Review

Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans

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Ochratoxin A (OTA) is a ubiquitous mycotoxin produced by fungi of improperly stored food products. OTA is nephrotoxic and is suspected of being the main etiological agent responsible for human Balkan endemic nephropathy (BEN) and associated urinary tract tumours. Striking similarities between OTA-induced porcine nephropathy in pigs and BEN in humans are observed. International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B). Currently, the mode of carcinogenic action by OTA is unknown. OTA is genotoxic following oxidative metabolism. This activity is thought to play a central role in OTA-mediated carcinogenesis and may be divided into direct (covalent DNA adduction) and indirect (oxidative DNA damage) mechanisms of action. Evidence for a direct mode of genotoxicity has been derived from the sensitive ^{32}P-postlabelling assay. OTA facilitates guanine-specific DNA adducts *in vitro* and in rat and pig kidney orally dosed, one adduct comigrates with a synthetic carbon (C)-bonded C8-dG OTA adduct standard. In this paper, our current understanding of OTA toxicity and carcinogenicity are reviewed. The available evidence suggests that OTA is a genotoxic carcinogen by induction of oxidative DNA lesions coupled with direct DNA adducts *via* quinone formation. This mechanism of action should be used to establish acceptable intake levels of OTA from human food sources.

Keywords: Carcinogenicity / DNA adducts / Genotoxicity / Kidney tumours / Ochratoxin A

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1 Balkan endemic nephropathy (BEN)

In the 1950s, a series of publications from Bulgaria [1], former Yugoslavia [2, 3] and Romania [4] described a kidney disease occurring in geographically limited areas of these three Balkan countries that was restricted to individuals from farming households. The progressive, untreatable course and fatal outcome in uraemia shortly after manifestation of symptoms made this kidney disease a major problem for renal pathology. In 1964, a group of experts from the World Health Organisation (WHO) reviewed critically the available data and provided the following description of the disease [5]: "…progressive and very gradually developing renal failure with insidious onset. It develops without a nephrotic syndrome and usually without hypertension. There is a marked anaemia, mild proteinuria, and trivial urinary deposit. The kidney concentration power is reduced in all cases and out of proportion to the degree of restriction

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Abbreviations: AA, arachidonic acid; **ALH,** ascending limb of Henle's loop; **BEN,** Balkan endemic nephropathy; **BSO,** buthionine sulphoximine oxide; **CD,** collecting duct; **CIT,** citrinin; **COX,** cyclooxygenase; **DA,** Dark Agouti; **DB,** debrisoquine; **DT,** distal tubule; **GSH,** glutathione; **IARC,** International Agency for Research on Cancer; **LOX,** lipoxygenase; **MAPK,** mitogen-activated protein kinase; **MDCK,** Madine–Darby canine kidney cells; **MESNA,** mercaptoethane sulphonate; **NDGA,** nor-dihydroguaretic acid; **OAT,** organic anion transporter proteins; **OK,** opossum kidney; **OTA,** ochratoxin A; **OTC,** ochratoxin C; **PAT,** patulin; **PCT,** proximal convoluted tubule; **PIA,** penicillic acid; **PST,** proximal straight tubule; **PTWI,** provisional tolerable weekly intake; **RCC,** renal cell carcinoma; **ROS,** reactive oxygen species; **WHO,** World Health Organisation; **UTT,** urinary tract tumours

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of the glomerular filtration"; "interstitial nephropathy of non-inflammatory origin with heavy damage of the tubular epithelium and only late and secondary destruction of the glomeruli leading eventually to an extreme renal contraction…The last stage shows marked fibrosis, especially in the outer zone of the cortex, where there is a complete absence of the tubules and many fibrotic glomeruli…". "At this stage chronic and/or acute inflammation is not frequent". The disease was then recognised as a new nosological entity and was referred to thereafter as BEN. The clinical features and pathomorphological changes of BEN have now been well defined [6, 7]. Earliest histopathological changes of BEN are characterised by tubular degenerative and regenerative processes of epithelial cells, without reduction in organ size. There are no signs of inflammation and glomerular lesions. In chronic cases, the size of the kidney is considerably reduced and diffuse interstitial fibrosis without inflammatory cells, are the most prominent findings. At late stage, hyalinisation of glomeruli accompanied by enzymuria, frequent headaches, lumbar pain, asthenia, anaemia, loss of weight, absence of hypertension, xanthodermia, polyuria accompanied with a red tongue, thirst and a bitter taste, impaired renal function without nephrotic syndrome and without oedema occurred [8]. Characteristic biochemical changes have also been noted [9, 10] that include mild proteinuria (0.15–0.5 g/24 h), glucosuria, mononuclear cell infiltration (20–250 leucocytes/mm^3), increased blood urea nitrogen concentration and elevated serum creatinine, increased levels of some urinary enzymes (γ-glutamyltransferase, alkaline phosphatase, lactate dehydrogenase), increased urinary pH, hypochromic-microcystic (iron deficiency) anaemia and increased plasma IgM and IgG levels.

In villages of Bulgaria with hyperendemic incidence of BEN, the age-adjusted rates of BEN (1965–1974) were $506/10^5$ and $315/10^5$ for females and males (female/male ratio: 1.61), respectively [11]. From 1958 to 1972, the average annual mortality rate from BEN in the population of Slavonski Kobaš was 5.6 *per* 1000 with an yearly peak of 10.6 *per* 1000 in 1965 [12]. As in Bulgaria, the ratio of sick women to sick men in Croatia was 1.65. In Kaniza, a village in Croatia hyperendemic for these diseases, an incidence rate of $503/10^5$ was found for the period 1975–1979, and in the next three 5-year periods, it ranged between 212 and $246/10^5$ [13]. While for some time no epidemiological studies were carried out in Romania, recent investigations in this country report a number of hot spots for BEN [14]. The highest incidence of BEN was noticed in the early 1980s, and then it started to decline [15–17]. It was also observed from 1957 to 1960 that BEN patients show an average age at death of 45 years, while nowadays their life expectancy is similar to the rest of the population [18].

An association between BEN and urinary tract tumours (UTT) was recognised in the three affected countries [11, 19–24]. Although a few cases of BEN have been diagnosed before the age of 50, most occur in the range 50–60 years and tumours may be detected even later [11]. Recent trends in Serbia demonstrate a shift of 3.5 years in the median age during the 1970s to 1980s and 7.5 years in the 1980s to 1990s [25]. Similarly, a shift of 5 years of the median age for UTT occurred in the 1980s to 1990s. Data from Bulgaria [11] show that the most affected organs are the renal pelvis and ureter, with combined age-adjusted incidence for these two organs of 74.2 and $43.5/10^5$ for females and males, respectively. These values are much higher than those from Trieste, Italy, where rates are already among the highest in the world (2.3 and $5.6/10^5$ for females and males). In the endemic area of Slavonski Brod district in Croatia, the incidence of UTT during 1974–1989 reached the extremely high rate of $66.4/10^4$ [26]. In 1995–2002, in the endemic region of Croatia, specific mortality from these carcinomas was 14 times higher than in Brodsko-Posavska county, and as many as 55 times higher than in the rest of Croatia [18]. In BEN, the early symptoms of UTT are tubular and/or glomemular lesions followed by parenchymal damage and fibrosis and marked membrane thickening and hyalinisation [27, 28]. There may also be an association with increased urinary bladder cancer incidence [29], although many confounding factors such as tobacco smoking may interfere in the analysis of data for this organ. In view of the very intimate association between BEN and UTT, the term 'endemic uropathy' has been proposed to qualify this set of urinary tract diseases in this region of the world (Bozic, Z., personal communication).

The clustering of BEN to specific families suggested that a genetic predisposition may be involved. According to Toncheva *et al.* [30], such a genetic factor for BEN could be due to genes located in a region between 3q25 and 3q26. Such genes may be involved in transforming growth factor-β(TGF-β), genetic heterogeneity of xenobiotic-metabolising enzymes, or defects in the host's immune system. Genetic alterations predisposing BEN patients to UTT could be germline mutations in tumour-suppressor genes [31] and/or acquired somatic mutations in oncogenes. However, studies on migrants [21, 28] strongly implicate environmental factors. Very recently, genetic polymorphisms of some xenobiotic-metabolising enzymes have been associated with BEN. A significant higher risk for BEN (OR 2.41) in individuals carrying the CYP 3A5*1 allele is observed [32]. Andonova *et al.* [33] have shown that carriers of at least one GSTM1 wild-type allele (positive conjugators) were more prevalent among BEN patients compared to controls (OR 7.92). These data are particularly interesting in light of ochratoxin A (OTA) involvement, which is a nephrotoxic mycotoxin biotransformed into genotoxic derivatives by these metabolising enzymes (see Section 5.4).

Nikolic *et al.* [34] demonstrated that the very intimate link between BEN and UTT can be explained by insult from an environmental contaminant. The intake of the

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agent at high doses causes nephropathy and early appearance of renal failure (BEN) during the third and the fourth decade of a patient's life. However, at low doses of the potential causative agent, the nephropathy is not recognised, but UTT still develops. Under these conditions, the patient may die from UTT even though kidney damage is minimal and subclinical, so that most of the patients (75%) in a BEN settlement may show no symptoms of renal failure at the time of nephrectomy.

In 1972, on the basis of a series of epidemiological observations, Akhmeteli [35] suggested that fungal toxins were involved in the aetiology of BEN.

BEN affects inhabitants in rural areas but not those from towns in the vicinity. This could be explained by the fact that rural populations consume homegrown and home-stored food, while urban populations consume commercial foods produced by factories. The disease often affects many members of one family, while neighbouring families may be free from the disease for several generations. All members of a family share the same food over many years, and some foodstuff may be repeatedly contaminated by moulds, while neighbours may not be exposed to this factor. This could be due to poorer exposure to the sun, leading to inferior conditions for storage of food grain and, consequently, conditions promoting contamination with moulds. In one village, one part may be more affected than others even though socio-economic conditions are similar. This can be explained by variations in fungi and their metabolites in a limited area, as was demonstrated by Linsell and Peers [36].

In view of the similarities between BEN and OTA-induced porcine nephropathy (see below), Krogh [37] suggested that OTA may be involved in the aetiology of BEN.

2 OTA contents in food and biological samples

2.1 OTA in food

The highest amounts of OTA in food of plant origin were found mainly in Eastern Europe, including rye flour from Poland (5410 μg/kg) and barley from Czechoslovakia (3800 μg/kg) [38]. Surveys of foodstuffs in the endemic area of former Yugoslavia demonstrated that 8–12% of the cereals were contaminated with OTA [39–41]. Various commodities (like wheat, maize, barley, beans, potatoes, bread) and animal feed also contain OTA. Higher mean OTA concentration and higher frequency was found in samples from endemic regions compared to controls [42, 43]. A similar study of food contamination conducted in Bulgaria demonstrated that, except for wheat and wheat flour, a higher percentage of the staple food was contaminated by OTA in the endemic area than in the nonendemic area [44, 45]. In a recent reanalysis of the data obtained by Petkova-Bocharova *et al.* [45], the contamination of food consumed by affected families was compared with that of food consumed by unaffected families in the endemic and control areas. The results showed a striking difference, demonstrating that, in Bulgaria, affected families are not only much more frequently exposed to the mycotoxins OTA and citrinin (CIT) than the control families but also to higher contamination levels of both toxins [46]. Vrabcheva *et al.* [47] found that in the BEN endemic area, wheat samples could also be contaminated by OTA, and at higher levels than in the control nonendemic region. Higher exposure levels to OTA have been confirmed in the BEN households than in the within village controls and in the controls in BEN free villages [48].

Comparison between crops from ecological farms (farms in which no pesticides or fungicides were used) and conventional farms in Poland show clearly higher OTA contamination in crops from ecological farms, about six times [49]. Similar results were reported in Denmark for the harvest years 1992–1999 [50]. Inappropriate farm management practices were associated with higher OTA amounts [51].

