methylcellulose (w/v)/0.1% Tween 80 (v/v) at dose levels of 500, 1000 and 2000 mg/kg bw. Bone marrow was collected 24 and 48 h after treatment. Cells were examined for the presence of micronucleated PCEs and the ratio of PCE/total erythrocytes was calculated. A positive control group was administered cyclophosphamide in 0.5% methylcellulose (w/v)/0.1% Tween 80 (v/v) and bone marrow collected at 24 h only. A negative control group was administered 5% methylcellulose (w/v)/0.1% Tween 80 (v/v).

The dose levels were based on a previous range-finding study (Monsanto study PCR 1205, EHL 97004), in which no deaths were observed in mice at a dose level of 2000 mg/kg.

**Results**

There were no deaths or clinical abnormalities and no changes in bodyweights, frequency of micronucleated PCEs or PCE/total erythrocyte ratios in any NC-00779-treated groups compared to the test groups. In the cyclophosphamide-treated group, there was a significant increase in the incidence of micronucleated PCEs in both male and female mice.

**Conclusion**

Under the conditions of this assay, NC-00779 did not induce an increase in micronuclei in the polychromatic erythrocytes of mice.

**OTHER STUDIES**

*HUMAN STUDIES*

**1. Azzam SM, Kisicki JC & Gao X(1997). Single Dose Tolerance of NC-00723 in Healthy Male Subjects. Sponsor: Monsanto Company. Sponsor Study no: PCR 1035**

Test chemical: NC-00723: source: Monsanto Company, batch 95MP028-3
Subjects: Healthy male human volunteers

**Study design**

NC-00723 was prepared in water in a single dose of 240 mL. Healthy adult male volunteers (6/dose) were used. For each dose, a single subject was tested for tolerance at that dose before the other volunteers receiving the compound. To be included in the study, subjects must be healthy, between 18 and 40 years old and within 20% of their ideal bodyweight. Exclusion criteria included being female, a smoker, having a current illness, having a history of alcohol or substance abuse, taking any medication within 2 weeks of the study, being on chronic medication which could not be stopped during the study or being unable or unwilling to comply with the protocol. Before treatment, subjects were screened with a medical history, physical examination, eye examination, ECG, urine drug screen and clinical laboratory evaluation.

Subjects arrived at the test facility on the evening before dosing commencing. They were then reweighed and it was confirmed they met the criteria. There were three dosing regimes used in the study; 0.1, 0.25 or 0.5 mg/kg bw in 240 mL of mineral water. Subjects fasted for 8 h before dosing and for 4 h after dosing. Water (240 mL) was consumed at 1 and 2 h post dose.

Pre-dose, an ECG was done and haematological parameters, including haematocrit, haemoglobin, platelet count, erythrocyte count and total and differential white cell count were assessed. Clinical chemistry parameters, including albumin, alkaline phosphatase, ALT, AST, calcium, chloride, cholesterol, carbon dioxide, creatinine, GGT, glucose, inorganic phosphorus, lactic dehydrogenase, magnesium, potassium, sodium, total bilirubin, total protein, triglycerides, blood urea nitrogen and uric acid were analysed. A urinalysis for pH and specific gravity was done.

The sitting and standing blood pressure, temperature, pulse and respiratory rate were measured before blood being taken and for up to 48 h after dosing. Blood was collected at intervals up to 48 h after dosing. The plasma was separated by centrifugation and frozen. Urine was collected for 12 h before dosing, and for 48 h after dosing. Subjects were encouraged to drink water to facilitate urine collection. A split sample was obtained from each void and frozen. Subjects were monitored for adverse experiences and any unusual symptoms, and encouraged to report these. A physical examination, including routine lab tests was done at around 48 h after dosing.

**Results**

Subjects had a mean age of 28 years (range 19 to 38), were Caucasian had a mean height of 182 cm (170 to 192 cm) and a mean weight of 81 kg (61 to 107 kg).

There were no treatment-related changes in pulse rate or blood pressure, and no change in haematology, clinical chemistry or urinalysis parameters. Two subjects experienced mild headaches one before dosing and one after a dose of 0.1 mg/kg bw/day. At 0.5 mg/kg bw/day, another two subjects had mild headaches, one before dosing and one after, and one had lower back pain. These signs resolved without further treatment and were not attributed to dosing with NC-00723.

The maximum plasma concentrations of NC-00723 were seen at 0.5 h after dosing for 0.1 and 0.25 mg/kg bw, and at 0.58 h after dosing for 0.5 mg/kg bw. Plasma concentrations were approximately linear with the maximums being 18.43, 66.12 and 97.31 ng/mL for 0.1, 0.25 and 0.5 mg/kg bw respectively. Plasma levels of NC-00751 peaked at around 1 h, and were 59.74, 199.70 and 319.94 ng/mL. These concentration distributions were linearly related to dose.

Excretion of NC-00723 by the urine accounted for 0.91, 1.55and 0.91 % of the administered dose for 0.1, 0.25 and 0.5 mg/kg bw/day. Excretion of NC-00751 accounted for 12.2, 14.8 and 13.8% of the dose for the respective dose levels. These findings support the extensive and rapid metabolism of NC-00723 to NC-00751.

**Conclusion**

Neotame administered in single doses of up to 0.5 mg/kg bw was well tolerated in human subjects.

**2. Holt PR & Kirkpatrick D (1997). A Pharmacokinetic Study of [$^{14}$C] NC-00723 in Healthy Male Subjects. Lab no: LCRC/EQ/030. Sponsor: Monsanto Company. Sponsor Study no: PCR 1039**

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| Test chemical: | NC-00723: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester.<br>Radiolabelled: Huntingdon Life Sciences, batch MRH/NTS31/37/2; activity 19.97 mCi/mmol, 52.77 μg/mg; purity 98.6–99.8%<br>Non-radiolabelled: Monsanto Company, batch 95MP028-3 |
| Reference chem: | NC-00751: N-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine; Monsanto Company, batch IP-1123-27-5<br>NC-00754: N-(3,3-dimethylbutyl)-L-aspartic acid; Huntingdon Life Sciences; batch MRH/NTS 39/68, activity 93.61 μCi/mg, 20.43 mCi/mmol, purity >98% |
| Subjects: | Healthy male human volunteers |
| GLP: | UK, EC, OECD, US FDA |

**Study design**

Both a pilot and a main study were performed. For both studies, radiolabelled NC-00723 was prepared in water and placed into flasks. In the pilot study, the flask contained 60 mL of NC-00723 solution, with approximately 18.5 mg NC-00723, while in the main study, each flask contained approximately 17.7 to 17.9 mg NC-00723.

In the pilot study, a single healthy male volunteer was fasted overnight, and given the contents of the flask orally. The flask was then rinsed three times with distilled water, with the rinsate ingested through the same straw used for the initial dose each time. A similar procedure was followed for the main study, with six subjects being used. Whole blood, urine and faeces were collected from each subject for 7 days after dosing. Blood samples were taken at intervals up to 168 h after dosing. At each sample time, 1 mL of blood was used to determine the PCV. Three samples at each time point were used to determine the concentration of radioactivity in the whole blood. The rest of the available blood was centrifuged and the plasma collected. The radioactivity in the plasma, and the concentration of NC-00723 and NC-00751 were determined. Any unused plasma was stored frozen. Urine was collected over the 12 h before dosing, and for intervals up to 168 h after dosing. Samples were collected into an individual container, and the weight and volume recorded. Citric acid was added to give 0.04 mL per mL of urine. Samples divided into 5 mL and 100 mL aliquots, frozen and shipped to Huntingdon Life Sciences for analysis. Faeces were collected between 12 h before dosing and 168 h after dosing and stored frozen.

**Results**

All seven subjects commencing the study completed it. All were Caucasian, non-smokers, with a median age of 23 years (range 21 to 25 years). The median weight was 75.8 kg (range 72.8 to 79.4 kg), with all within10% of their ideal bodyweight for height and frame size. NC-00723 was well tolerated.

There were no clinically significant changes in biochemical, haematological or physiological parameters. One subject reported mild diarrhoea and headache within 3 days post-dose; however, the relationship to treatment was not ascertained.

The total recovery of radioactivity in the urine and faeces was 90.7 to 108.3%. In 6/7 subjects, 27.8 to 35.8% of the dose was recovered in the urine, with 58.4 to 78.9% of the dose excreted in the faeces. One subject showed a different excretion pattern, with 50.6% excreted in the urine and 45.1% excreted in the faeces. Urinary excretion was rapid, with 94% of the total urinary excretion occurring in the first 24 h. Faecal excretion was more variable, but the majority occurred within 96 h of dosing.

There was rapid absorption of NC-00723, with maximum mean radioactivity in the plasma of 0.299 μg equivalent/mL at 0.75 h after dosing. Radioactivity had decreased to 0.013 μg equivalent/mL by 8 h after dosing. No NC-00723 was found beyond 2.5h after dosing, while NC-00751 was found for 10 h after dosing. The maximum concentrations of both compounds was seen within 1 h after dosing, with the maximum concentration of 95.7 ng NC-00723/mL seen at 0.4 h and 236 ng/mL NC-00751 seen at 1 h. Concentrations decreased monoexponentially, with the rate of decline of NC-00723 faster than NC-00751.

At early sampling times, these two compounds accounted for more than 87% of the total radioactivity; later other metabolites increased in importance. Radioactivity tended to be found in plasma rather than whole blood; it was not associated with cellular components.

