HOW ASPARTAME PREVENTS THE TOXICITY OF OCHRATOXIN A

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ABSTRACT — The ubiquitous mycotoxin ochratoxin A (OTA) is found as a frequent contaminant of a large variety of food and feed and beverage such as beer, coffee and win. It is produced as a secondary metabolite of moulds from Aspergillus and Penicillium genera.

Ochratoxin A has been shown experimentally to inhibit protein synthesis by competition with phenylalanine its structural analogue and also to enhance oxygen reactive radicals production. The combination of these basic mechanisms with the unusual long plasma half-life time (35 days in non-human primates and in humans), the metabolisation of OTA into still active derivatives and glutathion conjugate both potentially reactive with cellular macromolecules including DNA could explain the multiple toxic effects, cytotoxicity, teratogenicity, genotoxicity, mutagenicity and carcinogenicity.

A relation was first recognised between exposure to OTA in the Balkan geographical area and Balkan Endemic Nephropathy (BEN) with a high incidence (nearly 50 times higher than normal) of urinary tract tumours. Exposure rates of OTA are measurable in blood of humans and animals and are established in several countries including Scandinavia, Germany, France, Italy, Canada, Japan and Northern Africa mainly Tunisia and Egypt. The impact of OTA exposure in non-endemic areas in the world is not known, the rates of exposure being not correlated with the disease records, especially in developed countries, due to lack of well-designed epidemiological studies, genetic polymorphism and maybe to dietary contents of radical scavengers and antioxidants. However the incidence and mortality rates of renal cancer are increasing in European countries and Northern Africa which could be a global resultant of human exposure to natural compounds in food such as mycotoxins and especially ochratoxin A.

In addition to special care to prevent the growth of moulds and detoxification measures there was a need for the prevention of the OTA-induced toxic effects once the toxin is ingested. For this purpose several compounds have been studied including some therapeutic agents such as piroxicam which cannot be proposed for a large scale use in humans for preventive purpose. Among other compounds, Aspartame, already used as sweetener has shown a real effectiveness in vivo confirmed largely in vitro.

When rats exposed to OTA (289 μg/kg) by oral route every two days are given 25 mg/kg similarly for several weeks, all the toxic effects including genotoxicity are very efficiently prevented as shown for example by the disappearance of DNA-adducts in tissues excised from treated animals. Aspartame is also effective in washing out the toxin when given afterwards to animals intoxicated by the same OTA doses for several weeks.

In vitro, provided that it is added in cell culture medium before OTA it prevent significantly the inhibition of protein synthesis and lipid peroxidation induced by the toxin.

Obviously the molecular mechanism mediating the preventive effect of Aspartame is the delivery of phenylalanine by cleavage of the peptide but also the direct effect of the peptide on the binding capacity and transport of the toxin in vivo and in vitro. As a matter of fact when Aspartame is given to animals or added in culture medium the amount of peptide found unchanged (10-15%) may account for a preventive effect as entire peptide.

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by fungi of Aspergillus and Penicillium genera contaminating foodstuffs (wheat, maize, barley, beans, dried fruits, coffee, cocoa and beverages such as beer and win. It is also found in humans and animals blood urine and milk and tissues (Krogh 1987; Kuiper-Goodman

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and Scott 1989, Pohland et al. 1992)

In many Balkan areas, high concentrations (2-40 ng/ml) of OTA in blood as well as in food (1 to 35 μg/kg) have been tentatively related to a human chronic interstitial nephropathy (Balkan Endemic Nephropathy) which is most of the time associated to urinary tract tumours (Castegnaro et al. 1987, Petkova-Bocharova et al. 1988. The nephropathy observed in these people (a tubulo-interstitial nephritis with reduced size and function of the kidney) is very similar to the porcine nephropathy induced by feeding animals with feed containing ochratoxin A producing fungi or artificially contaminated by pure ochratoxin A. A similar situation is being observed now in Northern Africa where high incidence of exposure to OTA is likely producing the same human health problems, Bacha et al. 1993, Achour et al. 1993). The health situation in geographical regions of low OTA exposure rates needs to be explored for possible disease causation by the toxin in countries like Germany, Scandinavia, France, Italy, Canada and Japan (Bauer and Gareis 1987, Breihoitl et al. 1991, Creppy et al., 1991, Kuiper-Goodman et al. 1993; Kawamura et al. 1993).