In a duplicate diet study over 1 month, in which participants from the high BEN incidence area in Bulgaria were asked to store an equivalent part of the food consumed, it was recently demonstrated that the average weekly intake of OTA varies from 130 to 6489 ng, corresponding to 1.86–92.7 ng/kg body weight (bw) if one considers an average weight of 70 kg for an adult or a daily intake of 0.27–13.2 ng/kg bw [52, 53]. The provisional tolerable weekly intake (PTWI) established by the Joint FAO/WHO Expert Committee on Food Additives and Contaminants is 100 ng/kg bw/week. This assessment has used nephrotoxic effects in pigs to establish acceptable intake levels for OTA. Two of the nine volunteers from Beli Izvor, over 1 month, were very close to this PTWI (92.7 and 91 ng/kg bw/week, respectively) with weekly peaks up to 200 ng/kg bw. It is important to point out that the food collection for this study was performed during autumn/early winter. With OTA being a mycotoxin which develops during storage, the levels found in the food are probably much lower than levels which would have been found in spring/summer.

It is known from other countries that meat may also contain OTA through secondary contamination. Pepeljnjak and Blazevic [54] reported the presence of OTA in a variety of smoked meat products. Data provided by the WHO [55] show that countries with the highest frequency of OTA-contaminated sample of animal feed were Denmark (57.6%), Canada (56.3%) and Yugoslavia (25.7%); all in the range of 30–27.5 mg/kg. These data are limited in that they were derived from studies published in the early 1970s. More recently, OTA has been detected at significant levels in food of animal origin such as poultry, bacon and other pork products as well as cow's milk [38, 56–58]. Little is known about OTA contamination from food in Romania. A survey of slaughtered pigs demonstrated that 98% of serum contained OTA in the range 1.67–13.4 ng/mL. Kidney, liver

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and muscles were also contaminated. This implies contamination of animal feed and most probably all of the human food chain [59]. Stoev *et al.* [60, 61] analysed the serum and renal tissue concentrations of OTA in pigs from farms in endemic areas of Bulgaria. An average of 1.32 ± 1.25 μg/kg was found in renal tissue, and serum concentrations varied considerably between 27 and 249 ng/mL (66.8–616 nM).

2.2 OTA in human blood

Hult *et al.* [62] developed a method of OTA analysis in pig blood that has been adapted and applied to OTA analysis in human sera from former Yugoslavia [63]. With a detection limit of 1 ng/mL serum, 7% of human blood samples collected in the endemic area were positive for OTA; the highest levels being 40 ng/mL serum. Nine years later OTA was still detected in blood samples from the endemic area and also from a nonendemic area, although at lower levels [64]. In 1985, the frequency of contamination and OTA levels were higher in the nonendemic area, although the sampling seasons were different. A very high amount of OTA in serum (1.8 μg/mL) was found in one of the Croatian samples [65]. Samples were collected in winter in the endemic area but during the following summer in the nonendemic area, which would have allowed mould growth and OTA production during the spring period. In this later study, the reported LOD was high (5 ng/mL) and the percentage of samples exceeding that level was unusual for European countries. A 10-year study in Croatia on the presence of OTA in human serum yielded similar data [41]. Recently, the exposure of the general population in Croatia to OTA was investigated in five cities: two on the Adriatic coast and three inland. Osijek (Slavonski Brod) differed significantly from the other cities with all samples showing OTA plasma concentrations above 0.2 ng/mL. The number of samples with OTA concentrations between 0.2 and 1.0 ng/mL was also significantly higher in this region than in other cities, and the number of positive samples with OTA above 1.0 ng/mL plasma was higher than in any other city. This difference is probably due to the higher consumption of fresh and dried pork by the population of Osijek [66]. In Bulgaria, the exposure of populations to OTA was further supported by a very high prevalence of OTA levels exceeding 2 ng/mL in the blood of BEN-affected families [67–69]. These data have been confirmed some 20 years later in the same area of Bulgaria [53, 70]. Recently, Vöö *et al.* [71] compared OTA blood concentration of 25 patients with BEN and 15 healthy controls from a nonendemic area. OTA was present in serum samples of all BEN patients but was undetectable in the serum of healthy controls suggesting that OTA could have a role in the pathogenesis of BEN.

When comparing OTA levels in blood from BEN patients to those from healthy persons in other European countries using analytical methods with an LOD of 0.01 ng/mL (Table 1), the BEN population is more frequently exposed to levels exceeding 2 ng/mL. For healthy individuals the highest mean blood concentration in Denmark was 1.8 ng/mL, but only half of the population was exposed to OTA. When most of the population was exposed to OTA (Czech Republic, Germany, Italy, Switzerland, Sweden, UK), the average concentrations were typically much lower (0.17–1.09 ng/mL).

Variation of serum concentrations of OTA over time within individuals has been observed in several countries, as exemplified by ten-fold differences in people from Croatia [41] and Bulgaria [53, 70]. Ruprich and Ostry [86, 87] also found large differences in OTA concentrations on repeated sampling of sera from four Czech blood donors over 2 months (37 to <0.33 ng/mL). In Japan, large variations (31–825%) were found for OTA in the serum of three Japanese males analysed at an interval of more than 3 months [88]. Considerable variation of OTA serum concentration over 3 years was observed in 20 persons in a city of Germany, with extreme variations in two persons of 0.3–1.3 ng/mL [89]. Part of the variation may be explained by toxicokinetics. Once OTA reaches the bloodstream, it is bound to serum proteins (>99%), which facilitates its passive absorption in the nonionised form, but hinders its glomerular filtration. OTA binds strongly to albumin (binding saturation above several hundred micrograms *per* millilitre of serum) [90], but also strongly to other small proteins (20 000 Da), for which binding saturation is reached with an OTA concentration of 10–20 ng/mL [91]. The fraction of OTA bound to proteins constitutes a mobile reserve of OTA that can be released as soon as the free OTA fraction decreases. This delays elimination and thus increases the risk of accumulation of OTA in tissues. In a recent study, OTA consumption, blood concentration and excretion were monitored in Bulgarian volunteers for a 1-month period [53]. For some individuals, regular and continuous OTA intake equivalent to 1.2–2.2 ng/kg bw/day led to a relatively high steady state OTA blood concentration (1.46 ng/mL) and low OTA elimination. For others, variable intake (1 wk high, 1 wk low) led to a constant relatively low OTA blood concentration (0.5 ng/mL), which was in the same range as volunteers having a regular very low OTA intake (in some cases below the LOD). Taken together, these data indicate that OTA blood level is relatively stable over a period of 1 month for a given individual and is regulated by urinary excretion. Thus, variation of OTA food intake is not directly reflected by a variation of OTA blood concentration: (i) high OTA intake is not always reflected by high serum OTA concentration and (ii) the highest serum concentration is not related to the highest OTA consumption. In a 1-month duplicate diet study in the UK no correlation between plasma concentrations and consumption of OTA was observed [92].

Studer-Rohr *et al.* [93] have shown intraindividual fluctuations of OTA plasma concentration in humans. Some

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Table 1. OTA blood levels in healthy persons from European countries, using conventional HPLC techniques for analysis

Country	% Positive samples	OTA amount (ng/mL)	Reference
Denmark	54.2	0.1–13.2 (1.8)[a)]	[72]
France (Alsace)	19.4	0.1–11.8	[73]
France (Rhône Alpes)	14.6	0.1–4.3	[73]
Germany (1977–1985)	56.5	0.1–14.4 (0.6)	[74]
Germany (1988)	68	0.1–8.4 (0.75)	[75]
Germany (1999)	98.1	0.06–2.03 (0.27)	[76]
Italy (1992)	100	0.1–2.0 (0.53)	[77]
Italy (1994)	97	0.12–57.2 (0.56)	[78]
Poland (1983–1985)	7.2	1–40 (0.28)	[79]
Spain	53	0.5–4.0 (0.71)	[80]
Switzerland	100	0.06–6.02	[81]
Sweden (1989)	12.8	0.3–7.0 (0.20)	[82]
Sweden (1990–1991)	100	0.09–0.94 (0.17)	[56]
UK	100	0.4–3.11 (1.09)	[83]
Czech Republic	93.1	0.1–13.7 (0.24)	[84, 85]

a) The value in brackets correspond to the average value.

volunteers, followed over a 2-month period, showed rather constant OTA plasma concentrations, while for others high plasma OTA variability was observed. Kinetic experiments in humans indicated that during the first 6 days OTA is mainly distributed within the body and only a minor fraction is excreted. During the following 69 days, the OTA plasma concentration decreased, while a larger amount of OTA (and metabolites) was excreted *via* the urine. In the same study, the plasma half-life of OTA determined in one individual was about 20 h during the six first days and 35.6 days from day 6 onwards.

Regional variations in OTA plasma concentrations have also been observed in healthy humans within Canada [94], Switzerland [81], Sweden [82], Croatia [66], Germany [76, 95] and France [73], which may be explained by differences in diet or climate. In general, there was significantly higher OTA plasma concentrations in regions where more local food products are consumed [73, 82]. Correlation between plasma OTA concentration and specific types of food consumed has been shown for cereal products, wine, beer and pork in Norway [96] and for certain cereal products, sausages, red grape juice, chocolate with nuts and coffee in Germany [95]. Seasonal variability has also been recorded. OTA blood concentration is higher in the summer: (i) in Croatia (59% positive, mean 0.39 ng/mL in June *vs.* 36% positive, mean 0.19 ng/mL in December) [66]; (ii) in Tuscany, Italy (0.65 *vs.* 0.43 ng/mL) [78] and (iii) in Spain (1.75 *vs.* 0.97 ng/mL only in women) [97].

2.3 OTA in urine

OTA is excreted in kidney tubules using organic anion transporter proteins (OAT) [98–100]. OTA is then reabsorbed in all nephron segments using OAT or other transporters [101]. This delays elimination and thus increases the risk of OTA accumulation in tissues.

OTA has been found more often in the urine of people living in BEN-endemic villages than in those in non-endemic villages, and the highest amounts were seen in patients with BEN or UTT [69]. In Bulgaria, OTA could be found in urine in up to 98% of persons from an area with BEN [53, 69, 70, 102]. The relationship between OTA in the urine with OTA intake is also complex. In general, the elimination of OTA for human is low (average value between 20 and 80 ng/day) and independent of the dose ingested. However, when the intake level of OTA is below 100 ng/kg bw/week, OTA elimination increases dramatically and is multiplied 10–50-fold for an average intake of 100 ng/kg bw/week [53]. This observation is in accordance with the observation that OTA uptake is dependent on the free OTA concentration, which is severely limited by binding of OTA to serum albumin.

Calculations on OTA renal clearance demonstrate higher and lower than expected rates for high and low OTA plasma concentrations, respectively. This may be interpreted as representing a filtration-mediated reabsorption process with low capacity for OTA. At high OTA plasma concentration, reabsorption is saturated leading to excretion rates greater than expected [93]. In a 1-month duplicate diet study in the UK, OTA in urine was found to be a better indicator of OTA consumption than OTA plasma concentrations, even though OTA concentrations in urine were two orders of magnitude lower. Urine concentrations in 46/50 samples ranged from <0.01–0.058 ng/mL [92]. OTA was found in all Bulgarian participants in the range of 0.016–0.860 ng/mL [53, 70]. In Italy 22/38 urine samples from healthy people contained OTA in the range of 0.012–0.046 ng/mL [103]. OTA was detected in 60% of urine samples (88 samples) from healthy people in Hungary in the range of 0.0006–0.065 ng/mL [104]. In one human volunteer dosed with tritiated OTA, only 42–54% of the radioactivity remained in unchanged OTA, indicating OTA meta-

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bolism [93]. The presence of OTA metabolites in human urine was confirmed by various studies. The nonchlorinated OTB metabolite and hydroxylated metabolites (4-OH-OTA) were detected in Bulgarian urine [53]. The 4-OH-OTA metabolites in the range of 0.04–21 ng/mL were also found in the urine of 24/54 children under 5 years of age in Sierra Leone [105]. In another study conducted in Sierra Leone, in dry and wet seasons, OTA was detected in the urine of 55/231 boys (0.07–72 ng/mL) and 55/203 girls (0.08–148 ng/mL) and 4-OH-OTA was present in 167/434 up to 37 ng/mL [106]. In both studies [105, 106], OTB was also found at a maximum concentration of 218 ng/mL.