The urinary metabolites were only analysed up to 72 h after dosing, due to the low levels of radioactivity found after this. Shortly after dosing, most of the radioactivity was present as NC-00723 and NC-00751, with other metabolites found later after dosing. NC-00723 was found until 8 h, however at all time periods NC-00751 levels were higher than NC-00723, with NC-00751 making up 17.23 to 39.08% of the dose. Three minor radioactive compounds were found. C3 made up 3.2% of the dose, and was found from 12 h after dosing. C1 and C2 each made up less than 0.6% of the dose.

In the faeces, no NC-00723 was found in any sample analysed. NC-00751 made up a total of 39.9 to 70.5% of the dose over 0 to 96 h. NC-00754 made up 4.9% of the dose excreted in faeces, with two minor metabolites each making up less than 1%.

Based on this study, NC-00723 is rapidly absorbed and excreted in humans. For most individuals, the majority of excretion occurs via the faeces, with the remainder excreted in the urine. There is rapid and extensive metabolism, initially (and mainly) to NC-00751, with other metabolites only formed in small quantities.

**3. Azzam SM, Weston IE & Gao X(1997). Assessment of the Dose Related Pharmacokinetic Profile of NC-00723 in Solution Administered to Healthy Male Subjects. Sponsor: Monsanto Company. Sponsor Study no: PCR 1111**

Test chemical: NC-00723: Monsanto Company, barch 95MP028-3
Subjects: Healthy male human volunteers

**Study design**

NC-00723 was prepared in water in a single dose of 240 mL. Healthy adult male volunteers were used. To be included in the study, subjects must be between 18 and 55 years old, within 15% of their ideal bodyweight, be able to abstain from alcohol for 48 h before the study and during the study, and be able to understand and sign the consent form. Exclusion criteria included being female, a smoker, having a current illness, having a history of alcohol or substance abuse, having a positive hepatitis B or HIV test, taking any medication within 2 weeks of the study or being on chronic medication which could not be stopped during the study or being unable or unwilling to comply with the protocol. Before treatment, subjects were screened with a medical history, physical examination, eye examination, ECG, urine drug screen and hepatitis B and HIV screen, as well as clinical laboratory evaluation.

Subjects arrived at the test facility on the evening before dosing commencing. They were then reweighed and questioned to ensure there had been no changes that would make them ineligible to participate. Each subject was then assigned a study number. There were three dosing regimes used in the study; 0.1, 0.25 or 0.5 mg/kg bw in 240 mL of mineral water. The container was rinsed twice with 30mL water after dosing, with the subject ingesting the rinsate. Each subject received each dose level in a randomised protocol, with 72 h separating each dose.

Pre-dose, an ECG was done. Haematological parameters were assessed, including haematocrit, haemoglobin, platelet count, erythrocyte count and total and differential white cell count.

Clinical chemistry parameters, including albumin, alkaline phosphatase, ALT, AST, calcium, chloride, cholesterol, carbon dioxide, creatinine, GGT, glucose, inorganic phosphorus, lactic dehydrogenase, magnesium, potassium, sodium, total bilirubin, total protein, triglycerides, blood urea nitrogen an uric acid were analysed. A complete urinalysis including a microscopic examination was done.

Pre-dose, and 1, 4, 8, 12 and 24 h post dose, the sitting and standing blood pressure, temperature, pulse and respiratory rate were measured before blood being taken. Blood was collected at intervals up to 24 h after dosing. The plasma was separated by centrifugation and frozen. Urine was collected for 12 h before dosing, and for 84 h after dosing. Subjects were encouraged to drink water to facilitate urine collection. Subjects were monitored for adverse experiences and any unusual symptoms, and encouraged to report these. A physical examination, including an ophthalmological examination was done after the last urine sample was collected. Routine lab tests were done on day 11 after the first dose. Any abnormalities were followed up medically.

**Results**

Twelve healthy male subjects were enrolled. There was no treatment-related increase in pulse rate or blood pressure, and no change in haematology, clinical chemistry or urinalysis parameters. One subject experienced a mild headache after a dose of 0.25 mg/kg bw/day. This resolved without further treatment and was not attributed to dosing with NC-00723.

The maximum plasma concentrations of NC-00723 were seen at 0.5 h after dosing for all doses. Plasma concentrations were related to dose, with the maximums being 26.59, 60.55 and 125.19 ng/mL for 0.1, 0.25 and 0.5 mg/kg bw respectively. Plasma levels of NC-00751 peaked at around 1 h, and were 98.71, 226.82 and 422.60 ng/mL. These concentration distributions are closely related to dose.

Excretion of NC-00723 by the urine accounted for 1.3, 1.1 and 1.1% of the administered dose for 0.1, 0.25 and 0.5 mg/kg bw/day. Excretion of NC-00751 accounted for 22.0, 22.0 and 19.2% of the dose for the respective dose levels. These findings support the extensive and rapid metabolism of NC-00723 to NC-00751, with relatively rapid excretion (NC-00723 excreted by 12 h, with NC-00751 found until 84 h after dosing).

**4. Combs ML, Kisicki JC & Gao X(1998). Effect of Repeated Ingestion of NC-00723 in Solution Administered to Healthy Male Subjects. Sponsor: Monsanto Company. Sponsor Study no: PCR 1145**

Test chemical: NC-00723: source: Monsanto Company
Subjects: Healthy male human volunteers

**Study design**

Healthy adult male volunteers were used. To be included in the study, subjects must be between 19 and 55 years old, within 15% of their ideal bodyweight, be able to abstain from alcohol for 48 h before the study and during the study, and be able to understand and sign the consent form. Exclusion criteria included being female, a smoker, having a current illness, having a history of alcohol or substance abuse, having a positive hepatitis B or HIV test, taking any medication within 2 weeks of the study or being on chronic medication which could not be stopped during the study or being unable or unwilling to comply with the protocol.

Before treatment, subjects were screened with a medical history, physical examination, eye examination, ECG, urine drug screen and hepatitis B and HIV screen, as well as clinical laboratory evaluation.

Subjects arrived at the test facility on the evening before dosing commencing. They were then reweighed and questioned to ensure no changes had occurred which would make them ineligible to participate. NC-00723 was prepared at 0.25 mg/kg bw in 180 mL of water. Following ingestion, the container was rinsed twice with 30 mL of mineral water, which was also ingested. After an overnight fast, subjects were dosed with this preparation hourly for 8 doses. Subjects were required to remain fasting until after the $6^{th}$ dose. A standardised meal (low-fat, around 500 calories: low-fat yoghurt, a granola bar, fresh fruit and juice) was then provided, and the $7^{th}$and $8^{th}$ doses were given after the meal.

Pre-dose, an ECG was done. Haematological parameters, including haematocrit, haemoglobin, platelet count, erythrocyte count and total and differential white cell count was examined as part of the screening test, immediately pre-dose and at 48 h after the first dose.. At the same times, clinical chemistry parameters, including albumin, alkaline phosphatase, ALT, AST, calcium, chloride, cholesterol, carbon dioxide, creatinine, GGT, glucose, inorganic phosphorus, lactic dehydrogenase, magnesium, potassium, sodium, total bilirubin, total protein, triglycerides, blood urea nitrogen an uric acid were analysed. A complete urinalysis including a microscopic examination was done.

Pre-dose, and 1, 4, 8, 12 24 and 48 h post dose, the sitting and standing blood pressure, temperature, pulse and respiratory rate were measured before blood being taken. Blood was collected at intervals up to 48 h after the first dose. The plasma was separated by centrifugation and frozen. Urine was collected for 12 h before dosing, and up to 168 h after dosing. Subjects were encouraged to drink water to facilitate urine collection. Subjects were monitored for adverse experiences and any unusual symptoms, and encouraged to report these. A physical examination was done after the last urine sample was collected.

**Results**

Twelve subjects completed the study. They were all Caucasian, with a mean age of 34 years (22 to 50 years), height of 177.7 cm (163.8 to 188.0 cm) and 79 kg (66 to 89.5 kg). There was no treatment-related change in pulse rate or blood pressure, and no change in haematology, clinical chemistry or urinalysis parameters. One subject experienced a mild headache with onset 6 days after dosing, which persisted for around 10 h. This resolved without further treatment and was not attributed to dosing with NC-00723.

Maximum plasma NC-00723 levels occurred from 0.35 to 0.58 h after dosing throughout the dosing period. After the first dose, the maximum NC-00723 level was 65.90 ng/mL. This increased until after the $5^{th}$ dose, the plasma NC-00723 level was 79.35 ng/mL. Plasma concentrations following dosing then began to decrease, and the level after the final dose peaked at 67.36 ng/mL. The minimum plasma levels through the dosing period peaked at 34.74 ng/mL after the $5^{th}$ dose.

Following this, minimum plasma NC-00723 were much lower, being 33.17 ng/mL after the $7^{th}$ dose and 30.00 ng/mL after the final dose. Plasma NC-00751 levels peaked at 0.62 to 0.80 h after dosing. After the first dose, the maximum NC-00751 level was 225.28 ng/mL.

This increased throughout the dosing period, with the highest level achieved after the final dose at 875.94 ng/mL. The minimum plasma levels increased throughout the study, with the minimum levels after the 8$^{th}$ dose being 743.40 ng/mL.

NC-00723 was excreted in the urine, with 4.93 mg, or 3.12% of the total dose excreted. The main metabolite, NC-00751, was also excreted in the urine, with 37.13 mg or 23.41% of the administered NC-00723 excreted. Overall, subjects tolerated a series of 8 doses of NC-00723 at 0.25 mg/kg bw per dose over a 7 h period with no detectable adverse effects. The plasma NC-00723 levels reached a steady state within this time, but the plasma NC-00751 levels were still increasing at the end of the dosing period. This reflects the extensive metabolism to NC-00751 occurring in humans.