When introduced in the human body, OTA binds strongly to plasmaproteins (with a half-life time of 35 days at least,) and is metabolised into hydroxylated compounds, one of which, the (4R)-4-hydroxyochratoxin A being as cytotoxic and immunosuppressive as the parent compound in vivo and in vitro (Creppy et al. 1983ac). Different types of conjugations also occur which lead to very reactive derivatives mainly when glutathion is involved. OTA can be cleaved into phenylalanine and OAlpha the chlorinated dihydroisocoumarin moiety found not to be toxic, but retaining the genotoxicity, (Follmann et al. 1995).

The main molecular mechanism of action of ochratoxin A is inhibition of protein synthesis by competition with phenylalanine (Phe) in the reaction catalysed by phenylalanyl-tRNA synthetase (Heller and Rosen 1977, Bunge et al. 1978, Creppy et al. 1979a, 1979b). OTA is also able to inhibit all the reactions in which phenylalanine, its structural analogue is involved such as those of phenylalanine hydroxylase, (Creppy et al. 1990) or tyrosine hydroxylase leading to DOPA for tyrosine-OTA which is also cytotoxic (Creppy et al. 1983c).

Toxic effects of ochratoxins are also related at least partially to the oxidative processes, and to the disruption of the intracellular calcium homeostasis induced by OTA, (Khan et al. 1989), and finally to the inhibition of mitochondrial respiration and ATP production (Meisner and Chang 1974, Wei et al. 1985). All these reactions could result from the inhibition of protein synthesis.

The prevention of this public health problem is first avoid fungal growth on commodities, second avoid consumption of concentrations of OTA above NOEL by a very strict survey and regulation and also applicable and realistic detoxification procedures.

When ochratoxin is already ingested, the properties required for a good preventing agent for detoxification other than being harmless is the prevention of absorption and transportation via the blood to organs in particular to the kidney, prevention of tissue distribution and accumulation, prevention of metabolism into reactive compound mainly in relation to oxidative pathways. It is also implicitly desirable to increase the OTA elimination into urine and faeces.

Many substances have been studied for this purpose including sodium bicarbonate, phenylalanine and several drugs already used in therapeutic such as Proxamic (Baudrimont et al. 1995a) or Indomethacin.

This paper summarises data obtained along several years of investigation on the mechanism whereby Aspartame a structural analogue of both phenylalanine and OTA prevents the nephrotoxicity and genotoxicity induced by subchronic exposure to the toxin.

**MATERIALS AND METHODS**

Aspartame is the Nasparyl-phenylalanine methyl ester. It is thus a structural analogue of both phenylalanine and ochratoxin A. It is provided by Sigma Chemicals St Louis, USA or by Searle NutraSweet. Ochratoxin A was from Sigma.

Because the tissue distribution of the peptide (aspartyl-phenylalanine) and its elimination were still uncertain, a sensitive detection method of Aspartame using HPLC and fluorescence after derivation with ortho phthaldeyde has been recently developed. This permits following Aspartame in body fluids and organs. Several studies have then been conducted to determine it protective effects in nephrotoxicity and genotoxicity in vivo according to the methodology.
described by Baudrimont et al. (1994, 1995b). In addition and to explore shorter times rats 150 g to 170 g were given OTA and Aspartame every two days in alternance, for 10 days. 24 h after the last Aspartame dose, urine blood and tissues (liver, kidney brain) of animals were analysed for OTA and Aspartame, after extraction or directly. control animals were treated with Aspartame alone and or the saline solution serving as vehicle.