2.4 OTA in human milk

OTA has also been detected in human milk samples [107, 108]. Trace amounts of OTA in human milk (0.017–0.030 ng/mL) were measured in 4/36 samples [107]. In contrast, Rosner *et al.* [76] did not detect OTA in 27 samples of human milk (<0.06 ng/mL). In Norway, a positive correlation between high intake of liver paste and cake with OTA in milk has been established. In this study, 17/80 samples contained OTA in the range of 0.01–0.182 ng/mL [109]. In Sweden, 23/40 human milk samples contained 0.01–0.04 ng/mL OTA [56]. In Hungary, 38/92 samples of human milk were contaminated with OTA in the range of 0.2–7.3 ng/mL [108]. Concentrations of OTA in human milk from Italy were higher: 9/50 and 22/211 samples reached 1.7–6.6 and 0.1–12 ng/mL, respectively [110, 111]. OTA concentrations in human milk of three Italian mothers over 1 wk were compared with OTA in various foods [112]. Milk was found to contain OTA in the range of 0.08–0.54 ng/L even though OTA levels in food were quite low (0.03 μg to 1 μg/kg). In Australia, 2/100 milk samples contained OTA (3–3.6 ng/mL) [113]. In Sierra Leone, the incidence of OTA in human milk was high (35/40) with positive samples in the range 0.2–337 ng/mL, and frequent co-occurrence with aflatoxins was observed [114]. In another study, 30% of human milk samples were contaminated with OTA [105]. OTA was also detected in human milk from Brazil in 2/50 samples (0.01 and 0.2 ng/mL) [115]. The presence of OTA in milk represents a high risk of exposure for the infant. Postupolski and Karlowski [116] found OTA in breast milk from Polish women at levels similar to those found in milk samples from Germany, Sweden and Switzerland.

3 Acute and subacute toxicity of OTA

The toxicity of OTA varies widely, depending on the animal species, sex and route of administration. The LD_{50} after oral administration ranges from 0.2 mg/kg bw in dogs to 30.3 mg/kg in male rats (female rats being more sensitive). OTA is more toxic when administered intraperitoneally than orally. Di Paolo *et al.* [117] have described a case of acute intoxication in a man with oliguria and tubulonecrosis which might have been due to inhalation of *Aspergillus ochraceus*. It has recently been demonstrated [118–120] that OTA is present in spores and airborne dust, thus supporting this hypothesis. Brera *et al.* [121] and Iavicoli *et al.* [122] in Italy and Degen *et al.* [123] in Germany have demonstrated exposure to OTA in workplaces from dust and a relation to plasma OTA levels. Similarly, we have isolated in dust collected in 'silo', *penicillia* and *aspergilli* capable of producing OTA (Pfohl-Leszkowicz *et al*., Personal communication, 2001; [124]).

OTA is very toxic to numerous animal species, the kidney being the main target organ (for reviews, see [125, 126]). Other toxic effects observed include cardiac and hepatic histological abnormalities, aberration of coagulation factors in the rat, accompanied by haemorrhage and thrombosis in the spleen, brain, liver, kidney and heart [127], lesions of the gastro-intestinal tract and lymphoid tissues in the hamster [128], myelotoxicity in mice [129, 130], and intestinal fragility and kidney lesions in chickens [131]. Prior *et al.* [132] noted decreased feed intake in chickens exposed to feed contaminated with 0.5 ppm OTA. When feed is contaminated with 2 ppm OTA, signs of ochratoxicosis appear in poultry (weight loss, decreased egg production, increase of water intake, diarrhoea, and excessive urine excretion which is a characteristic of a renal disorder) [133–137]; haematological modifications have also been noted [138]. At 4 ppm OTA, mortality increases dramatically [134, 135]. Haazele *et al.* [139] have demonstrated that vitamin C has beneficial effects in hens exposed to OTA. For the related mycotoxin CIT, which is frequently found as a cocontaminant of food sources [140–142], the LD_{50} depends on animal strain and sex and on the route of administration. It varies from 20 mg/kg bw by subcutaneous injection in the rabbit to 112 mg/kg bw in the mice after intraperitoneal administration (for a review, see [143]). CIT could increase the toxic and carcinogenic effect induced by OTA (see below).

3.1 Nephrotoxicity

The nephrotoxicity of OTA is well documented. OTA is nephrotoxic to birds and mammals [144, 145], but not to adult ruminants [146]. Studies in Denmark, Hungary, Scandinavia and Poland have demonstrated that OTA plays a major role in the aetiology of porcine nephropathy [147]. This nephropathy has been induced in pigs administered feed contaminated with 4000, 1000 or 200 μg of OTA *per* kg (4, 1 or 0.2 ppm) [148]. With doses of 1 and 4 ppm, the kidney loses its colour and necrosis is observed within 3–4 months. Ultrastructural investigations of kidney in animals exposed to 0.8 ppm OTA identified a process of condensation of cellular material with disappearance of membranes and continuous desquamation in the lower part of

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the proximal convoluted tubules (PCTs). In target cells, peroxisomes lose membrane integrity and leak their contents into the cytosol [149]. Water intake and urinary excretion by animals increases. Weight loss occurs at doses of more than 2 ppm OTA [150]. Recently, ultrastructural and toxicological investigations in spontaneous cases of porcine nephropathy have been carried out [61]. These studies show striking similarities between the described OTA-induced porcine nephropathy in pigs [151–153] and BEN in humans. In both diseases, major kidney lesions were observed in the epithelial cells of the proximal tubules. Many cell organelles in the damaged epithelial cells were decreased and exhibited loss of membrane integrity and the brush border was reduced in height and density. A large number of apical vesicles, lysosomes and peroxisomes with granular material were seen, and many nuclei had condensation of chromatin and disappearance of the nuclear envelope. At a later stage, thickening of the basement tubular membranes of the proximal tubules and a large number of collagen fibres in the interstitium dominated the ultrastructure picture. OTA was found in 100% of investigated serum samples collected from the same pigs (48.34 ± 6.7 to 84.2 ± 41.17 ng/mL) [61]. In these pigs, renal damages were characterised by impairment of proximal tubular function, manifested by an increase in urinary excretion of glucose and protein, and by increases in urea and creatinine concentration in blood, whereas the levels of serum protein and glucose were decreased. The serum levels of potassium, sodium, aspartate-aminotransferase (ASAT) and glutamate dehydrogenase (GLDH) were increased [60, 152] and an increase of gamma-glutamyl transpeptidase (GGT) and leucine aminopeptidase were also observed [154]. Stoev *et al.* [155] induced porcine nephropathy with a diet containing 90 and 180 μg/kg OTA. The characteristic renal lesions were similar to those observed in spontaneous cases of porcine nephropathy in Bulgaria, but were a little different from the classic Danish porcine nephropathy. According to the authors, the enhanced toxicity of OTA may be due to a synergistic effect of penicillic acid (PIA). It must be noted that no analysis of CIT was performed and that CIT was found in human Bulgarian food by two authors [45, 47]. Stoev *et al.* [156] induced porcine nephropathy with 800 μg/kg OTA for 1 year. The characteristic renal lesions were similar to the classic Danish porcine nephropathy but rather different from the spontaneous cases of porcine nephropathy in Bulgaria. These results suggest strongly for a role of OTA in the aetiology of porcine nephropathy.

OTA also induces glomerulonephrosis, tubulonephrosis, focal tubular epithelial cell proliferation and multiple adenoma like structures in the renal parenchyma in broiler chicks exposed to 0.5 mg OTA *per* week for 4 wk [157]. Stoev *et al.* [158] observed a nephropathy in poultry for which the severity does not justify the levels of OTA exposure. The authors suggested that the nephropathy may have a multitoxin aetiology.

OTA induces nephropathy in rats [159], chickens [131] and more general poultry, as demonstrated by Hamilton *et al.* [160] in their investigations on potential etiological factors in nine outbreaks. Huff *et al.* [161] demonstrated that OTA is not only nephrotoxic to chickens but it is also hepatotoxic. In young ducklings, OTA induces renal and liver hypertrophy [162]. The sensitivity of ducks and turkeys is similar but the quail is much more sensitive [163].

3.2 Immunotoxicity and myelotoxicity

Lea *et al.* [164] demonstrated that OTA inhibits the proliferation of T and B lymphocytes *in vitro* and abolishes the production of IL2 and its receptors. OTA suppresses the activity of killer cells as well as the production of INF in mice [165]. Recently, Heller *et al.* [166] demonstrated *in vitro* that OTA and crude *A. ochraceus* toxins inhibit the secretion of the proapaototic cytokine, TNFα. Interestingly, induction of TNFα by OTA is stimulated by lipoxygenase (LOX) and epoxygenase pathways, whereas it is inhibited by the cyclooxygenase (COX) pathway [167, 168].

When administered to several animal species, OTA induces diverse effects on bone marrow and on immune responses that include lymphopenia in dogs [169], chickens [170], turkeys [171] and swine [151, 172, 173], and thymus regression and immunosuppression in mice [129]. The immune system of the pig is sensitive to OTA doses below 1 mg/kg (a 10% inhibition of the immune response is observed with 0.06 mg/kg of OTA) under normal breeding conditions [174, 175]. Doses of 2.5 mg OTA/kg feed administered to pigs reduces the phagocytic activity of macrophages and the production of IL2 [176], confirming the findings of Lea *et al.* [164] and Holmberg *et al.* [177]. The same decreases have been observed previously in mice [129, 178], turkeys [171] and broiler chickens [179, 180]. The effects of OTA on humoral immunity depend on the route of administration and the species. Broiler chickens fed diets containing 4 mg of OTA/kg of feed had decreased IgG, IgA and IgM concentrations [136, 137], whereas Igs in cows were not affected by diets containing 390–540 μg of OTA/kg of feed [181]. Stoev *et al.* [182] demonstrated that pigs administered feed moulded by *A. ochraceus* which contained, among other toxins, 1–3 ppm OTA, were more susceptible to salmonellas. *Salmonella choleraesuis* was isolated from the faeces and liver of all the treated animals. In the same experiment, animals immunised against *S. choleraesuis* and ingesting feed containing 1 ppm OTA presented haemorrhagic diarrhoea. This experiment demonstrated both an immunosuppresive effect and a delayed immunisation related to OTA and ingestion of other toxins. Müller *et al.* [183, 184] showed that severity of experimental pneumonia was considerably increased when pigs were fed daily subtoxic amounts of OTA (5–50 μg/bw for 28 and 34 days). Severe and rapid progression of coccidiosis occurred in chicks fed OTA-contaminated feed [185].