**5. Combs ML, Kisicki JC & Gao X(1998). A Comparison of the Profile of NC-00723 in Solution and Capsules Administered to Healthy Subjects. Sponsor: Monsanto Company. Sponsor Study no: PCR 1112**

Test chemical: NC-00723: source: Monsanto Company
Subjects: Healthy human volunteers

**Study design**

Healthy adult male and females volunteers were used. To be included in the study, subjects must be between 18 and 55 years old, within 15% of their ideal bodyweight, be able to abstain from alcohol for 48 h before the study and during the study, and be able to understand and sign the consent form. Exclusion criteria included being pregnant, a smoker, having a current illness which was determined would interfere with the results, having a history of alcohol or substance abuse, having a positive hepatitis B or HIV test, taking any medication within 2 weeks of the study, with the exception of the contraceptive pill, or being on chronic medication which could not be stopped during the study or being unable or unwilling to comply with the protocol. Before treatment, subjects were screened with a medical history, physical examination, eye examination, ECG, urine drug screen, pregnancy screen and hepatitis B and HIV screen, as well as clinical laboratory evaluation.

NC-00723 was provided by the sponsor in gelatin capsules containing 10 mg NC-00723. For the capsule test, subjects ingested 2 capsules with 300 mL of an unsweetened beverage. For the solution test, two capsules were opened and the contents dissolved in 240 mL unsweetened beverage which was ingested. The container was rinsed twice with 30 mL of the same beverage, with the rinsates ingested by the subjects. The empty capsules were also ingested. A 2-way crossover study design was used, with the doses separated by one week. An equal number of males and females were used for each order of administration.

Subjects arrived on the evening before the test and fasted overnight. Before administration of the test article, a physical examination was done, the bodyweight was determined and a drug and pregnancy test were performed. Subjects received the test article at 9 am and remained fasting for 4 h after dosing. Water was allowed freely from 2 h after dosing. Subjects remained at the test facility during the collection phase, and were provided with standardised meals.

Blood samples were taken for the levels of NC-00723 and NC-00751 up to 24 h after dosing. Vital signs (including sitting blood pressure, temperature, pulse and respiratory rate) were taken at screening and at intervals until 24 h after dosing before blood collection.

Clinical laboratory tests were repeated on days 9 and 10 after the initial dose. Bodyweight and the ECG were measured on days 8 and 10 after the initial dose

**Results**

Twenty-six subjects commenced the study, however two stopped the study after the first dose. One dropped out for personal reasons; the other had an adverse reaction to venipuncture, and dropped out. Neither of these was considered related to treatment. Twelve male and twelve female subjects completed the study. The females had a mean age of 39 (25 to 53) years, weight of 65.3 (55.5–78.6) kg, and height of 165 (154–173) cm. The males had a mean age of 37 (26–55) years, weight of 76.8 (52 to 97.3) kg and height of 176 (165–190) cm.

There were no treatment-related changes in pulse, blood pressure or on any of the clinical laboratory evaluations. A number of mild adverse experiences, including headache, vomiting, sore throat and light headedness were seen; these were not considered treatment related, and resolved without any medical intervention.

The pharmacokinetic properties following administration of NC-00723 in capsule or solution are presented in the table below.

**Pharmacokinetic properties for plasma NC-00723 following capsule or solution administration.**

| | **Capsule administration** | | **Solution administration** | |
|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** |
| **Cmax (ng/mL)** | 87.53 | 145.64 | 77.69 | 109.77 |
| **AUC (0-t)** | 65.30 | 110.21 | 56.62 | 74.82 |
| **AUC (0–24)** | 66.14 | 111.69 | 57.48 | 75.53 |
| **AUC (0–∞)** | 68.09 | 118.0 | 62.00 | 77.29 |
| **Tmax (h)** | 0.58 | 0.56 | 0.48 | 0.50 |
| **Elimination half life (h)** | 0.64 | 0.63 | 0.70 | 0.60 |

**Pharmacokinetic properties for plasma NC-00751 following capsule or solution administration.**

| | **Capsule administration** | | **Solution administration** | |
|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** |
| Cmax (ng/mL) | 299.21 | 300.88 | 265.00 | 273.64 |
| **AUC (0-t)** | 795.82 | 804.55 | 683.02 | 713.49 |
| **AUC (0–24)** | 799.57 | 807.82 | 686.60 | 717.13 |
| **AUC (0–∞)** | 800.80 | 812.29 | 690.05 | 705.68 |
| **Tmax (h)** | 0.96 | 0.98 | 0.94 | 0.88 |
| **Elimination half life (h)** | 1.80 | 1.64 | 1.67 | 1.71 |

Based on these findings, it was concluded that exposure of subjects to NC-00723 and NC-00751 from capsules is at least as great as that from solution. This indicated that capsule administration would be suitable for further studies. When the difference in bodyweight between males and females was taken into consideration, there were not significant differences in the pharmacokinetics of NC-00723 between the sexes.

**6. Combs ML, Kisicki JC & Gao X(1998). Two Week Tolerance Study of NC-00723 Administered to Healthy Male and Female Subjects. Sponsor: Monsanto Company. Sponsor Study no: PCR 1113**

Test chemical: NC-00723: source: Monsanto Company
Subjects: Healthy human volunteers
Study dates: Clinical phase: 15 August 1997 to 19 September 1997

**Study design**

Healthy adult male and females volunteers were used. To be included in the study, subjects must be between 19 and 55 years old and females must be either postmenopausal or using a medically accepted contraceptive method during the study. Subjects must be within 20% of their ideal bodyweight, be able to abstain from alcohol for 48 h before the study and during the study, and be able to understand and sign the consent form. Exclusion criteria included being pregnant or breastfeeding, a heavy smoker (more than 1 pack per day), having a current illness which was determined would interfere with the results, having a history of alcohol or substance abuse, having a positive hepatitis B or HIV test, taking any medication within 2 weeks of the study, with the exception of the contraceptive pill, or being on chronic medication which could not be stopped during the study or being unable or unwilling to comply with the protocol, having given a blood donation within 30 days of treatment, having an unusual diet or being unable to comply with the provided menu during the study period or having a bodyweight less than 50 kg or more than 110 kg.. Before treatment, subjects were screened with a medical history, physical examination, eye examination, ECG, urine drug screen, pregnancy screen and hepatitis B and HIV screen, as well as clinical laboratory evaluation.

NC-00723 was provided by the sponsor in blister pack containing 5 gelatin capsules containing 10 mg NC-00723 or placebo per capsule. Subjects were dosed at 0, 0.5 or 1.5 mg/kg bw/day by consuming a mixture of placebo and treatment capsules to yield the appropriate dose. Dosages were calculated using the following weight ranges: 50–65 kg, 65–80 kg, 60–95 kg and 95–110 kg. The study was a double blind trial, with a code required to allow identification of treated subjects. The test material was administered in divided doses three times daily (7 am, midday and 5 pm). Subjects attended the clinic for each of these doses, and to eat a standardised meal. Water was allowed freely at all times. The first morning dose following at least an 8 h overnight fast.

Before the first dose, the bodyweight was determined. The sitting blood pressure, temperature, pulse rate and respiratory rate were determined before the morning dose on days 1, 3, 5, 7, 9, 11 and 14. Blood for clinical pathology was taken pre-dose on days 1, 3 and 7. Blood samples were taken to determine NC-00723 and NC-00751 levels on days 1, 2, 3, 4, 7, 11 and 15. The plasma was separated and stored frozen for later analysis. A urine drug screen was done pre-dose on days 1 and 7. An ECG was done pre-dose on days 1 and 3, and a physical examination (including bodyweight) was done on day 7. Subjects were monitored for adverse experiences or unusual symptoms. After treatment, a physical examination was done. The vital signs, bodyweight, an ophthalmological examination ECG and clinical laboratory tests were done after the last blood sample was collected. Any detected abnormalities were followed up medically.

**Results**

Of the 74 subjects commencing the study, 72 successfully completed the study. The elimination of the other two subjects was unrelated to NC-00723 administration (a failed drug screen, and administrative difficulties). There were 70 Caucasians, 1 Hispanic, 1 Middle Eastern and 2 Asian subjects commencing the study. The mean age for females was 33 (20–53) years, height was 167 (149–178) cm and weight 66.5 (52.2–89.9) kg. For males, the mean age was 31 (20–53) years, height 180 (170–193) cm and weight 79.9 (51.8–102.6) kg. On clinical chemistry tests, one subject had high triglyceride levels pre-test, pre-dose and at the end of the study. This was considered to be unrelated to treatment, but the individual was referred to their doctor for follow up. There were no treatment-related changes in clinical pathology parameters, heart rate, blood pressure, respiratory rate, temperature, weight or ECG.

A range of clinical symptoms were documented throughout the study. The most common finding was headache, which occurred as 8 headaches in 5 control subjects, 16 headaches in 7 subjects at 0.5 mg/kg bw/day and 10 headaches in 4 subjects at 1.5 mg/kg bw/day. Diarrhoea was seen in control and 0.5 mg/kg bw/day subjects, but was not seen at 1.5 mg/kg bw/day. Abdominal pain was seen in 1 subject at 0.5 mg/kg bw/day and 2 subjects at 1.5 mg/kg bw/day. No abnormalities required medical intervention, and most were considered mild to moderate, although 4 headaches were documented as severe.