The efficacy of Aspartame to eliminate OTA from the body and mainly from the kidney was investigated. A group of animals treated for 6 weeks with OTA (289 μg/kg/48 h) were afterwards treated by Aspartame (25 mg/kg/48 h) and compared to OTA alone treated ones for the contents of OTA.

Several parameters have been investigated to evaluate OTA nephrotoxicity and protective effects of Aspartame. These are enzymuria, proteinuria, glucosuria. The enzymes assayed were GGT, LAP, LDH, ALP.

In order to assess the preventive effect of Aspartame on the genotoxic effects of OTA two parameters were investigated, DNA-ductuct formation by the post labelling method with enrichment by nuclease P1, and karyomegaly formation in renal tissue cells. After 6 weeks treatment with a combination of OTA and Aspartame.

Using a blood concentration (10 μg/ml, i.e. 34 μM) the effects of Aspartame on OTA binding to human plasma proteins have been investigated in the two following conditions, A19 is combined with the toxin to mimic the gastric environment, and Aspartame is added to plasma before OTA is added to the lower compartment of a diffusion cell. Only bound QTA is assayed by HPLC and fluorescent detection.

To assess the effect of Aspartame on the cytotoxicity of OTA on vero cells, the cells were cultured and treated as previously described by Baudrimont et al. 1997 in the presence or in the absence of OTA and or Aspartame.

RESULTS AND DISCUSSION

Aspartame is the N-aspartyl-phenylalanine methyl ester. It is thus a structural analogue of both phenylalanine and ochratoxin A. Comparing the metabolism of Aspartame in humans and animals Ranney et al. (1976) showed that similarly in both species Aspartame is completely cleaved into aspartate phenylalanine and methanol within a few hr. They have found that the major portion of the 14C label was expired (10-24%) within 24 hr while 0.7-11% are found in faeces in urine and about 10% is incorporated into proteins. The plasma peak occurs after 4-7 h.

Recently, Moller (1991) reported a very meaningful study on Aspartame metabolism in humans. Six human males each received 0.56 g phenylalanine (Phe) in the form of 1.0 g aspartame or 12.2 g bovine albumin in 200 ml water or water alone. Venous blood samples collected before consumption and during the following 4 hr were assayed for plasma levels of large, neutral amino acids (LNAAs), aspartate, insulin and glucose. The area under the curve for plasma Phe was 40% greater, although not significant, after aspartame compared with albumin intake. The indicated increased clearance rate of plasma Phe following the intake of albumin may be caused by a significant increase in insulin, on which aspartame had no effect. There was a significant effect of aspartame on plasma tyrosine but not on tryptophan, valine, isoleucine or leucine. Plasma aspartate was significantly increased at 0.25 hr after the aspartame intake. The percentage Phe/LNAAs decreased slightly in response to albumin but increased 55% after aspartame and remained significantly increased for 2 hr. Tyrosine/LNAAs increased and tryptophan/LNAAs decreased modestly after aspartame intake. The study showed that the intake of aspartame at a not unrealistically high level produced a marked and persistent increase of the availability of Phe to the brain, which was not observed after protein intake. The study indicated, furthermore, that Phe was cleared faster from the plasma after consumption of protein compared with aspartame.

In our investigations, Aspartame was given to animals alone or combined with OTA and assayed afterwards by HPLC. The results showed that 10 to 12% of Aspartame is distributed unchanged in blood urine and organs (kidney, liver, brain and testicles). When given alone to rat, Aspartame (25 mg/kg) results in level of 73±6 μg/g, 1.8±0.1 μg/g, 156±9 μg/g, 34±2 μg/g 66±5 μg/ml 19±2 μg/ml in respectively kidney, liver, brain, testicle, urine and serum. In the presence of OTA the levels are as follows for the same organs and fluids, 68±5 μg/g, 2.1±0.1 μg/g, 105±9 μg/g, 25±0.6 μg/g 45±3 μg/ml and 11±0.2 μg/ml. The presence of μg quantities of Aspartame had not been reported before due to the sensitivity of the methods used previously in the seventies.