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Administration of a diet contaminated by OTA (130, 305, 790 μg/kg) and PIA (2000–5000 μg/kg) to broiler chickens induced significant lower haemagglutination inhibiting antibody titers in the experimental chickens immunised with vaccine against Newcastle disease. Degenerative changes and depletion of lymphoid cells were observed in bursa Fabricci, thymus, spleen, and Peyer's patches of intestinal mucosa [186]. In BALB/c mice, a single intraperitoneal dose of 5 μg/kg of OTA suppresses the immune response to sheep erythrocytes. This effect can be prevented by simultaneous treatment with phenylalanine at twice the OTA dose [187]. Müller *et al.* [130] have demonstrated that mice immunised against *Pasteurella multocida* are less resistant to OTA than nonimmunised mice. Intraperitoneal dosing of 20–80 mg/kg of OTA to female B6C3F1 mice on alternate days over an 8-day period resulted in a dramatic dose-related decrease in thymic mass (down to 33% of controls), histological evidence of nephrotoxicity restricted to the inner cortex and myelotoxicity as evidenced by bone marrow hypocellularity, decreased marrow pluripotent stem cells (CFU-S), granulocyte-macrophage progenitors (CFU-GMs), and decreased ^{59}Fe uptake in the marrow and spleen of exposed mice [129]. Subchronic oral exposure of female Balb/c mice to OTA at exposure levels similar to those found in food decreased the proportion of mature CD4+ or CD8+ lymphocyte cells, suppressed antibody production and the number of thymocytes [188]. The same authors studied the effects of prenatal exposure to OTA on the immune system of BALB/c mice. Exposure of the dams to relatively low levels of dietary OTA altered the absolute and relative numbers of lymphocyte subpopulations in lymphoid organs, but did not suppress immune function in the offspring [189]. Recently, a study using rat showed that oral exposure to low OTA doses during 4 wk decreased the lymphocyte population and thymus weight [190, 191].

Singh *et al.* [192] studied the effect of 0.5 and 2.0 ppm OTA on broiler chicks. Highly significant reductions in cell-mediated immunity were indicated by diminished skin sensitivity, graft *versus* host reactions and T lymphocyte counts. On the other hand, only the overall haemagglutinin titers differed significantly between the various treatment groups. Total lymphocyte counts, total serum protein, serum albumin and serum globulin levels were significantly depressed on the 21st day of intoxication. The number of splenic macrophages was drastically reduced in both the intoxicated groups compared with controls. The weights of the thymus, bursa of Fabricius and spleen of intoxicated birds were significantly reduced.

3.3 Teratogenicity

OTA is a potent teratogen in mice [193], rats [194–196], hamsters [197, 198], chickens [199], and rabbit [200], but not in pigs [201]. Maternal OTA can cross the placenta, accumulate in foetal tissues and induce malformation, mainly in the central nervous system after administration to pregnant animals (for a review see [202]). Studies of teratogenicity have demonstrated a direct foetal effect of OTA [195, 203–207]. Recent investigations show that gestation days 6 and 7 are the most critical for the induction of OTA-mediated teratogenicity in rats [208]. A single oral dose of 2.75 mg/kg bw OTA was found to be the minimum effective teratogenic dose in pregnant Wistar rats and was shown to cause internal hydrocephaly, microphthalmia, enlarged renal pelvis and renal hypoplasia in the foetuses [208].

4 Carcinogenicity

OTA is carcinogenic in rodents [209]. The first studies on OTA carcinogenicity were performed on rats [210], trout [211] and mice [212, 213] by oral or intraperitoneal administration. Only after oral administration tumours were induced in the kidney of mice and rats and hepatoma in the trout. When these studies were evaluated by International Agency for Research on Cancer (IARC) [214, 215] it was found that the evidence for carcinogenicity was either inconclusive or limited. The results of the mouse carcinogenicity study were subsequently confirmed by a second study by the same group [216], which also demonstrated a synergistic effect of CIT when it was administered simultaneously.

The carcinogenic potency of OTA to mice was confirmed by Bendele *et al.* [217]. Groups of B6C3F1 mice (50 males and 50 females) were given a feed containing 40 μg OTA for 24 months. In treated male mice, 31 were affected (14/49 had renal carcinomas and 26/49 renal adenomas). All males showed nephropathy but only a small number of females. None of the females had renal carcinoma or adenoma. A small number of both males and females had hepatocellular neoplasm.

In a study by the US National Toxicology Program (NTP), three doses of OTA (210, 70, 21 μg/kg bw) were administered to male and female F344N rats [218]. At 210 μg/kg bw renal tubular adenomas and carcinomas were observed (72% for males and 16% for females) after 2 years. At 70 μg/kg bw, 39% of the males and 4% of the females developed renal adenomas or carcinomas. The females were less susceptible than the males to OTA carcinogenicity. In addition, non-neoplastic renal modifications (hyperplasia, cell proliferation, cytoplasmic alteration, and karyomegalies) were observed. All animals treated with 70 or 210 μg/kg bw doses presented karyomegalies. A high rate of metastases from renal cell carcinomas (RCCs) mainly to lung, liver and lymph nodes was also observed in both males and females. In view of these results, IARC [209] evaluated the experimental evidence for carcinogenicity as sufficient, and classified OTA as 'possibly carcinogenic to humans'. These results in rats were confirmed by a study at IARC using Lewis and Dark Agouti (DA) rats.

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Male DA rats were very sensitive and female DA rats were resistant. In addition, male DA rats were much more sensitive than either male or female Lewis [219]. This difference in sensitivity may be due to differences in biotransformation capacity [220]. Since 2-mercaptoethane sulphonate (MESNA) protects rats against nephrotoxicity and carcinogenicity induced by oxidative stress by increasing free thiol groups in kidney [221, 222], the potential protective effect of MESNA on renal toxicity and carcinogenicity induced by OTA was examined in a long term rat study [223]. MESNA significantly decreased karyomegalies in kidney of all OTA-treated animals but had no beneficial effect on renal tumour incidence. Even a significant increase in renal tumour formation was observed in male DA rats [223].

In a recent study [224], OTA (100 µg/day) was administered to male Fisher-344 rats by gavage, in which the feed was homogenised with artificially fermented wheat rich in OTA and OTB and free of any other mycotoxins. No renal tumours were found in control rats. For the OTA treated rats, the first renal tumour was discovered at 75 wk and 20% of the rats developed renal carcinoma and renal parenchyma. Some large tumours were associated with metastasic nodules located exensively along the abdominal mesenteries, and occasionally carcinoma extended to the lungs. Other histopathological changes in kidney were observed such as karyomegalic nuclei in tubular epithelia, predominantly in the cortico-medullary region [224]. A commission of the Deutsche Forschungsgemeinschaft (Commission for the investigation of Health Hazards of Chemical compounds in the work area 2003) has classified OTA in category 2 "a substance which is considered to be a carcinogen for man because sufficient data from long-term animal studies or limited evidence from animal studies substantiated by evidence from epidemiological studies indicated that it makes a significant contribution to cancer risk. Limited data from animal studies can be supported by evidence that it causes cancer by a mode of action that is relevant to man and by results on in vitro tests and short-term animal studies" [225]. In addition to renal carcinoma, Schwartz [226] in a correlational study hypothesised that OTA exposure may be related to increased incidence of testicular cancer. Although no epidemiological studies have been done to confirm this hypothesis, implication in such a cancer is substantiated by high amounts of OTA and derivatives in testis and time- and dose-dependent DNA adduct formation [190, 227]. Mammary glands could also be a potential target. Indeed, a statistically significant increased incidence of fibroadenomas of the mammary gland was observed in high-dosed female rats (28/50; 56%) compared to controls (17/50). An increased incidence of multiple mammary fibroadenomas (two *per* animals) was observed in high-dosed female rats (14/50 compared to controls (4/50). No mammary tumour was observed in male rats [218]. Incidence of mammary proliferative lesions was also increased in OTA-treated female Lewis rats compared to controls (6/19; 31.6%) [228].

5 Toxicokinetic

OTA (Fig. 1) consists of a *para*-chlorophenolic moiety containing a dihydroiso-coumarin group that is amide-linked to L-phenylalanine. In the following sections, the structural aspects of the toxin are discussed with reference to the mechanism(s) of toxicity and carcinogenicity. The first part of this section deals with OTA transport. OTA is rapidly absorbed both from stomach and the small intestine in rats and mice. Absorption from the duodenum can take place against a concentration gradient [229], highlighting the presence of OAT proteins for OTA transport [98–100]. Following absorption, the concentration of the toxin and its metabolites depends on a number of factors including dose, route and duration of administration, and also on species-specific factors such as half-life and the degree of serum binding [128, 202]; for recent reviews see Ringot *et al.* [230] and O'Brien *et al.* [231]. Enterohepatic circulation also appears to be a factor in the kinetics of OTA. The reabsorption of OTA by the kidney has also been proposed to facilitate the residual persistence of the toxin and hence renal toxicity in rodents [127, 232]; for a review see Gekle *et al.* [233].

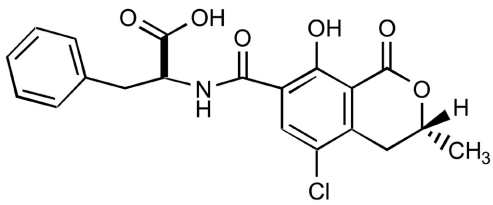

Figure 1. Structure of OTA.

5.1 Absorption

The pK_a values of OTA are in the range 4.2–4.4 and 7.0–7.3, for the carboxyl group of the phenylalanine moiety and the phenolic group, respectively. These acidic moieties play an essential role in OTA adsorption. At the physiological pH conditions of the duodenal chyme both the monoanion (OTA^-) and the dianion (OTA^{2-}) are present, whereas the fully protonated toxin is mainly present in acidic solutions, such as in the upper parts of the gastrointestinal tract. In most animal species, OTA is passively absorbed in its non-ionised and monoanion (OTA^-) forms from the stomach and, particularly, from the proximal duodenum [229, 234, 235]. The passive absorption is highly favoured by the high binding affinity of OTA to plasma proteins (see below). Recently, Berger *et al.* [236] have used an *in vitro* model of the human intestinal epithelium based on the culture of CaCo-2 cells in a bicameral system to study the mechanism of OTA transport across the human intestinal mucosa. Using conditions mimicking the *in vivo* situation in the duodenum (apical pH 6, basolateral pH 7.4) these authors

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showed that the apical to basolateral passage direction of OTA was predominant. They also showed that carrier systems like the H^+-dipeptide cotransporter, the organic anion carrier (oat/OAT) and the organic anion polypeptide carrier (Oatp) are not implicated in OTA transport across CaCo-2 cells, which again suggests that the absorption is the result of a diffusion phenomenon. On the other hand, and in accordance with the results of Kumagai and Aibara [229], Berger *et al.* [236] showed that the intestinal absorption is limited by the efflux of the toxin at the apical (AP) pole of the CaCo-2 cells and suggested the role of a multidrug resistance-associated protein (probably MRP2 isoform, now named ATP-Binding Cassette C2, ABCC2) in the efflux of OTA (secretion of xenobiotics out of the cells). These transporters counteract absorption, contribute to protective tissue barriers and play an important role in the elimination process. Recently, BCRP, another member of the ATP-dependent membrane transporter, has been implicated in OTA efflux [237]. Expression of BCRP in humans was found in various organs, including the intestines, liver, blood vessels, lactating mammary gland and kidneys [238, 239]. The percentage of OTA absorbed differs between species: 66% in pigs, 56% in rats and rabbits and 40% in chicken [240]. Bioavailability may vary in the presence of food components, many of which are BCRP substrates, as well as inhibitors. Indeed, Sergent *et al.* [241] have shown that flavonoids (quercetin, chrysin, genistein and biochanim) and resveratrol increase OTA absorption and cellular accumulation. OTA is subsequently transported *via* the portal system and distributed to different tissues and organs.

5.2 Distribution

5.2.1 Blood transport

It is well known that OTA binds strongly to proteins [65, 128, 240]. Once OTA reaches the bloodstream, it is 99% bound to serum proteins (mainly albumin), which facilitates its passive absorption in the nonionised form and partly explains its long half-life in the body [242, 243]. Studies *in vivo* reveal that the lifetime of OTA in living systems is dependent on the presence of serum albumin and that this binding is species- and sex-dependent [244, 245]. The role of albumin in the kinetics of OTA distribution has been illustrated by Kumagai [244] in a study with albumin deficient rats that were able to clear OTA from systemic circulation 20–70-fold faster than normal rats. It was suggested that albumin binding delays OTA elimination by limiting the transfer from the blood to the hepatic and renal cells.