On analysis of blood samples, NC-00723 was below the level of quantification in morning samples at all time periods. Plasma NC-00751 levels were approximately proportional to dose. In males, a steady state of plasma NC-00751 was reached after 24 h; there was therefore no increase in levels throughout the study. In females, the levels reached a steady state after 72 h, with no further increase after this. After a dose of 0.5 mg/kg bw/day, males plasma NC-00751 levels ranged from 3.91 to 7.79 ng/mL and females had levels from 1.60 to 4.50 ng/mL. After 1.5 mg/kg bw/day, males plasma levels ranged from 8.69 to 13.34 ng/mL and females from 7.47 to 13.22 ng/mL.

**Conclusion**

Neotame administered in doses of up to 1.5 mg/kg bw for a period of 2 weeks was well tolerated in human subjects.

**7. Combs ML, Kisicki JC & Weston IE(1998). Thirteen Week Tolerance Study of NC-00723 Administered to Healthy Adult Male and Female Subjects. Sponsor: Monsanto Company. Sponsor Study no: PCR 1114**

Test chemical: NC-00723: source: Monsanto Company
Subjects: Healthy human volunteers
Study dates: 6 January 1998 to 27 May 1998

**Study design**

Healthy adult male and females volunteers were used. To be included in the study, subjects must be between 18 or 19 and 55 years old and females must be either postmenopausal or using a medically accepted contraceptive method during the study. Subjects must be within 20% of their ideal bodyweight, be able to abstain from alcohol for 48 h before the study and during the study, and be able to understand and sign the consent form.

Exclusion criteria included being pregnant or breastfeeding, a heavy smoker (more than 1 pack per day), having a current illness which was determined would interfere with the results, having a history of alcohol or substance abuse, having a positive hepatitis B or HIV test, taking any medication within 2 weeks of the study, with the exception of the contraceptive pill, or being on chronic medication which could not be stopped during the study or being unable or unwilling to comply with the protocol, having given a blood donation within 30 days of treatment, or having a bodyweight less than 50 kg or more than 110 kg. Before treatment, subjects were screened with a medical history, physical examination, eye examination, ECG, urine drug screen, pregnancy screen and hepatitis B and HIV screen, as well as clinical laboratory evaluation, which included thyroxine levels and coagulation times.

NC-00723 was provided by the sponsor in blister pack containing 4 gelatin capsules containing 10 mg NC-00723 or placebo per capsule. Subjects were dosed at 0, 0.5 or 1.5 mg/kg bw/day by consuming a mixture of placebo and treatment capsules to yield the appropriate dose. A total of 144 subjects (24/sex/group) were used. If subjects dropped out, they were replaced by subjects of the same gender. Average weights were considered to be 70 kg for females and 80 kg for males; females received test article at 0, 30 or 110 mg/day and males at 0, 40 or 120 mg/day. The study was a double blind trial, with a code required to allow identification of treated subjects. The test material was self-administered in divided doses three times daily (7 am, midday and 5 pm). Subjects recorded the dates and times of administration in a daily log book. Subjects attended the clinic on a weekly basis to receive seven days of test material, and were also supplied with 3 extra days test material to allow flexibility in attendance at the clinic. The first morning dose following at least an 8 **h** overnight fast.

On study day 1, before the first dose, the sitting blood pressure, temperature, pulse rate and respiratory rate, haematology, clinical chemistry, pregnancy test, urinalysis, screen for alcohol or drugs of abuse, ECG and bodyweight were determined. On the mornings of study days 7, 14, 28, 42, 56, 84 and 92 subjects were instructed not to take the morning dose or eat until after clinic attendance and blood sampling. Haematology, clinical chemistry and urinalysis, as well as a physical examination, were done at these times. Additional blood samples were collected into EDTA tubes to obtain plasma for analysis of levels of NC-00723: this was not done. Vital signs were determined weekly before morning dose throughout the study (at the times of attendance at the clinic to obtain additional test material).

On the morning of day 92 (after the completion of treatment), blood was taken for clinical pathology tests, clotting times, thyroxine, pregnancy test and possible analysis of compound levels in the plasma. Urine was collected for urinalysis and alcohol and drug screen. A physical examination, including vital signs, bodyweight, ophthalmological examination and ECG was also done.

**Results**

A total of 151 subjects were enrolled: of these 9 dropped out after the start of the study, with 7 of these being replaced. The reasons for dropping out were unrelated to treatment, and included: moved location (1), flu-like symptoms (1 subject in placebo group), 'personal reasons' (1), lost to follow-up (1) and positive drug screen (5). The majority of subjects were Caucasian, with a small number of American Indians, Negroes, European/Middle Eastern and Hispanic subjects.

Females age was 35 (19–65) years, weight was 64.8 (50.8–86.7) kg and height 166 (150–178) cm. Males age was 34 (19–54), weight was 82.3 (56.3–107.6) and height was 180 (165–192) cm.

There were no treatment-related changes throughout the study in pulse rate, blood pressure, respiratory rate, temperature, bodyweight, ophthalmology or haematology parameters. There were statistically significant differences between females at 0.5 mg/kg bw/day and the placebo group in chloride levels. Additionally subjects at 1.5 mg/kg bw/day had lower carbon dioxide levels and triglyceride levels than subjects in the placebo groups. None of the changes in clinical chemistry results were considered of biological significance or to be treatment related.

Of the 151 subjects enrolled in the study, 82 experienced at least one adverse reaction during the study period. Most of these were determined to be mild or moderate severity, and ranged across the three treatment groups with no dose relationship or statistically significant differences between groups. Headache was the most common adverse experience, occurring in 16, 15 and 13 subjects at 0, 0.5 and 1.5 mg/kg bw/day. Of these, 60% were determined to be unrelated to treatment, with the relationship of the other 40% being uncertain. No serious adverse event occurred during treatment.

Based on these findings, dosing with NC-00723 at up to 1.5 mg/kg bw/day for 91 days was well tolerated, with no significant changes.

**8. Combs ML, Lee M & Morrison DN (1998). Effect of Multiple Doses of NC-00723 Compared to Placebo on Plasma Glucose and Insulin Concentrations in Non-Insulin Dependent Diabetes Mellitus (NIDDM) Subjects. Sponsor: Monsanto Company. Sponsor Study no: PCR 1115**

Test chemical: NC-00723: source: Monsanto Company
Subjects: Human volunteers with NIDDM

**Study design**

Subjects with NIDDM were used in the study. To be included in the study, subjects must be between 18 and 55 years old and females must be either postmenopausal or using a medically accepted contraceptive method during the study. Subjects must have bodyweight of less than 135 kg, and be able to understand and sign the consent form. Subjects must provide verification from their physician that they had been diagnosed with NIDDM according to specified criteria. The management of the diabetes must have been stable (either diet controlled or oral hypoglycaemic agent) for 30 days before the study, and any other medical condition being managed with medication must have been stable (no medication change for 30 days) before study. Exclusion criteria included being pregnant or breastfeeding, a heavy smoker (more than 1 pack per day), having a current illness which was determined would interfere with the results, having a history of alcohol or substance abuse within the last year, having a positive hepatitis B or HIV test, using insulin to control diabetes, being unable or unwilling to comply with the protocol, having given a blood donation within 30 days of treatment. Before treatment, subjects were screened with a medical history, physical examination, eye examination, ECG, urine drug screen, pregnancy screen and hepatitis B and HIV screen, as well as clinical laboratory evaluation, which included plasma glucose and insulin levels.

NC-00723 was provided by the sponsor in blister pack containing 5 gelatin capsules containing 10 mg NC-00723 or placebo per capsule. Subjects were dosed at 0, 0.5 or 1.5 mg/kg bw/day by consuming a mixture of placebo and treatment capsules to yield the appropriate dose. A total of 36 subjects (6/sex/group) were enrolled, with 34 completing the study.. Average weights were considered to be 100 kg, with the test article given at 0, 60 or 150 mg/day. The study was a double blind crossover trial, with a code required to allow identification of treated subjects. The test material was self-administered in divided doses three times daily (7 am, midday and 5 pm) for 43 doses (14 days). Each subject received each dose, with the order randomised between subjects, and a 72-h period allowed between study periods. Subjects recorded the dates and times of administration in a daily log book.

On day 2 of the study, subjects completed a questionnaire to confirm that they did not meet any of the exclusion criteria. A urine sample was collected for a drug and pregnancy screen, and a physical examination, including bodyweight, ECG and sitting vital signs was done. Blood was taken for haematology and clinical chemistry, and subjects were supplied with the test article. They were asked to return on day 8 for more test article and a compliance check.

On the evening of day 14, subjects were admitted and given a low fat evening meal, and an evening snack. They then fasted overnight, with water available until one h before dosing. Fasting continued until 3 h after dosing on day 15, with water allowed from 1 h after dosing. Blood was taken for plasma glucose and insulin levels 15 min before dosing and 0, 15, 30, 45, 60, 75, 90, 120, 150 and 180 min after dosing. Subjects also returned on day 18 of study period 3, when haematology, clinical chemistry and urinalysis was done after an 8 h fast. A physical examination, including vital signs and ECG was also done at this time.

**Results**

Thirty-seven subjects commenced the study, with 3 withdrawing or being dropped. One withdrew due to a dislike of the blood sampling procedure, one commenced elective medical treatment, and the third had a myocardial infarction, while on the placebo treatment. Females were 54 (32–64) years, 91.2 (61–127) kg and 161 (150–177) cm, while males were 52 (37–64) years, 104.3 (69–133) kg and 175 (167–187) cm.