These quantities of Aspartame however seem extremely important for its mechanism of antagonism in OTA binding to human plasma proteins, transport, elimination, metabolism etc.
The efficacy of Aspartame to eliminate OTA from the body and mainly from the kidney has been investigated. Animals treated for 6 weeks with OTA (289 μg/kg/48 h) has been afterwards treated by Aspartame (25 mg/kg/48 h) and compared to OTA alone treated ones. The results showed lower blood and kidney tissue concentrations, after 10 days treatment, Table 1 and Fig. 1.

**Effects of Aspartame on the binding of OTA on human plasma proteins in vitro**

Ochratoxin concentrations used in these experiments are very high, exceeding those found in natural contaminations. They have been put on purpose to show the very high efficacy of Aspartame to prevent the OTA binding on human plasma proteins. The efficacy is higher when Aspartame is present in plasma prior to OTA and decreases when the toxin concentrations increase. Aspartame is also capable of displacing OTA from blood plasma proteins.

**Effects of Aspartame on protein synthesis inhibition induced by OTA**

In Vero cells, cultured with 5% foetal calf serum, the IC50% value increases from 15 μM to 33 μM in presence of Aspartame (10 μM) added in the culture medium before OTA. In case Aspartame is added before OTA severe hr are needed before any protection could be seen which is indeed weak. Aspartame is less effective than free phenylalanine. It may be that this protecting agent needs to be metabolised before being efficient and enters the cells more slowly than OTA.

**Effects of Aspartame on OTA-induced nephrotoxicity in rats**

Several parameters have been investigated to evaluate OTA nephrotoxicity and protective effects of Aspartame. These are enzymuria, proteinuria, gluco-
suria. In all cases beneficial effects of Aspartame have been observed, Creppy et al. 1995.

**Effect of Aspartame on the OTA-induced genotoxicity**

Two parameters have been investigated, DNA-adduct formation by the post labeling method with enrichment by nuclease P1, and karyomegaly formation in renal tissue cells. After 6 weeks treatment with a combination of OTA and Aspartame, the karyomegaly was prevented in 80% of animals and 20% of animals showed a few big nucleus as compared to animals treated with OTA alone. DNA lesions (adducts) were absent showing a very interesting property of

![OTA concentration graph](image)

Fig. 1. Curative attempt with Aspartame Influence of Aspartame on the concentration of OTA in the kidney of rats treated during 6 weeks with OTA (289 mg/kg/48 h) then with Aspartame (25 mg/kg) for 10 days.

<table>
<thead>
<tr>
<th>OTA concentrations μg/ml</th>
<th>Day 0 of curative treatment</th>
<th>Day 10 of curative treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTA for 6 weeks and NaHCO₃ for 10 days</td>
<td>3.61 ± 0.72</td>
<td>1.01 ± 0.34</td>
</tr>
<tr>
<td>OTA/6 weeks and ASPARTAME/10 days</td>
<td>0.48 ± 0.11a</td>
<td>a</td>
</tr>
</tbody>
</table>

*Significantly different from OTA-NaHCO₃, p<0.01*

*Table 1.* Attempt of curative treatment with Aspartame. Influence of Aspartame on the OTA blood levels in rats treated for 6 weeks with the toxin (289 μg/kg/48 h) and then for 10 days with Aspartame (25 mg/kg).
Aspartame which phenylalanine does not possess. The beneficial effect of Aspartame is partially and probably be due to the peptide itself.

**Proposed mechanism of action of Aspartame**

Aspartame is absorbed from stomach and intestine into the blood both unchanged (10 to 12% of the given dose) and cleaved form (aspartate and phenylalanine mainly).

The cleavage is time dependent. That gives rise to the pool of phenylalanine which is much more slowly cleared from the body than the one provided by food proteins.