Il'ichev *et al.* [246, 247] have concluded that OTA binds to HSA as a dianion (OTA^{2-}) at two sites, each one containing one OTA molecule. A high affinity site (site I) is situated in domain IIA of HSA, and contains cationic residues (histidine (His242), Lys199 and probably Arg257) that stabilise the anionic groups on OTA by electrostatic interactions. HSA binding by OTA lowers the pK_a of the phenolic group, thereby facilitating the formation of an ion pair between the phenolate of OTA and protonated R257 [246–249]. The second binding site (site II) has a lower affinity for OTA and is situated in domain IIIA of the protein, but this secondary site has not been fully characterised.

Stojkovic *et al.* [91] demonstrated a strong binding of the toxin to two small serum proteins (20 000 Da) that bind OTA more specifically than albumin. Because such small molecules can pass through the glomerular membrane, the authors concluded that this binding could be relevant to the nephrotoxic effect of OTA in mammals. The specific binding on this protein was saturated at an OTA concentration of 10–20 ng/mL serum, whereas for HSA binding saturation is only attained above several hundred micrograms *per* millilitre of serum. In addition to these reported proteins, other, not yet characterised, blood proteins seem to be involved in OTA transport [90]. Hagelberg *et al.* [128] examined the relationship between the plasma binding properties of OTA and its toxicokinetics in several animal species. After oral or intravenous administration of 50 ng OTA/g bw, the fraction of the toxin that remained unbound varied considerably and was 0.02% in humans and rats, 0.08% in monkeys, 0.1% in mice and pigs and 22% in fish.

The fraction of OTA bound to HSA and other serum macromolecules constitutes a mobile reserve of mycotoxin that can be released to the tissue. Heussner *et al.* [245] have also reported species-dependent differences in the binding characteristics of OTA to proteins present in renal cortical homogenates from pig, mouse, rat, and human of both sexes. Using a modification of a classical receptor-binding assay, these authors described the presence of at least one homogeneous OTA-binding component. This component appeared to have low affinity but high capacity for ^{3}H-OTA, which could be competed for by a range of substances known to have affinity for steroid receptors and/or for various organic anion transporters previously reported to be responsible for the transport of OTA [99]. The generated binding-capacity ranking of human > rat > pig ≥ mouse correlates well with the biological half-lives determined by other authors (human > rat ≥ pig > mouse) and also with the toxicity ranking for experimental animals *in vivo*.

The wide species difference in serum half-life of OTA was recently reviewed by Petzinger and Ziegler [250]. Plasma clearance rates for the monkey (0.17 mL · $kg^{-1}h^{-1}$) were almost ten-fold slower than those of the rat (0.91 mL · $kg^{-1}h^{-1}$). In addition, Stander *et al.* [251] determined the half-life of OTA in vervet monkey to be 456–504 h after intravenous administration. The half-life after oral ingestion of OTA (p.o.) is shorter than after intravenous injection (i.v.), most probably because of the transit through the liver and the subsequent elimination by the bile, before entering the systemic blood circulation. Monkey has the longest elimination half-life ($t_{1/2}$ = 35 days after i.v.; $t_{1/2}$ = 21 days after p.o.), which is about four to five times higher than that of rat ($t_{1/2}$ = 7 days, i.v.; $t_{1/2}$ = 5 days, p.o.). Among

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all the studied models, humans appear to have the longest serum half-life, estimated to be 35 days [252].

5.2.2 Tissue-specific distribution

In animals, the concentration of the toxin and its metabolites in tissues and plasma depends on the animal species, the dose administered, the form of OTA administered (crystalline or naturally occurring in feed), the diet composition as well as the health status of the animal. These factors are important to assess in determining the occurrence of OTA in animal tissues. Generally, the elimination half-life of OTA is longer in blood than in tissues, which may be related, in part, to the higher binding affinity of the toxin to blood proteins [93, 128, 253]. The tissue distribution in pig, rat, chicken and goat, after acute treatment, is kidney > liver > muscle > fat [254, 255] or kidney > muscle > liver and fat [72, 256, 257]. Kane *et al.* [258] observed a slightly different tissue distribution in rats after ingestion of low dose (144 μg/kg bw/day during 12 days) OTA (lung > liver > kidney > heart > fat > intestine > testes > muscle > spleen > brain). OTA was still present 8 days or more after injection in the blood, myocardium, lung, large intestine, liver, kidney, urine and skin [259]. Daily oral exposition of chicken to OTA during 4 wk leads to a similar tissue distribution (liver > kidney). After i.v. injection to rat and mice, the distribution was lung > liver > heart > kidney [259, 260]. Recently, we demonstrated that OTA tissue distribution is dose- and sex-dependent [190, 191, 261–263]. DA rats (male and female) received wheat artificially contaminated with increasing doses of OTA (2.5–100 μg/kg food, corresponding to an intake range from 40 ng to 8.5 μg/kg bw) every day during 4 wk. At low dose OTA (172 ng/kg bw) the distribution in female tissue was brain > lung > liver and undetectable amounts were found in kidney; whereas in male the distribution was liver > lung > kidney > testis = brain. For intermediate dose (494 ng/kg bw) the distribution in female tissues was lung = brain > liver = kidney; whereas in male the distribution was liver = lung > kidney > testis > brain. When the intake was 3.5 μg/kg bw, the distribution in female tissue was liver > kidney = lung > brain, whereas in male the distribution was liver = lung > kidney = testis > brain. Finally for the highest dose tested (8.5 μg/kg bw) the distribution in female tissue was lung > liver > kidney > brain; whereas in male the distribution was lung = liver > kidney = testis > brain. Except in brain, male rats accumulate higher amounts of OTA in liver, kidney and lung than female rats [190, 191]. In an *in vitro* study with proteins from various rat organs and cultures of different renal cell lines, Schwerdt *et al.* [90] reported the potential of OTA to bind to different cytosolic and organellar proteins in a highly specific fashion. One OTA binding protein is a 62 kDa organellar protein present in the cells originating from the proximal tubules. Although this protein is of low abundance, its binding to OTA is suspected to contribute OTA accumulation in kidney cells, enhancing its nephrotoxic effect.

Several studies have shown in utero transfer of OTA in mammalian species such as mice, rat and swine [202, 264]. The amounts of OTA found in kidney and blood of offspring exposed both by placenta and lactation were higher (four- to five-fold) compared to offspring exposed only by placenta or only *via* milk [264]. A linear relationship was found between OTA in the milk of rabbit and OTA in the plasma of suckling rabbits, indicating the effective transfer of OTA from milk to offspring [255]. The distribution in the tissue of rabbit was kidney > liver > mammary gland > muscle [255]. OTA crosses the placenta more readily at days 8 and 9 than at day 10 of gestation, with radioactive labelled OTA appearing within 20 min in uterine wall and placental and foetal tissues [265]. The differences observed in OTA foetal uptake after different durations of gestation suggest that the transfer is influenced by the developmental stage of the placenta, which is considered to be completely developed after day 9 of gestation. In humans, the OTA concentration in foetal serum was reported to be twice the maternal concentration, thus suggesting an active placental transfer of OTA [81]. Miraglia *et al.* [266] showed that the OTA levels in placenta and in funiculum were higher than those found in the corresponding maternal serum. Additionally, these authors suggested that genetic, environmental and pathological factors may contribute to OTA transport in the placenta. Little is known on the mechanism of OTA transfer across the placenta. Foetal blood is separated from the maternal blood circulation by polarised cells, which possess carrier-mediated transport pathways, like the renal proximal tubules and the intestinal epithelial cells. Carrier proteins facilitate the supply of the essential compounds to the developing foetus. Cha *et al.* [267] showed the role of hOAT4, isolated from human placenta and localised in the trophoblast membrane facing the foetal blood, in the mediated transport of various organic anions including OTA. BCRP seems also implicated in OTA secretion in milk as demonstrated in human [268].

5.3 Elimination

Both biliary and renal routes are involved in the excretion of OTA in rats and mice, with the biliary route predominating, although this may also be dependent on the dose and the route of administration [202, 253, 269, 270]. In humans and nonhuman primates (vervet monkeys), the parent OTA is excreted mainly *via* the kidney [93, 251]. The relative contribution of each excretory route is influenced by the route of administration, the dose, the degree of binding with plasma proteins and the enterohepatic circulation of OTA; for reviews see [230, 271].

Since OTA is characterised by a high plasma protein binding potential, its glomerular filtration is limited. Instead, OTA undergoes tubular elimination to the urine. OTA is also reabsorbed at all nephron segments [233]. These phenomena involve the carrier-mediated transport of

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OTA across the tubular membranes by several transporter proteins. OTA is a substrate for uptake transport *via* OAT [272] and for efflux transport *via* the multidrug resistant protein-2 (MRP-2) [273] and BCRP [237]. The regulation of these transporters are sex-dependent [274–276].

Early studies indicated that multispecific OAT [98, 277] mediates the uptake of OTA across the basolateral membrane of the proximal tubule. Human hOAT1 (predominantly present in kidney) and hOAT3 (also present in liver and brain) have been reported to be involved in OTA transport [100]. The hOAT1 protein is localised in the basolateral membrane of intact renal tubule [278] and its classical substrates are polyaromatic hydrocarbons (PAHs). In kidney, hOAT3 is located in the basolateral membrane of the proximal tubule [279] and it can transport lipophilic organic anions such as estrone sulphate. Kusushura *et al.* [280] reported that rOat3 isolated from rat brain also mediates the transport of OTA. Sex-specific differences in mRNA expression levels were observed for Oat2 (*Slc22a7*) and Oat3 (*Slc22a8*) but not for Oat1 (*Slc22a6*) [281–283]. Bahnemann *et al.* [284] have shown by using the nonfiltering toad kidney model that OTA exits cells across the luminal (apical) membrane by facilitated diffusion. Leier *et al.* [273], using recombinant human MRP2 (ABCC2), showed a role for the apical MRP2 efflux pump in OTA excretion into the lumen of proximal tubules. The MRP2 may also be involved in hepatobiliary elimination of OTA-conjugates [236]. In hepatocytes, it is situated in the apical (canalicular) membrane and is responsible for the biliary excretion of glutathione (GSH), glucuronide and sulphate conjugates of xenobiotics [285].

Babu *et al.* [286] demonstrated the role of human hOAT4 (localised in the luminal membrane of the proximal tubule) in OTA transport at the apical side of the proximal tubule. The affinity constant (Km) of OTA for human OAT4 was determined to be 23 μM. Subsequent to its excretion, OTA is reabsorbed in all nephron segments [233] and this reabsorbance is dependent on pH [101]. At pH 6, the estimated fractional reabsorption (FR) values expressed in percentage of the OTA amount infused during microinfusion at different sites were: PCT 14.8%, proximal straight tubule (PST) 27.4%, ascending limb of Henle's loop (ALH) 13.6%, distal tubule (DT) 11.6%, collecting duct (CD) 24.6% and terminal collecting duct (TCD) 22.0%. At pH 8, FR values were as follows: PCT 0%, PST 25.9%, ALH 14%, DT 3.2% and CD 8.2%. As reabsorption in PST and ALH is pH independent, reabsorption is thought to be carrier-mediated. Microperfusion experiments *in vivo* (rats) indicated that the H^+-dipeptide cotransporter [101, 287] mediates OTA reabsorption; *in vitro* studies also showed that transporters like human hOAT4 [286] and rat kidney-specific rOAT-K2 [288] are involved in OTA reabsorption at this nephron segment. Thus, hOAT4 mediates both the efflux of OTA into the tubule lumen and its reabsorption at the apical side of the proximal tubule. Recently, Kobayashi *et al.* [274] showed that mOat2 is involved in OTA uptake in mouse kidney. In fact, mOat2 is predominantly present in the kidney of the mouse and more specifically in the apical membranes of the tubules and of the ALH, whereas in rat, the rOat2 is predominantly expressed in the liver. The localisation of mOat2 suggests its specific role in ALH reabsorption. However, the role of OAT2 in renal OTA transport in different mammalian species remains unclear. Notably, its localisation is reported to be apical in rat and mouse kidney, but basolateral in human kidney [289]. The reabsorption in DT and CD takes place mostly by passive nonionic diffusion [101, 287]. An *in vitro* study with Madine–Darby canine kidney cells (MDCK) suggested the additional role of the H^+-dipeptide cotransporter in OTA reabsorption from the CD [290]. The reabsorption of filtered and secreted OTA retards its excretion and may lead to the accumulation of the toxin in the renal tissue and thus contribute to its renal toxicity. These toxicokinetic features result in an accumulation of OTA in renal tissue, where the highest concentrations have been detected in the papilla and in the inner medulla.