There were no changes in heart rate, respiratory rate, temperature, blood pressure, bodyweight, ECG, haematology, clinical chemistry or urinalysis during treatment. On urinalysis, glucose and ketones were present at all stages; this was related to NIDDM rather than compound administration. There were no adverse experiences related to treatment, and no differences in plasma glucose or insulin during treatment.

**9. Harry J & Aikens PJ (1998). An Investigation of a Urinary Metabolite in Healthy Male Subjects after administration of $^{14}C/^{13}C$- NC-00723 Sponsor: Monsanto Company. Sponsor Study no: PCR 1215**

Test chemical: NC-00723: radiolabelled: source: Huntingdon Life Sciences, batch no: MRH/MRO56/33/1, activity 57.26 μCi/mg; purity>98.7%
$^{13}C$--NC-00723, batch SPS/MON47/29, purity 99.4%
Reference chem:NC-00784: 3,3-dimethylbutanoyl-L-carnitine
Subjects: Healthy human volunteers
GLP: UK, EC, OECD, US FDA

**Study design**

An accurately measured weight of radiodiluted $^{13}C/^{14}C$-NC-00723 was dissolved in a known weight of water and the concentration verified. The test material was then dispensed into sequentially numbered flasks, each containing 37.5 mg NC-00723. Six healthy male subjects were fasted overnight and given 2 doses of radiolabelled NC-00723 separated by 6 h. The dose for each subject was approximately 0.5 mg/kg bw, contained in 240 mL between test solution and rinsate. Urine was collected for each subject from -12–0, 0–12, 12–24, 24- 36, 36–48, 48–60, 60–72, 72–84, 84–96, 96–108 and 108–120 h after the first dose. Citric acid was added to each urine sample to give 0.04 mL citric acid per mL urine. Two 10 mL aliquots of urine were taken and stored frozen, as was the remainder of each sample. Faeces were collected as voided for 12 h pre-dose and 120 h after the first dose. The collection times were noted and the samples stored frozen. Urine and faeces were analysed for radioactivity using liquid scintillation counting. The metabolites in urine were determined qualitatively and quantitatively using HPLC and thin layer chromatography. Mass spectral analysis was also used.

**Results**

All six subjects completed the study. They were all Caucasian, non-smokers and most were within 10% of their ideal weight. The mean age was 30.8 (20–39) years and weight was 75.4 (70.4–87.6) kg. NC-00723 was well tolerated, with no clinically significant adverse experiences or changes in haematological, biochemical or physiological parameters reported. The total recovery of radioactivity in urine and faeces was 88.5 to 97.8% of the administered dose, with 35.6 to 41.8% recovered in urine and 52.7–60.3% recovered in faeces. Urinary excretion was rapid, with 78.7% of the total urinary excretion occurring before 12 h after the first dose. A previously uncharacterised metabolite was recovered in the urine from 12 h after dosing. This metabolite made up 0.5 to 3.4% of the urinary excretion, and was identified as NC-00784 (3, 3-dimethylbutanoyl-L-carnitine).

*PHARMACOLOGY STUDIES*

**1. Allen JL (1997) NC-00723 Charcoal Propulsion Test in Rats (oral administration). Lab Study No: MTO/67/963576. Sponsor: Monsanto Worldwide Regulatory Affairs, Deerfield Illinois. Study No: PCR 1169**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire |
| Test chemical: | NC-00723, batch no. 96NK002-6 |
| Vehicle | 0.5% w/v methylcellulose/0.1% v/v Polysorbate 80 |
| Positive control: | Morphine sulfate |
| Test species: | Crl: CDBR Sprague Dawley rats; source: Charles River (UK) Ltd), Margate Kent UK |
| Study dates: | 8 November 1996 to 14 November 1996 |

**Study design**

Male rats were housed in groups of five in controlled conditions with free access to food and water. They were acclimatised for 2 days before random allocation to treatment groups, and for 3 days following allocation. NC-00723 was formulated in the vehicle, and samples analysed to determine appropriate concentration.

Rats were fasted overnight, and dosed orally with 0, 5 or 15 mg/kg bw NC-00723 or 100 mg/kg bw morphine sulfate, using 10/group. After 30 min rats were given 1 mL of a 5% charcoal solution. Forty-five min after charcoal administration, rats were killed and the gastrointestinal tract removed. The distance the charcoal had travelled from the pyloric sphincter to the caecum was measured, and expressed as a percentage of the total gut length.

**Results**

The charcoal had travelled 53, 60 or 53% of the gut length at 0, 5 or 15 mg NC-00723/kg bw respectively. In rats treated with morphine sulfate, the charcoal had travelled 19% of the gut length. Therefore in this trial, NC-00723 at up to 15 mg/kg bw did not have any effect on gut motility, whereas the positive control significantly decreased gut motility.

**2. Allen JL (1997) NC-00723 Assessment of Hexobarbital sleeping time in Rats. Lab Study No: MTO/68/963571. Sponsor: Monsanto Worldwide Regulatory Affairs, Deerfield Illinois. Study No: PCR 1168**

| | |
|---|---|
| Laboratory: | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire |
| Test chemical: | NC-00723, batch no. 96NK002-6 |
| Vehicle | 0.5% w/v methylcellulose/0.1% v/v Polysorbate 80 |
| Positive control: | Chlorpromazine hydrochloride in sterile water |
| Test species: | Crl: CDBR Sprague Dawley rats; source: Charles River (UK) Ltd), Margate Kent UK |
| Study dates: | 8 November 1996 to 14 November 1996 |

**Study design**

Rats were housed in groups of five in controlled conditions with free access to food and water. They were acclimatised for 2 days before random allocation to treatment groups, and for 3 days following allocation. NC-00723 was formulated in the vehicle, and samples analysed to determine appropriate concentration.

Rats were then fasted overnight, and dosed orally with 0, 5 or 15 mg NC-00723/kg bw or chlorpromazine hydrochloride at 15 mg/kg bw, using 5/sex/group. At 30 min after dosing, rats were given hexobarbital sodium by intraperitoneal injection at 100 mg/kg bw (females) or 150 mg/kg bw (males). When animals lost their righting reflex, they were placed on a heating pad, and the time of onset of sleep and duration of sleep (as determined by the loss and reappearance of the righting reflex) was recorded. Rats were killed and discarded after the reappearance of the righting reflex.

**Results**

Following NC-00723 administration, sleeping times in males were 47.4, 44.4 or 46.9 and in females were 78.6, 81.0 or 94.9 min at doses of 0, 5 or 15 mg/kg bw. Following chlorpromazine hydrochloride administration, sleeping times were 71 min in males and 140 min in females. Thus in males, NC00723 had no effect on sleeping times. In females, there was a slight, dose-related increase in sleeping times, however the times noted were within historical control ranges for the sex and strain of rats, and were significantly less than the times seen following administration of the positive control. It was therefore concluded that NC-00723 had no effect on sleeping times following hexobarbitol administration.

**3. Allen JL (1997) NC-00723 and NC-00751. Effects on the Isolated Guinea Pig Ileum. Lab Study No: MTO/66/963751. Sponsor: Monsanto Worldwide Regulatory Affairs, Deerfield Illinois. Study No: PCR 1170**

| | |
|---|---|
| Lab: | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire |
| Test chemical: | NC-00723, batch no. 96NK002-6 |
| | NC-00751, batch no. IP-1281-47-1 |
| Vehicle | DMSO |
| Test species: | Male Dunkin Hartley guinea pigs Sourc: David Hall, Newchurch UK |
| Study dates: | 7 November 1996 to 14 December 1997 |

**Study design**

Male guinea pigs were killed, and the terminal ileum removed. Sections of around 2 cm in length were suspended in an organ bath and attached to a transducer coupled to an amplifier and recorder to facilitate the measurement of isotonic contractions. These contractions were measured following an equilibration period.

Using separated tissues for each agonist, submaximal contractions with acetylcholine, histamine, 5HT or barium chloride were obtained. The effect of the vehicle, NC-00723 (at 20, 60 or 200 ng/mL) and NC-00751 (at 60, 200 or 600 ng/mL) on both basal tone and submaximal contractions was determined. This was compared with the effect of specific antagonists. Atropine, pyrilamine, cyproheptadine and verapamil were used as antagonists for acetylcholine, histamine, 5HT and barium chloride respectively. Tests were conducted over an 8-min cycle. At T0, the agonist was added. At one minute, the sample was washed. The vehicle/test article/reference standard was added at 3 min, and the agonist added at 5 min. At 6 min the sample was washed, and the cycle could be repeated at 8 min.

**Results**

The vehicle, NC-00723 and NC-00751 had no effect on either the basal tone of guinea pig ileum or the effect of the agonists. In contrast, the specific antagonists significantly reduced the contractions produced by the agonists.

**4. Allen JL (1997) NC-00723 Cardiovascular, Respiratory and Renal Evaluation in the Anaesthetised Dog following Intraduodenal Administration. Lab Study No: MTO/55/962434. Sponsor: Monsanto Worldwide Regulatory Affairs, Deerfield Illinois. Study No: PCR 1167**

| | |
|---|---|
| Laboratory | Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire |
| Test chemical: | NC-00723, batch no. 96NK002-6 |
| Vehicle | 0.5% w/v methylcellulose/0.1% v/v Tween 80 |
| Test species: | Male Beagle dogs; source: Interfauna |
| Study dates: | 6 March 1996 to 17 February 1997 |

**Study design**

Dogs were quarantined for 2 weeks after arrival, then housed individually in controlled conditions. Food was freely available during the quarantine period; once dogs were individually housed, they were provided with 400 g/day. Water was freely available at all times. Dogs were acclimatised to kennel conditions for 7 days. NC-00723 was prepared in vehicle for concentrations of 5 or 15 mg/mL.