The so provided phenylalanine plays a role as detoxicating agent (prevention of protein synthesis inhibition induced by OTA etc...)

The unchanged Aspartame (10 to 12%) of the given dose (which is much more than ochratoxin A concentrations in the body in case of natural contamination)-prevents OTA binding to plasma proteins, -enhances OTA elimination, -enhances OTA metabolism, especially to less toxic and genotoxic metabolites. This mechanism is confirmed by the decrease of OTA distribution in organs such as kidney brain liver and testicles and finally by the prevention of OTA-induced nephrotoxicity and genotoxicity.

**Table 2.** Influence of Aspartame (34 μM) on OTA binding to plasma proteins in static diffusion cell. A₁₉ introduced into plasma (upper compartment) just before dialysis. For each value the experiments were conducted in triplicate (OTA=Ochratoxin A).

<table>
<thead>
<tr>
<th>OTA concentration (μM)</th>
<th>Time of dialysis (min)</th>
<th>OTA bound concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>30</td>
<td>215 ± 11 ng/ml</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>431 ± 18 ng/ml</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>772 ± 25 ng/ml</td>
</tr>
<tr>
<td>250</td>
<td>30</td>
<td>442 ± 21 ng/ml</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>739 ± 17 ng/ml</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>1231 ± 71 ng/ml</td>
</tr>
<tr>
<td>1240</td>
<td>30</td>
<td>556 ± 15 ng/ml</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1175 ± 71 ng/ml</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>1744 ± 112 ng/ml</td>
</tr>
</tbody>
</table>

**Table 3.** Influence of Aspartame (34 μM) on OTA binding to plasma proteins in static diffusion cell. Aspartame and OTA simultaneously introduced into the lower compartment. For each value the experiments were conducted in triplicates.

<table>
<thead>
<tr>
<th>OTA concentration (μM)</th>
<th>Time of dialysis (min)</th>
<th>OTA bound concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>30</td>
<td>163 ± 42 ng/ml</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>488 ± 41 ng/ml</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>774 ± 43 ng/ml</td>
</tr>
<tr>
<td>250</td>
<td>30</td>
<td>491 ± 39 ng/ml</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>983 ± 48 ng/ml</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>1296 ± 61 ng/ml</td>
</tr>
<tr>
<td>1240</td>
<td>30</td>
<td>539 ± 41 ng/ml</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1347 ± 921 ng/ml</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>1899 ± 89 ng/ml</td>
</tr>
</tbody>
</table>

OTA alone | OTA+A₁₉
---|---
ng/ml | ng/ml

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Aspartame has the advantage to be a sweetener, already used all over the world which is not toxic. Its efficacy covers all the aspects of OTA toxicology, absorption distribution, metabolism, elimination and specific toxic effects such as nephrotoxicity and genotoxicity.

ACKNOWLEDGEMENTS

The author is grateful to Prof. G. Dirheimer for useful discussion and for the first DNA-adducts determination in his laboratory.

<table>
<thead>
<tr>
<th>Treatment MDA protein</th>
<th>concentration (pmol/mg of % increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>646.0±22.1</td>
</tr>
<tr>
<td>Controls (NaHCO₃)</td>
<td>635.5±17.5</td>
</tr>
<tr>
<td>Aspartame (500 µM)</td>
<td>647.4±23.5*</td>
</tr>
<tr>
<td>OTA (50 µM)</td>
<td>963.4±14.0**</td>
</tr>
<tr>
<td>OTA (50 µM)+aspartame (500 µM)</td>
<td>833.1±8.3***</td>
</tr>
</tbody>
</table>

Aspartame (50 µM) and OTA (50 µM) were used. Results recorded after 24 h incubation.

*Not significantly different from controls (NaHCO₃), p=0.01 (Wilcoxon Rank Sum Test).

**Significantly different from controls (NaHCO₃), p=0.01.

***Significantly different from OTA alone-treated cells, p=0.01.

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