5.4 Metabolism of OTA

The metabolism of OTA has been extensively studied by the team of Størmer during the past 25 years [291] using *in vitro* and *in vivo* assays in liver of different animals [292–295]. The major metabolites are shown in Fig. 2 and include: hydroxylated derivatives 4(*R*)-, 4(*S*)- and 10-OH-OTA (numbering in Scheme 1 for OTA) and OTalpha (OTα), which lacks the phenylalanine moiety. The 4(*R*)-OH-OTA metabolite was mainly formed following incubation in presence of human and rat liver microsomes, whereas 4(*S*)-OH-OTA was essentially formed *via* pig microsomes [293, 296, 297] and in the urine of rat [269, 298]. The 10-OH-OTA metabolite was detected after *in vitro* incubation of OTA with rabbit liver microsomes [294]. Studies *in vivo* show that cleavage of the peptide bond of OTA to yield OTα occurs in homogenates from pancreas and small intestine, but not in liver [295, 299]. This proteolytic activity is due to α-chymotrypsine and carboxypeptidase [300].

Other researchers have more recently studied the biotransformation of OTA. *In vitro* incubation of OTA in the presence of microsomes or treatment of cell lines with OTA leads to the formation of at least 20 derivatives [296, 297, 301, 302]. Our laboratory has demonstrated that after *in vitro* incubation in the presence of microsomes or cell cultures the formation of OTA metabolites is both time- and dose-dependent. In the presence of pig liver microsomes the kinetics are linear during the first 10 min, and then a plateau is reached at 25 min. At least ten different OTA derivatives have been separated by HPLC with fluorescence detection from cell culture (human bronchial epithelial (BEAS-2B), opposum renal cells) and *in vitro* incubation

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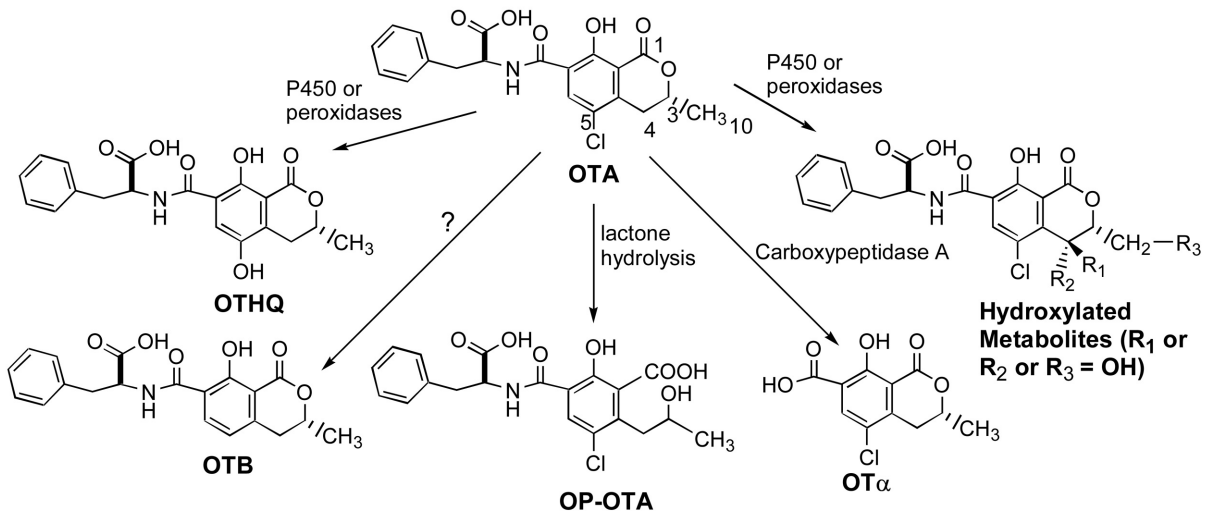

Figure 2. Metabolism of OTA.

(pig kidney microsomes). Coelution of these derivatives with synthetic OTA metabolite standards indicate that under our *in vitro* conditions, the open-lactone ring form of OTA (OP-OTA) is formed exclusively after incubation with cortex microsomes. Pig liver microsomes (male/female), and female cortex and medulla microsomes induced formation of 4(*R*)-OH-OTA; but not 4(*S*)-OH-OTA. In contrast, male pig microsomes from cortex and medulla generated both 4(*S*)- and 4(*R*)-OH-OTA. These results show that the relative quantity of metabolites depends on the origin of the microsomes (male or female, liver or kidney) and on the cofactor used: NADPH, arachidonic acid (AA), or both simultaneously [302]. The 4(*R*)- and 4(*S*)-OH-OTA derivatives are formed in the presence of kidney microsomes [301, 303–305], pig seminal vesicle microsomes [306], monkey kidney cells [307], hepatocytes [308] and in BEAS-2B cells [306]. Gross-Steinmeyer *et al.* [309] reported the formation of these metabolites in hepatocytes, and also the formation of six unknown OTA derivatives in the supernatant of the hepatocytes.

The nonchlorinated OTB analogue was also formed from OTA following incubation with rabbit kidney microsomes pretreated with PB [305] and in monkey kidney cells [307]. The OTB derivative has also been detected in kidney of pig fed OTA [301]. While little is known about the mechanism of OTB formation *in vivo*, it is also generated from OTA photochemically [310, 311]. Under photochemical conditions, it is expected that OTA interacts with a solvated electron, which initiates C–Cl bond homolysis to afford an OTA carbon-centred radical and chloride; H-atom abstraction by the radical provides a route to OTB [311]. This reaction is akin to reductive dehalogenation of alkyl- and aryl-halides that are catalysed by CYP450 enzymes [312]. As pointed out by Guengerich, ferrous CYP450 should be a good one-electron-reducing agent and that transfer of an electron to a substrate may be competitive with oxidation even in the presence of molecular oxygen [312]. It is also important to point out that OTA stimulates the production of superoxide ($O_2^{\bullet-}$) that will lead to the liberation of free ferrous ion (Fe^{2+}). The interaction of OTA with Fe^{2+} may also cause reductive dehalogenation of OTA to yield the OTB metabolite [313].

The other nonchlorinated metabolite of OTA shown in Scheme 1 is the hydroquinone analogue OTHQ [314]. Recent biological studies confirm the presence of OTHQ in the urine of rat following administration of OTA by gavage [315]. On the basis of analogy to the metabolism of pentachlorophenol (PCP), which generates benzoquinone/hydroquinone redox couples through an oxidative dechlorination process [316], it is expected that a two-electron reduction of the quinone of OTA (OTQ) by the action of NADPH:quinone reductase, for example, generates the hydroquinone metabolite OTHQ. Chemical studies have also provided indirect evidence for the intermediacy of OTQ in the oxidation of OTA [313].

In terms of specific CYP450 isoforms responsible for OTA metabolism, Ueno [317] demonstrated that in rat CYP1A2 is involved in 4(*R*)-OH-OTA production, whereas 4(*S*)-OH-OTA is formed by CYP2B. We observed using BEAS-2B cells expressing specific human CYP450 isoforms that 4(*R*)-OH-OTA is formed by several CYP450 isoforms (1A2, 2B6, 2C9, 2D6, 2A6), whereas 4(*S*)-OH-OTA is formed by only CYP2D6 and 2B6 [307, 318]. Implication of CYP1A1/1A2, 2B1 and 3A1/3A2 in the metabolism of OTA has also been pointed by Omar *et al.* [319]. Formation of the OH-OTA derivatives is modulated by inducers or inhibitors of CYP450, and prostaglandin synthase, which has similar activity to COX and LOX. Indeed, pretreatment of animal with phenobarbital (PB) increased 4(*R*)-OH-OTA production in hepatocytes [308], rat liver [292, 319], rabbit kidney, and in BEAS-2B cells [305]. We have also observed these hydroxylated metabolites in kidney of pig fed OTA

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[301] and have noted that inhibition of the LOX pathway increases formation of 10-OH-OTA [306]. The CYP isoforms implicated in OTB formation are CYP 2A6 [318] and CYP 2C9 [305], both of which are involved in the biotransformation of coumarins [320], which may be relevant given that OTA contains an isocoumarin moiety.

Specific CYP450 isoforms have also been implicated in the genotoxicity and mutagenicity of OTA. Fink-Gremmels and coworkers have recently demonstrated that in cells expressing CYP2C9, OTA exerts increased cytotoxicity, as measured by the neutral red assay [321] and mutagenicity [322]. The CYP3A4 isoform has also been suggested to be involved in the biotransformation of OTA [323]. CYP3A enzymes are particularly relevant for xenobiotic metabolism because of their broad substrate specificity and abundant expression in the human liver, intestine and kidney. The CYP 3A5*1 allele was more prevalent in BEN patients with a frequency of 9.38% compared to 5.36% in controls and was associated with a higher risk for BEN (OR 2.41). Our studies have also shown that in male DA rats that are highly susceptible to OTA-mediated renal carcinogenesis that the OTA-toxifying enzymes (CYP450 2C11, 1A2, and 3A) were highly expressed in the liver [220]. The induction of CYP2C11 in the DA rat is also highly relevant. Patients suffering from BEN have been reported to be more frequently extensive metabolisers of debrisoquine (DB) due to CYP3A activity [32]. In male rats CYP450 2C11 is a DB-metabolising phenotype suggesting a correlation between the high susceptibility of rat in relation to DB hydroxylation capacity (due to CYP 2C11) [220]. A close relationship exists between CYP3A and CYP2C enzymes. Indeed, CYP450 2C is induced by sex hormones [324–326] which are under the control of CYP450 3A.

CYP450 enzymes are primarily responsible for the oxidation of most xenobiotics in liver [327], whereas much lower CYP450 activities are expressed in other tissues. In the kidney, lung and brain, several xenobiotics are co-oxidised by COX or LOX [328, 329]. Because OTA induces renal tumours in rats [217–219] and catalyses formation of reactive oxygen species (ROS) and stimulates lipid peroxidation in the kidney [330, 331], its biotransformation could be due to co-oxidation by several enzymes; notably those enzymes implicated in the metabolism of AA, such as COX, LOX and epoxygenase, which is related to CYP450 2C11 [332, 333]. Systems that we have used to study OTA metabolism, such as pig seminal vesicle microsomes as well as BEAS-2B cells express high levels of COX and LOX enzymes [329, 334–336], as well as CYP450-epoxygenases that are found in the cortex and outer medulla of kidney and bladder cells [337, 338]. OTA is also known to stimulate GSH oxidation and superoxide generation and this activity correlates with the activity of LOX enzymes that also mediate these signals of oxidative stress [339]. In this regard, pretreatment of cells with indomethacin (0.1 μM) or nor-dihydroguaretic acid (NDGA) (inhibitors of LOX pathway) prior to OTA treatment modified the profile of OTA derivatives, as studied by HPLC with spectrofluorometry detection, suggesting a direct role for LOX enzymes in the bioactivation of OTA [306]. Notably, 4-(*R*)-OH-OTA was no longer present in the HPLC trace. However, three new metabolites of unknown structure were detected and appear to be significant because they were also generated exclusively in BEAS-2B cells specifically expressing CYP 2C9 [305], a CYP450 enzyme thought to be highly involved in OTA genotoxicity and carcinogenicity in rats [220]. The unknown metabolites coelute on HPLC with OP-OTA and the synthetic OTHQ standard provided by the Manderville laboratory. Interestingly, the OTA derivatives formed with pig microsomes are similar to those obtain under cell culture conditions where LOX and CYP450-epoxygenase activities were predominant compared to COX (*i. e.* cells pretreated with 10 μM indomethacin or epinephrine). The hypothesis for CYP450-epoxygenase involvement in formation of these three metabolites was suggested by their disappearance when CYP450-epoxygenase was inhibited (*e. g.* pretreatment with NDGA or 10 μM indomethacin).