The following parameters were recorded or derived in anaesthetised dogs to indicate the functional status of the cardiovascular, respiratory and renal systems. The general haemodynamic status was monitored by the systolic, diastolic and mean blood pressure and heart rate. The contractile status of the myocardium was monitored using the left ventricular systolic pressure, and the left ventricular dp/dt max. The electrical status of the myocardium was monitored using Lead II of the ECG. The general respiratory status was monitored by the respiratory rate, tidal volume and minute volume, while the renal function was determined by the urinary concentration of protein, sodium, potassium and chloride.

Dogs were fasted for 16 h, then anaesthetised with sodium thiopentone. Anaesthesia was maintained using α-chloralose and pentobarbitone sodium. A homeothermic blanket was used to maintain body temperature during anaesthesia. The trachea was cannulated, with the air flow used to measure the tidal volume and respiratory rate. Catheters were placed in the right femoral artery and in a cephalic vein to facilitate measurement of blood pressure and heart rate, and to allow maintenance of anaesthesia. A midline abdominal incision was made, and a catheter inserted 15 cm into the duodenum via the stomach. Catheters were also inserted into each ureter and the ends exteriorised to enable collection of urine. A catheter was placed into the left carotid artery and advanced into the left ventricle to measure the left ventricular systolic pressure. Dogs were placed on a saline drip during the experimental period.

Following catheterisation, a 60-min stabilisation period was used. Vehicle was then administered intraduodenally at 1 mL/kg. Two hours later, NC-00723 was given intraduodenally at 5 or 15 mg/kg bw. Physiological parameters were monitored continuously. Cardiovascular and respiratory parameters were measured every 5 min during stabilisation, every 10 min for 1 h after dosing, then every 15 min until 2 h post-dose. Urine was collected over each 20-min period throughout the experiment. In the last four animals tested, 1 μg/kg noradrenaline was given intravenously to verify the responsiveness of the parameters recorded. At the end of the study, all dogs were killed without further examination.

**Results**

There were no changes in arterial blood pressure following administration of the vehicle or either dose of NC-00723. Noradrenaline products a 14% increase in the blood pressure. NC-00723 had no effect on the heart rate, with noradrenaline producing a decreased heart rate. The cardiac output was increased after NC-00723 at 15 mg/kg bw in comparison to output following vehicle administration. This was not considered treatment related, as the output was not different to the output seen in other animals after 5 mg/kg bw, and there were no other related changes. No changes in respiratory parameters or the electrical condition of the heart were seen. Following both vehicle and NC-00723 administration, there was a general increase in urinary excretion. Additionally, potassium excretion increased after vehicle or NC-00723 at 5 mg/kg bw, although no change was seen after NC-00723 at 15 mg/kg bw. Overall, there were no changes in physiological parameters related to NC-00723 administration.

## Chemical and physical properties

### Technical grade active constituent

| | |
|---|---|
| Colour | White to off-white |
| Physical state | Powder |
| Melting point (for solids) | 80.9 to 83.4°C |
| pH (0.5% aqueous solution of Neotame at 20°C) | 5.80 |
| Solubility | 4.75% (w/w) in water at 60°C<br>soluble in ethanol and ethyl acetate |
| Octanol/water coefficient | $Log_{10}P = 0.917$ |
| Sensitivity to UV | |
| Stability | Stable in normal storage conditions |
| Degradation products | NC-00751 — hydrolysis of methyl ester group<br>NC-00764 — β-rearrangement of Neotame<br>NC-00777 — cyclisation of Neotame<br>NC-00779 — methyl ester hydrolysis of NC-00777 |

### Manufacturer details

Not stated

### Impurity profile for Neotame

| Ingredient | % |
|---|---|
| N-(3,3-dimethylbutyl)-L-α-aspartyl-L-phenylalanine | 0.42 |
| Other related substances | 0.05 |
| Lead | <0.0001 |
| Water | 4.5 |

**APPENDIX B**

**LIST OF CLINICAL CHEMISTRY, HAEMATOLOGY AND URINALYSIS PARAMETERS TESTED**

| Clinical chemistry | Haematology | Urinalysis |
|---|---|---|
| Albumin | Platelet count | Appearance |
| Alkaline phosphatase | MCV | Specific gravity |
| Bilirubin (total) | MCHC | Glucose |
| Calcium | MCH | Ketones |
| Chloride | Leucocyte total count | Sediment (microscopic) |
| Cholesterol (total) | Leucocyte differential count | Occult blood |
| Creatinine (blood) | Haemoglobin | Leucocytes |
| Gamma-glutamyl transpeptidase (GGTP) | Haematocrit (packed cell volume) | pH |
| Globulin | Erythrocyte count | Protein |
| Glucose (blood) | Blood smear | Bilirubin |
| Ornithine decarboxylase | Clotting time, prothrombin time, APTT | Chloride |
| Phosphorus (inorg) | | Potassium |
| Potassium | | Sodium |
| Protein (total) | | Nitrite |
| Albumin:globulin ratio | | Urobilinitrogen |
| Serum alanine aminotransferase (ALT) | | Osmolality |
| Serum aspartate aminotransferase (AST) | | Volume |
| Sodium | | |
| Triglycerides | | |
| Urea nitrogen (blood) | | |

**APPENDIX C**

## ORGANS/TISSUES FOR ORGAN WEIGHT DETERMINATION AND HISTOPATHOLOGICAL EXAMINATION

### Organs weighed

| Organ weighed | Tissues examined for histopathology | |
|---|---|---|
| Adrenals | Adrenals | Nerve (peripheral) |
| Brain | Aorta | Oesophagus |
| Heart | Blood smear | Ovaries |
| Lungs | Bone | Oviduct |
| Ovaries | Bone marrow | Pancreas |
| Testes | Brain | Pituitary |
| Seminal vesicles | Caecum | Prostate |
| Prostate | Colon | Rectum |
| Kidneys | Diaphragm | Salivary gland |
| Liver | Duodenum | Seminal vesicle |
| Salivary gland | Epididymis | Skin |
| Spleen | Eyes | Spinal cord |
| Gall bladder | (including optic nerve) | Spleen |
| Thymus | Gall bladder | Sternum |
| Pituitary | Harderian glands | Stomach |
| Thyroid | Head (3 sections: nasal cavity, paranasal sinus, tongue, oral cavity, nasopharynx, inner ear) | Testes |
| (w/parathyroid) | Heart | Thymus |
| Uterus | Ileum | Thyroid |
| | Jejunum | (w/parathyroid) |
| | Kidneys | Tonsils |
| | Lacrimal gland | Trachea |
| | Liver | Urinary bladder |
| | Lungs | Uterus |
| | Lymph nodes | (including cervix) |
| | Mammary gland | Vagina |
| | | Zymbal's gland |
| | Muscle (skeletal) | Gross lesions |

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3. Hawkins DR, Kirkpatrick D, Aikens PJ & Beeby TL (1995) $^{14}$C-NC-00723 Determination of the Distribution in Rats by Whole-Body Autoradiography. Laboratory Study No: NTS 29/943207. Sponsor Study No: PCR-958.
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**ATTACHMENT 4**

## Food Technology Report

### The use of Neotame as an intense sweetener and flavour enhancer

Currently the intense sweeteners approved in the *Food Standards Code* for use in Australia and New Zealand includes: saccharine, cyclamate, aspartame, acesulphame potassium, thaumatin, sucralose, and alitame. Intense sweetener approval is subject to satisfactory evaluation of its toxicology and safety assessment (Refer to Toxicology report Attachment 3). The development of a wide range of sweeteners has advantages for the food industry. Because of the different properties of individual sweeteners, one may be more suitable for a specific application than others.

Furthermore, there are possibilities of combining two or more sweeteners in a product. Improved food safety is one benefit of mixtures, since the required quantity of each component sweetener is then reduced. In addition, mixtures of sweeteners provide the food developer with many more opportunities, than with single sweeteners, for optimising such features as quality and stability of the taste profile of the product, by their mutual complementation and compensation characteristics.

The properties sought in an intense sweetener may be summarised as:

1. The same taste and functional characteristics as sucrose.
2. Low caloric density on a sweetness equivalency basis.
3. Non-carcinogenicity.
4. Metabolised normally or excreted unchanged.
5. No allergic, mutagenic, carcinogenic or other toxic effects in the body.
6. Chemical and thermal stability.
7. Compatibility with other food ingredients.
8. Economically competitive with existing sweeteners.

The applicant claims and provides supporting studies showing that Neotame has a clean sweet taste similar to sugar with no significant bitter, metallic or other off tastes. Moreover, this taste profile is maintained over the range of concentrations required in food and beverage applications. At sweetness levels typical in food applications, Neotame is 7,000 to 13,000 times sweeter than sugar. On a sweetness equivalency basis versus existing sweeteners, Neotame offers the potential to deliver improved cost structure due to its high sweetness potency and low usage levels.

The important property of Neotame is that it is stable under conditions of intended use. It is stable as a sweetener across a wide range of food applications and is particularly more stable in baked and dairy goods than other intense sweeteners.