The biotransformation of OTA into several derivatives is particularly important as half-life and route of elimination are metabolite-dependent [253]. Moreover, the toxicity of OTA derivatives are not equivalent. Notably, OP-OTA (ring opening lactone) is reported to be more toxic than the parent OTA [340, 341]. Hoehler *et al.* [342, 343] have also shown that several metabolites of OTA induce ROS formation in microsomes that stimulate hydrogen peroxide formation; a cofactor for the enzymatic activity of LOX as well as COX enzymes with peroxidase activities. It is well known that ROS production generated during peroxidase catalysis initiate free radical chain reactions which lead to epoxidation or oxidation of xenobiotics [344]. Liu and Massey [345] have also shown that epoxidation of aflatoxin (AFB_1) is catalysed by AA-derived peroxyl radicals, which are generated *via* interactions of lipid hydroperoxide, and prostaglandin G_2, with the heme group of COX. The initiation of this reaction may occur in the absence of oxygen, because thiyl radicals, formed from the oxidation of thiols by transition metals, may react directly with an unsaturated bond in lipid [346]. This mechanism could also be involved in OTA metabolism. Several studies clearly establish a link between lipid peroxidation and OTA metabolism [330, 331, 342, 343]. In cell cultures, the formation of OTA metabolites is modulated by several inhibitors of the GSH pathway and by antioxidants. For example, relatively large amounts of OTB and another dechlorinated derivative of OTA are formed in cells pretreated with acivicin and buthionine sulphoximine oxide (BSO) [347], suggesting that removal of GSH stimulates OTB production. The structure of several of these derivatives (OP-OTA, OTB, 4(*R*)-OH-OTA) identified by cochromatography have also been confirmed by MS [347, 348].

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The pharmacokinetics of OTA and five of its metabolites in rat was investigated by Li *et al.* [253] who demonstrated that OTA followed by OP-OTA have the longest half-lives. Some of these metabolites were isolated, from pig or rat tissues, notably OP-OTA, 4-OH-OTA, 10-OH-OTA and OTB [301]. 4-OH-OTA was excreted from urine, in addition to other metabolites [340, 341, 348, 349]. Gautier *et al.* [350] also detected 4-OH-OTA and an unknown OTA metabolite in urine. Mally *et al.* [315] found traces of OTB and OTHQ in urine of rat treated with 2 mg/kg OTA for 2 wk. Contrary to these reports, Zepnick *et al.* [351] did not detect any OTA metabolites apart from OTα in tissue and urine of rat treated orally with a single OTA dose of 0.5 mg/kg bw. To explain this discrepancy with previous *in vivo* studies, the authors stated that previous studies administered much higher doses of OTA to the animals, which may interfere with renal function or result in a higher yield of OTα formation due to slower or incomplete intestinal absorption of OTA. In fact, Castegnaro *et al.* [349] administered OTA by oral gavages to two strains of rats (DA and Lewis) at the respective doses of 0.5, 2.5 and 5 mg/kg bw. They observed the excretion of OTA and 4-OH-OTA. The amount of 4-OH-OTA excreted after 24 h was related to the dose of OTA administered in both strains of rats. Thus, the argument by Zepnick *et al.* [351] that the differences could be due to different OTA doses used in the various studies is inconsistent with the report by Castegnaro *et al.* [349], as one of the OTA doses was identical and the data demonstrated a dose relation between 4-OH-OTA excreted and the OTA administered in a concentration-dependent fashion. Clearly other reasons for the discrepancy in the data reported by Zepnick *et al.* [351] must exist.

A potential reason for the lack of OTA metabolites detected by Zepnick *et al.* [351] could be due to the method used for extraction of OTA and its metabolites. This hypothesis was tested using kidney of male pigs fed 18 days with OTA (18 μg/kg bw/day) and liver from rats treated with OTA [301]. Two methods of extraction were tested in parallel ending with the same method of detection, HPLC/spectrofluorimetry [352]. The first technique of extraction was the classical one using chloroform extraction after acidification; the second one was exactly that described by Zepnick *et al.* [351] that is based on ethanol protein precipitation. The extracts were compared with a series of OTA metabolite standards. With chloroform extraction, OTA, and metabolites corresponding to the elution time of 4(*R*)- and 4(*S*)-OH-OTA were found in the medulla and to a lower extent in the cortex of pig, and in liver of rats. In these extracts, compounds with retention similar to OTB, OTα, OTβ and OP-OTA were observed [352]. Other compounds of unknown nature were also detected. In contrast, using ethanol extraction, only a compound with retention time of the parent OTA was detected [352]. This indicates that the OTA metabolites are most probably lost during the deproteinisation step. Zepnick *et al.* [351] reported high recovery of OTA during their extraction under the above-mentioned conditions; this is based on the addition of OTA to the tissue and is not contradictory with our results. That OTA spiking to tissue was performed just before homogenisation indicates that OTA probably lacked sufficient time to bind proteins, as it does under *in vivo* conditions. Thus, the OTA analysed by Zepnick *et al.* [351] was free OTA (that from spiking) which was easily extracted. On the other hand, OTA bound to proteins requires very acidic conditions and $MgCl_2$ to liberate OTA and its metabolites for efficient extraction [353]. The need for acid is completely consistent with the known fact that OTA binds HSA through electrostatic interactions involving the phenolate anion of OTA [246, 247]; protonation of the phenolate reduces protein affinity of OTA.

6 Molecular mechanism

6.1 Genotoxicity and mutagenicity

OTA has long been considered nongenotoxic. The various standard tests using procaryotes are generally negative or very slightly positive. Ames tests carried out with different strains of *Salmonella typhimurium* (TA 98, TA 102) were negative for OTA mutagenicity [353, 354]. No induction of growth inhibition of various strains of *Bacillus subtilis* occurs after OTA treatment [355]. No recombination is observed in *Saccharomyces cerevisiae* D3 after OTA treatment [356, 357]. At doses of 5 and 10 μg/mL, OTA did not induce 8-azaguanine-resistant mutation in mammary cells of C3H mice [390]. In the absence of metabolic activation, no mutations were detected in cells from FM3A mice exposed to OTA [358].

More recent data have demonstrated the mutagenic potency of OTA in mammalian cells. A very potent mutagenic effect was seen in *S. typhimurium* TA 1535, 1538 and 100 exposed to supernatant of rat hepatocytes pretreated with OTA [359] and also in the presence of kidney microsomes in *S. typhimurium* TA 1535, 1538 and 98 [360]. Mori *et al.* [361] observed disorganisation of DNA synthesis in rat (ACI) and mouse (C3H) hepatocytes. OTA-induced unscheduled DNA synthesis was demonstrated in rat hepatocytes and in urinary bladder epithelial cells from pig [362] and in primary human urothelial cells [363]. The effect of OTA on DNA repair and induction of DNA damage was further investigated by detecting the occurrence of DNA damage by the alkaline single cell gel electrophoresis assay (comet assay). In MDCK cells, OTA-induced single-strand breaks in a concentration-dependent manner. When an external metabolising system (S9-mix from rat liver) was added this genotoxic effect was significantly stronger, thus implying a role of metabolisation [364]. In the same study, it was demonstrated that OTA-induced DNA damage is increased by blocking repair

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mechanisms. OTA also slightly increased the response in the SOS-spot test [365], although a dose–response relationship was not observed. In the absence of metabolic activation, 4 mM OTA-induced SOS repair in *Escherichia coli* PQ37 [366]. OTA induces chromosomal mitotic recombination in *Drosophila* [367]. A small, but dose-dependent, increase in sister chromatid exchanges were induced in CHO cells treated with OTA [202]. Sister chromatid exchanges were also induced in human lymphocytes in the presence of hepatocytes [359] and in cultured isolated porcine urinary bladder epithelial cells treated with OTA [368]. In another study, a statistical increase of structural chromosal aberration and sister chromatid exchanges associated with a decrease of the mitotic index was observed in bovine lymphocytes [369]. OTA-induced micronuclei in ovine seminal vesicle cells [370, 371], in Syrian hamster fibroblasts [372] and in human hepatic (HepG2) cells [373]; all in a dose-dependent manner. Significant dose-dependent increases in the frequency of micronucleated cells, DNA single-strand breaks and alkali-labile sites, as measured by the comet assay, were also obtained in primary kidney cells from both male rats and humans of both genders with OTA from 0.015 to 1.215 μM [374]. Single-strand DNA breaks have been observed in kidney, liver and spleen DNA of BALB/c mice treated intraperiteonally or orally with OTA [258, 375]. Manolova *et al.* [376] demonstrated the presence of chromosomal aberrations on chromosome X from human lymphocytes cultivated in the presence of OTA. Similar aberrations have been detected in BEN patients [377].

On the basis of structure–activity relationships, Malaveille *et al*. [378] proposed that the presence of the C-5 chlorine atom in OTA is one determinant of its genotoxic action. Xiao *et al.* [340, 341, 379] also demonstrated that the phenylalanine moiety and the *para*-chlorophenolic group are important for *in vitro* and *in vivo* toxicity. In contrast to the proposal by Rahimtula *et al.* [330] that Fe-chelation may be linked to OTA-toxicity, Xiao and coworkers showed that an O-methylated phenolic derivative of OTA that lacks metal chelation properties, was equally efficient in generating ROS production, suggesting that the toxicity of OTA is not linked to its chelation of Fe^{2+}. These authors concluded that the phenylalanine group is important for genotoxicity and demonstrated that some OTA derivatives, notably OP-OTA (Scheme 1), can bind macromolecules, protein and DNA [341].

The biotransformation of OTA is complex and involves several enzymes such as CYP450, but also GSH transferases [380] and LOX. The metabolites conjugated to GSH and/or UDP are excreted in bile and in kidney. At least 20 different metabolites of OTA including OTB, OH-OTA, OP-OTA, OTα, and metabolites of unknown structure have been detected. Based on all the data available we summarise the known and hypothetical pathways for OTA metabolism in Scheme 2.

Conversion of OTA into the quinone (OTQ) by an oxidative dechlorination pathway generates ROS by redox cycling that can lead to DNA breaks and LPO-derived exocyclic adducts (pathway 2). The quinone OTQ can undergo either a two-electron reduction by action of the NAD(P)H: quinone reductase to form hydroquinone OTHQ (pathway 3), or undergo a one electron-reduction to yield a semiquinone radical (pathway 4), which in turn could induce DNA breaks, LPO and exocyclic adducts (pathway 2). OTA was shown to induce oxidative damage due to the generation of hydroxyl radicals ($HO^{\bullet}$) by microsomes in presence of NADPH as a microsomal reductant and O_2 not requiring exogenous iron (pathway 2). Pathway 2 is thus inhibited by ROS scavengers such as MESNA and NAC and explains the reduction of OTA-induced karyomegalies after MESNA treatment. OTHQ could be formed directly by GST (pathway 5) and can be oxidised into OTQ (pathway 6). Indeed, GSTs are involved in dehalogenation: the first step is formation of an epoxide, in the second step the epoxide is converted into phenol. This can lead to OTHQ and/or OTB. Evidence to support the pathways outlined in Fig. 3 are presented in Section 6.2.