The applicant claims and provides supporting studies for the use of Neotame as a flavour enhancer at levels that would not be used to sweeten food. This desirable effect can lead to significant reduction in use levels of flavourings and food ingredients.

**Conclusion:** Neotame is a viable alternative to other available intense sweeteners and flavour enhancers and its use is technologically justified for use in food and beverages. It has the properties required of an intense sweetener and flavour enhancer. It offers the advantages of greater stability and lower usage levels in baked goods and dairy foods compared to other permitted intense sweeteners and flavour enhancers.

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**ATTACHMENT 5**

## Revised Dietary Exposure Assessment Report

## A406 – Permission for use of Neotame

A dietary exposure assessment for Neotame was conducted at Full Assessment.

At Full Assessment of A406, the conclusion to the dietary exposure assessment report was as follows:

> "The results indicate that the general population are likely to have dietary exposures to Neotame below the ADI for mean and high consumers. However, high consumers in the younger age groups appear to have the potential to exceed the ADI on a total diet basis. Of more concern is that high consumers of some individual foods also have the potential to exceed the ADI from these foods alone. Total dietary exposure calculations are conservative in that it is assumed Neotame is the only sweetener that is used, and it is in all food groups where the level of use is assigned. Therefore, in order to make the dietary models more realistic, information on the proposed use of Neotame in foods such as breakfast cereals, biscuits and cakes, and desserts is required.
>
> These results indicate that Neotame could be generally permitted in Standard 1.3.1 of the joint Australia New Zealand *Food Standards Code*. Due to the potential for high consumers of some individual foods to exceed the estimated ADI the use of Neotame should be monitored. ANZFA proposes putting it on the monitoring list for food additives and recommends an intense sweetener consumption survey in the near future to provide base line data on sweetener use."

It was noted also at Full Assessment that the dietary exposures were an overestimate due to the assumption that Neotame was in all breakfast cereals, biscuits and cakes, and desserts. Further data on use of Neotame in these food groups was requested. The applicant submitted further data on market share from the United States.

Additionally, the Acceptable Daily Intake (ADI) was revised since Full Assessment, from 0.3 mg/kg bw/day to 2 mg/kg bw/day.

Therefore, a revised dietary exposure assessment was conducted, in order to make the models and the results more realistic.

**Review of submitted comments on dietary exposure assessment and market share data**

The NutraSweet Company provided some comments about the dietary exposure assessment in Attachment B of their submission. They agree with ANZFA's statement that the use of Neotame in the major contributing categories of breakfast cereals, and cakes and biscuits is overstated. They indicated that the unrealistic assumptions led to the incorrect conclusion that the intake of Neotame for children exceeds the ADI. They state that the market for the use of intense sweeteners in these foods groups is very small. Reference was made to the market for aspartame, which has been used to represent Neotame, as uses will be similar, stating that beverages account for the majority of consumption of aspartame (70% and 85% in US and UK respectively).

The submission noted that the market share for reduced calorie/sugar free/lite cereals in the US was less than 1%, and the reduced calorie/sugar free/lite cookies and cakes was 2%. As these categories also contain reduced fat foods and the like, the market for intense sweetened foods would be even less. They also noted technological implications, i.e. lack of a suitable bulking agent, that limits use of intense sweeteners in these foods.

The market share data submitted were produced by the Kubo Group Limited for NutraSweet, using US sales data from Neilson Marketing Planning Service Research. The data provided summarised the percent of the total market that the reduced energy segment of the food group represented. These figures may still be an overestimate of market share as they contain reduced fat as well as intense sweetened foods. The figures as provided in the submission by the applicant are presented in Table 1. The figures presented are only those that are relevant to the revised modelling for the foods for which additional data were requested. Figures for other food categories were also included in the submission. However, as ANZFA's dietary modelling computer program, DIAMOND, already contains food groups separated into intense sweetened sub-groups for these foods, the additional figures were not required for revised modelling.

**Table 1: Market share data for reduced calorie food groups from the US**

| Food Group | Total Category Sales Volume (eq. Vol.) | Reduced Calorie Sales Volume (eq. Vol.) | Reduced calorie Share % |
|---|---|---|---|
| Frozen desserts | 5 335 703 956 | 289 541 741 | 5.4 |
| Pudding mixes | 63 470 113 | 5 282 494 | 8.3 |
| Cookies | 1 448 200 000 | 31 600 000 | 2.2 |
| Ready to eat cereal | 2 501 500 000 | 8 600 000 | 0.3 |

These data covers retail grocery sales in stores with annual sales over $2 million (US). Brands with less than 5% national retail distribution were not included. The data were in equivalent units to account for variations in package sizes. The data for the pudding mixes and frozen desserts were for the 52-week period ending 30 June 2000. The data for cookies and cereals were for the 52-week period ending 5 December 1998. The data for cookies and cereals is thought not to have changed over the past 2 years.

There are limited data available on market share for intense sweetened food groups for Australia and New Zealand. The US market for these foods is more developed than for Australia and New Zealand, and available data (from the US) are likely to overestimate the market share in Australia and New Zealand. The US figures were used for estimating market share for producing a revised dietary exposure assessment based on conservative consumptions.

**Dietary Modelling**

The revised dietary exposure assessment was conducted the same way as at Full Assessment, using ANZFA's dietary modelling computer program, DIAMOND. The dietary exposure was estimated by combining food consumption data, from national nutrition surveys (NNS) (1995 Australian NNS and 1997 New Zealand NNS), with proposed levels of use of Neotame in foods (as provided in the application, see Appendix 1).

The same concentrations of Neotame used for models at Full Assessment were used for the revised dietary exposure assessment. The ADI that the estimated dietary exposures were compared to had changed since Full Assessment.

The market share data were only used for food groups in the model that did not have coded intense sweetened sub-category for the food. The market share data submitted were rounded up to the nearest 5% for the purposes of the revised dietary exposure assessment. This is conservative and takes into consideration that the data supplied did not include total sales of foods across the US. Also, the data only included a value for cookies, and the DIAMOND food group also includes cakes. Despite the rounding being conservative, it is still far more realistic than assuming 100% of the foods in these groups contain Neotame. The food groups and market share used for the modelling are shown below in Table 2.

**Table 2: Market share levels used for the revised dietary exposure assessment**

| Food Group | Reduced calorie share % | Share used for modelling |
|---|---|---|
| Desserts, all types | | 10 |
| *Frozen desserts* | *5.4* | *10* |
| *Pudding mixes* | *8.3* | *10* |
| Cookies | 2.2 | 5 |
| RTE Cereal | 0.3 | 5 |

The dietary model uses the market share data to assume that only 10% or 5% of the market, and therefore of the foods in the groups, contain Neotame.

**Results**

The estimated dietary exposures to Neotame were re-calculated for Australian and New Zealand populations, and for specific age groups for both countries, including the market share data and the revised ADI. The results are displayed in Table 3 below.

The results indicate that for the whole population for both Australia and New Zealand, the estimated dietary exposures to Neotame were well below the ADI for mean respondents and consumers, and were 3 to 6% of the ADI for high consumers. Population results, as opposed to results for smaller age groups, generally give the best indication of dietary exposures over a lifetime.

These results are much lower than those derived at Full Assessment (see Appendix 2), due to the market share data and the revised ADI being included in the revised model.

The risk of exceeding the ADI for children is higher than that of others in the population because they consume more food per kilogram of body weight. When younger age groups were assessed, results indicate that high consumers in the 2-4 years and 5-12 years age groups for Australia do not exceed the ADI (11% and 9% respectively). No dietary exposures for these age groups were able to be determined for New Zealand.

This is contrary to the results at Full Assessment (Appendix 2), where there was a theoretical possibility that these age groups may exceed the ADI for high consumers. The market share data and the revised ADI included in the revised models resulted in a more realistic exposure estimate.

The estimated dietary exposures for the New Zealand population presented above are slightly lower than the Australian figures. This could be explained by the fact that for some of the intense sweetened food categories, there were no foods in the New Zealand dietary survey that fitted into the category, therefore these foods were not considered in the New Zealand model. However, the habitual consumption of these kinds of foods may not have been accurately represented in 24-hour recall survey. The Australian results indicate that these foods would not have made a significant impact on the estimated exposures for New Zealand if present in the model.

**Table 3: Revised estimated dietary exposures to Neotame (at Inquiry)**

| Country | Age group | Number of consumers of Neotame | Consumers as a % of total respondents[#] | Mean all respondents mg/d (%ADI*) | Mean consumers mg/d (%ADI*) | 95th percentile consumers mg/d (%ADI*) |
|---|---|---|---|---|---|---|
| **Australia** | Whole population | 12 495 | 90 | 1.1 (0.9) | 1.2 (1.0) | 7.0 (5.6) |
| | 2-4 years | 559 | 96 | 0.6 (1.7) | 0.6 (1.7) | 3.8 (10.7) |
| | 5-12 years | 1 426 | 95 | 0.9 (1.4) | 0.9 (1.5) | 5.3 (8.5) |
| | 13-18 years | 836 | 90 | 1.2 (1.0) | 1.3 (1.1) | 7.9 (6.5) |
| **New Zealand** | Whole population | 4 057 | 88 | 0.6 (0.4) | 0.7 (0.5) | 3.7 (2.5) |
| | 15-18 years | 206 | 84 | 1.0 (0.7) | 1.2 (0.9) | 7.6 (5.4) |

# Total number of respondents for Australia: whole population = 13 858, 2-4 years = 583, 5-12 years = 1 496, 13-18 years = 928; New Zealand: whole population = 4 636, 15-18 years = 246.
* ADI = 2 mg/kg bw/d.

Out of all Australian Neotame consumers (12 495) there were no people who had the potential to exceed the ADI. For New Zealand population (4 057 consumers), no people had Neotame dietary exposures that had the potential to exceed the ADI.

The assumptions and limitations that were documented as a part of the Full Assessment report are valid for the revised dietary exposure assessment.

**Major contributing foods**

The major contributors to total Neotame dietary exposures are shown in Table 4. These are displayed for the revised exposure assessment and the Full Assessment exposure assessment, and for the total population as well as for the younger age groups. The major contributors to total exposure to Neotame were water based flavoured drinks in all the models, making up 40% or more of the total.

**Table 4: Major contributors to total Neotame dietary exposures for Australia and New Zealand, and for different age groups**

| Country | Age group | Major contributing foods | Percent of total Neotame exposures | |
|---|---|---|---|---|
| | | | At Inquiry | At Full Assessment |
| **Australia** | Whole population | Water based flavoured drinks | 65 | 23 |
| | | Desserts | 6 | 7 |
| | | Electrolyte/sports drinks | 5 | 2 |
| | | Biscuits and cakes | 5 | 34 |
| | | Breakfast cereals | 4 | 29 |
| | 2-4 years | Water based flavoured drinks | 40 | 12 |
| | | Desserts | 18 | 12 |
| | | Breakfast cereals | 9 | 42 |
| | | Biscuits and cakes | 6 | 29 |
| | | Peanut butter | 6 | 1 |
| | 5-12 years | Water based flavoured drinks | 55 | 14 |
| | | Desserts | 13 | 9 |
| | | Breakfast cereals | 8 | 39 |
| | | Biscuits and cakes | 7 | 34 |
| | 13-18 years | Water based flavoured drinks | 61 | 18 |
| | | Breakfast cereals | 7 | 38 |
| | | Electrolyte/sports drinks | 6 | 2 |
| | | Desserts | 6 | 5 |
| | | Biscuits and cakes | 5 | 32 |
| **New Zealand** | Whole population | Water based flavoured drinks | 44 | 10 |
| | | Biscuits and cakes | 11 | 50 |
| | | Electrolyte/sports drinks | 11 | 2 |
| | | Desserts | 9 | 12 |
| | | Table top | 8 | 2 |
| | | Breakfast cereals | 4 | 20 |
| | 15-18 years | Water based flavoured drinks | 47 | 14 |
| | | Electrolyte/sports drinks | 21 | 6 |
| | | Table top | 9 | 3 |
| | | Biscuits and cakes | 8 | 45 |
| | | Breakfast cereals | 4 | 22 |
| | | Desserts | 2 | 7 |

Breakfast cereals, biscuits and cakes, and desserts were the highest contributors in all age groups for dietary exposure assessments conducted at Full Assessment because the Neotame concentration was assigned to the whole category of the normal counterpart of these foods (no intense sweetened versions) rather than a segment of the market for the food, for example pre-sweetened cereals rather than all breakfast cereals. When the more realistic revised dietary exposure assessments were conducted, and market share data were entered for these foods, they no longer appear as major contributing foods to total exposures.

**High consumers of individual foods**

Exposure estimates based on individual foods were conducted at Full Assessment. The conclusion made at Full Assessment was that there is the potential for high consumers of some individual Neotame-containing foods to exceed the ADI, based on 95$^{th}$ percentile food consumption figures, derived by the DIAMOND model, for each food group. These foods were water based flavoured drinks and desserts for both populations and tabletop sweeteners for New Zealand teenagers (15-18 years).

Exposure estimates based on individual foods were recalculated at Inquiry using the revised ADI. The results showed that no individual foods have the potential to exceed the ADI for the population, or younger age groups.

Data are still required on long-term use of foods containing intense sweeteners and market share for use of one sweetener versus another to confirm these estimated exposures.

**Conclusion and Recommendation**

The results from the revised dietary exposure assessment indicate that the general population, for both Australia and New Zealand, are likely to have dietary exposures to Neotame below the ADI for mean and high consumers.

The revised results do not change the overall conclusion made at Full Assessment in that Neotame could be generally permitted in Standard 1.3.1 of the joint Australia New Zealand *Food Standards Code*.

As Neotame is a new intense sweetener, ANZFA proposes putting it on the monitoring list for food additives and recommends an intense sweetener consumption survey in the near future to provide base line data on individual sweetener use. As dietary modelling is based on the assumption of market share, monitoring would test the market share values used.

## Appendix 1: Proposed use of Neotame in foods and levels of use used in dietary modelling

| Food Group | Food Description | Proposed use level mg/kg |
|---|---|---|
| Tabletop sweeteners | Tabletop sweetener | 900 |
| Breakfast cereals | Pre-sweetened cereals | 46 |
| Beverages; beverage concentrates; beverage mixes | Carbonated soft drink | 17 |
| | Pasteurised lemon tea | 8 |
| | Soft drink mix | 16 |
| | Iced tea drink mix | 12 |
| | Flavoured milks | 15 |
| | Fruit juice based | 25 |
| | Electrolyte drinks | 15 |
| | Cordial, as consumed | 17 |
| Desserts; dessert mixes; fillings, filling mixes; toppings; topping mixes | Frozen dairy desserts (ice cream) and novelties (ices) | 20 |
| | Gelatin desserts (jelly) | 19 |
| | Pudding desserts | 45 |
| | Yoghurt | 15 |
| | Pie filling | 30 |
| | Whipped toppings | 25 |
| Chewing gum | Chewing gum | 250 |
| Fruit and vegetable spreads; purees and sauces | Jams/Jellies | 100 |
| | Fruit purees | 100 |
| | Maple syrup | 70 |
| Salad dressings | French style dressing | 10 |
| Condiments | Relish | 30 |
| Peanut/nut spreads | Peanut butter | 15 |
| Confectionery; glazes; coatings | Icings, frostings, cookie fillings | 50 |
| | Hard candy | 60 |
| | Soft candy | 28 |
| Bakery products; bakery mixes | Cookies | 60 |
| | Cakes | 35 |
| | Cheese cake | 40 |

**Appendix 2: Estimated dietary exposures to Neotame at Full Assessment**

| Country | Age group | Number of consumers of Neotame | Consumers as a % of total respondents[#] | Mean all respondents mg/d (%ADI*) | Mean consumers mg/d (%ADI*) | 95th percentile consumers mg/d (%ADI*) |
|---|---|---|---|---|---|---|
| **Australia** | Whole population | 12 495 | 90 | 3.2 (18.5) | 3.5 (20.5) | 11.6 (69.5) |
| | 2-4 years | 559 | 96 | 2.3 (47.3) | 2.4 (49.3) | 7.7 **(154.8)** |
| | 5-12 years | 1 426 | 95 | 3.5 (37.9) | 3.6 (39.7) | 10.5 **(107.7)** |
| | 13-18 years | 836 | 90 | 4.1 (22.5) | 4.5 (24.9) | 13.7 (77.4) |
| **New Zealand** | Whole population | 4 057 | 88 | 2.8 (12.5) | 3.2 (15.0) | 10.9 (51.0) |
| | 15-18 years | 206 | 84 | 3.4 (17.0) | 4.1 (21.3) | 12.9 (68.0) |

# Total number of respondents for Australia: whole population = 13 858, 2-4 years = 583, 5-12 years = 1 496, 13-18 years = 928; New Zealand: whole population = 4 636, 15-18 years = 246.
* ADI = 0.3 mg/kg bw/d.

**ATTACHMENT 6**

## PUBLIC COMMENT RECEIVED AT INQUIRY

| | |
|---|---|
| Confectionary Manufacturers of Australasia Ltd | **Supports** the application |
| Goodman Fielder | **Supports** the application |
| Australian Food and Grocery Association | **Supports** the application. However, considered that ANZFA should re-examine its dietary modelling to reflect a more realistic or likely consumption pattern. |
| NutraSweet | **Supported** the application and provided a detailed submission which raised aspects of the toxicology report, dietary modelling and typographical errors that needed examination by ANZFA. |
| Australasian Soft Drink Association | **Supported** the application. |
| FTA Victoria | **Supports** the application |
| InforMed Systems | **Supports** the application |
| Unilever | **Supports** the application |
| Schweppes Cottee's | **Supports** the application |
| Dairy Bell Ice Cream (Aust.) Pty Ltd | **Supports** the application |
| SIAS Australia Pty Ltd | **Supports** the application |
| Merisant Manufacturing Australia Pty Ltd | **Supports** the application |
| Grocery Manufacturers Association of New Zealand | **Supports** the application |
| The Wrigley Company Pty Ltd | **Supports** the application |
| Holland and Knight LLP | **Does not** support the application.<br><br>Presented data which suggested that Neotame had adverse toxic effects on the dog liver and biliary system and effects on reproduction and development. Suggested that Neotame should not be approved until more reliable studies are conducted to address these concerns. |
| National Council of Women of Australia | **Does not support** the application.<br><br>Submitted a copy of a submission by Mr Arnold ward who raises questions about the authenticity of research carried out by Monsanto with aspartame and Neotame |

| | |
|---|---|
| Mr Chris Hart | **Does not support** the application on the grounds that Neotame is a synthetic clone of aspartame, there is overwhelming evidence that it is associated with ill effects and the controversy for approval of aspartame and Neotame in the USA. |
| Mr Graeme Pirie | **Does not support** the application.<br><br>Submitted internet addresses that described the toxic affect of aspartame and suggested that Neotame is even more toxic. |