6.2 DNA adduction

The process of chemical carcinogenesis is initiated by covalent binding of carcinogens or their reactive metabolites to DNA, thus forming DNA adducts [381]. There is a strong correlation between DNA adduct formation and the frequency of mutations [382]. To interact with cellular macromolecules and thus initiate cancers, most chemical carcinogens require metabolic activation [381].

The genotoxicity of OTA has also been established by its ability to promote DNA adduct formation. It has been shown that rats and mice treated with OTA show numerous DNA adducts [383]. The highest levels were found in kidney DNA where some adducts persist for more than 16 days, while adducts are repaired in liver and spleen after 5 days [304, 384]. Similar adduct patterns have been found in kidney and bladder tumours of BEN patients [385]. Single doses of OTA administered to mice [304, 384, 386] and rat by gavages or in feed [387] induced DNA adducts in kidney in a dose- and time-dependent manner. In cell culture (opossum kidney (OK) or BEAS-2B) and after *in vitro* incubation in the presence of pig and rat kidney microsomes, dose- and time-dependent DNA adducts were observed [348, 388, 389]. Petkova-Bocharova *et al.* [386] demonstrated that administration of OTA to pregnant mothers at day 9 of gestation-induced DNA adduct formation in foetuses and pups that were detectable several months after birth. Moreover, some of them developed renal carcinoma 2 years later (unpublished results).

In order to elucidate the metabolic pathway leading to adduct formation, a series of *in vitro* experiments have been undertaken. Only kidney microsomes were capable of indu-

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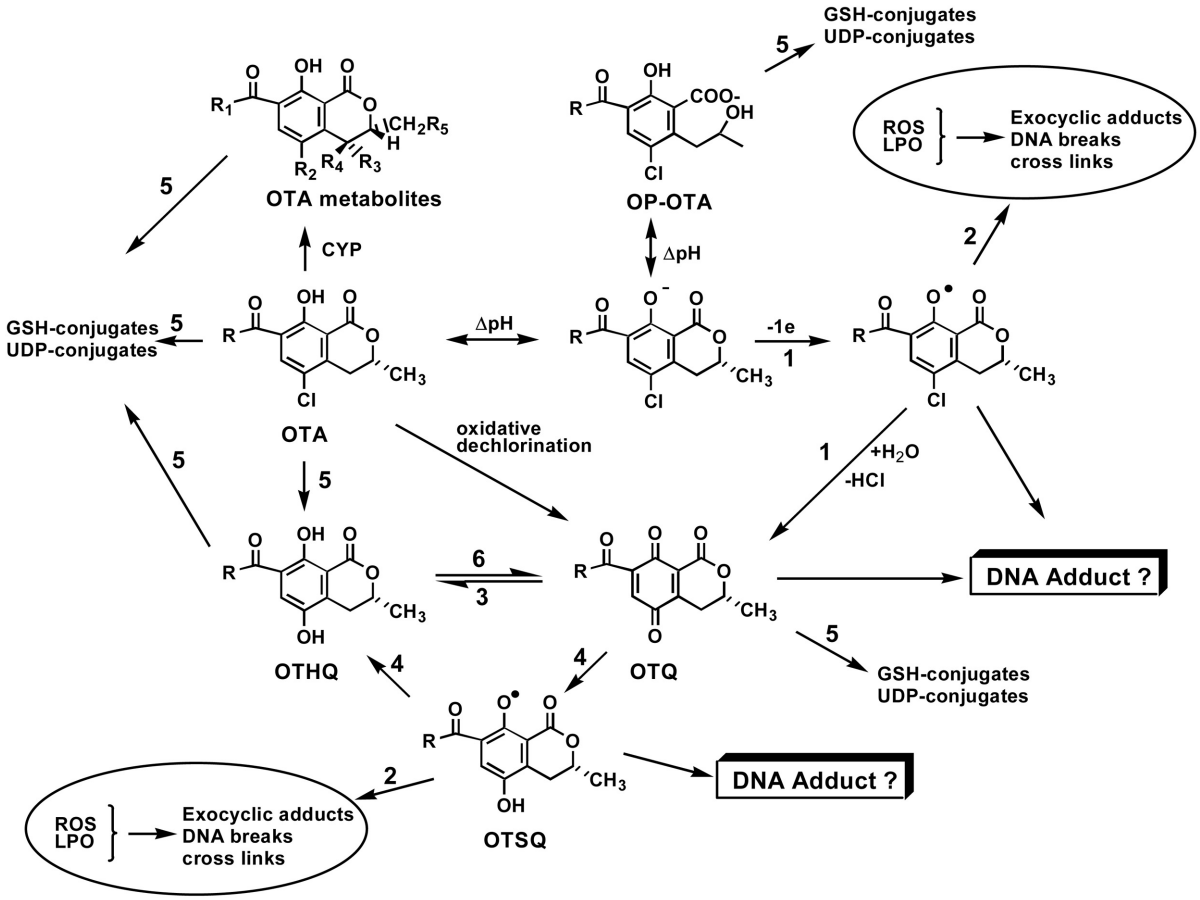

Figure 3. Proposed pathways for the bioactivation of OTA [223].

cing adduct formation [388, 390]. OTA alone does not yield DNA adducts by reacting directly with DNA and no adducts can be formed by *in vitro* incubation using hepatic microsomes [388]. This aspect has been confirmed by other researchers, notably Gross-Steinmeyer *et al.* [309] using ^{3}H-OTA did not find adducts in hepatocytes and Gautier *et al.* [391] found no evidence for OTA-mediated DNA adduction using liver microsomes for bioactivation. Because kidney is rich in peroxidases, the implication of an oxidative metabolic pathway was investigated. Administration of superoxide dismutase and catalase given prior to OTA treatment inhibited DNA adduct formation in mice kidneys [388]. Protection from OTA-induced genotoxicity by indomethacin and aspirin (inhibitors COX and LOX enzymes) in the urinary bladder and kidney of mice was also observed [392]. DNA adduct formation was also partially prevented by antioxidant vitamins, supporting the implication of a peroxidase pathway for the bioactivation of OTA [393]. Induction of LOX by vitamin A increases the formation of the main OTA-DNA adduct in kidney of mice treated by OTA [393]. The implication of the LOX pathways in OTA genotoxicity was also reinforced by the absence of OTA-DNA adduct formation when cells were pretreated with NDGA and a higher dose of indomethacin (10 μM), which inhibits all the AA biotransformation pathways. These findings correlate with a report by Degen *et al.* [371] in which the addition of 10 μM indomethacin to 16 μM OTA inhibited OTA-mediated micronuclei formation in ovine cells.

Implication of leukotriene C4 synthase (LTC4) has also been demonstrated by the use of etacrynic acid which specifically inhibits this enzyme which is a member of the group of nonheminic Fe enzymes and is capable of oxidising GSH to the oxidised form GSSG and simultaneously generating superoxide anion radicals [305, 306]. These pathways could partially explain OTA-mediated oxidative stress observed in rat [305, 331, 350, 394].

Use of microsomes from transgenic mice show that the formation of OTA-mediated DNA adducts are under control of several biotransformation enzymes such CYP450 1B1, 2C9, LOX, and COX [395]. Moreover, induction of NADPH-quinone-reductase regulated by the AH receptor decreases OTA genotoxicity, which reinforces the hypothesis of involvement of a quinone pathway in OTA genotoxicity (see below). In a carcinogenic study, we demonstrated that the susceptibility of male DA rat is due to CYP450 2C11, corresponding to human CYP450 2C9, which is able

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to metabolise DB [223]. Several other experiments implicate a role for CYP450 2C in the bioactivation of OTA. For example, BEAS-2B cells expressing specific human CYP450 isoforms demonstrate that CYP450 1A2, 3A4, 2D6, and 2C9 are responsible of the formation of several OTA-DNA adducts, whereas CYP450 2A6 and 2E1 decrease OTA genotoxicity [305, 318]. A close correlation between type of OTA metabolites formed and DNA adducts could be drawn.

To confirm the hypothesis of a quinone pathway in the metabolism of OTA [314] we have exposed rats to an acute dose of OTA and examined changes in OTA-mediated DNA adduction by the action of (i) NAC (*N*-acetylcysteine) a precursor of intracellular cysteine and GSH, and an ROS scavenger [396, 397]; (ii) BSO, shown to protect rat against *p*-aminophenol toxicity [398] and (iii) acivicin (alpha amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid), an inhibitor of GGT which blocks the cytotoxicity of hydroquinone-*S*-conjugates [399]. After treatment with MESNA or NAC, the same four adduct spots persisted in the cancer susceptible male DA and Lewis rats [223]. MESNA modified the DNA adduct patterns in kidney of both male rat strains leading to a reduction in spot number and total adduct level but did not prevent the formation of all DNA adduct spots. After treatment with acivicin, only one adduct persisted whose formation appeared to involve biotransformation by LOX, as this adduct is the major adduct in OTA-treated cells when the LOX pathway is enhanced [305, 306], and also in kidney of mice pretreated by vitamin A, a known LOX inducer [318]. Interestingly, the metabolic study performed by us in cells indicated that in the presence of acivicin large amounts of OTB and another dechlorinated OTA derivative are formed [347, 348]. Moreover, the cytotoxicity of OTA was not decreased by pretreatment by acivicin. In the same way, BSO also induced formation of these metabolites. Our data suggest that different mechanisms for OTA-induced karyomegalies, renal carcinogenicity and DNA adduction are involved [223]. Schaaf *et al.* [400] demonstrated that in primary rat proximal tubule cells and LLC PK1 cells, OTA induces a concentration-dependent elevation of ROS levels and an increase in the formation of 8-oxoguanine along with depletion of cellular GSH. In this paper, the authors expressed that *N*-acetylcysteine completely prevents OTA-induced increases in ROS levels as well as the formation of 8-oxoguanine and completely protects against the cytotoxicity of OTA. In contrast, our data show that *N*-acetylcysteine does not prevent OTA-induced DNA adduction.

In addition to the above findings, the DNA adducts which persisted in kidney of OTA-treated rats had similar chromatographic properties with those found in renal tumours from Bulgarian patients suffering from BEN/UTT [385] and in French patients with kidney tumours [395, 401]. These adducts were also detected in pigs which had developed OTA-related nephropathy [301, 402] and persisted in kidney of OTA-treated mice and rats [304, 383, 384, 388]. The main OTA-induced DNA adducts are formed *in vitro* by incubation in the presence of pig [301] and human microsomes [227].

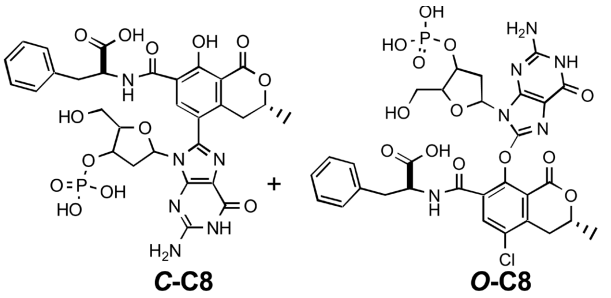

Figure 4. Structures of C8-OTA-3′-GMP adduct standards.

The Manderville laboratory has demonstrated that oxidative activation of OTA generates the phenoxyl radical and the OTHQ/OTQ redox couple, resulting from dechlorination of OTA [314, 403]. The OTQ electrophile can form a conjugate with GSH [404]. Recent biological studies confirm the presence of the hydroquinone (OTHQ) in the urine of rat following administration of OTA by gavage [315]. For other chlorinated compounds, the implication of formation of a quinone species in DNA adduct formation was demonstrated notably using ^{32}P-postlabelling [405, 406]. Utilising the photochemistry of OTA, the Manderville laboratory were able to isolate and characterise the two C8 deoxyguanosine adducts (O-C8-dG-OTA and C-C8-dG-OTA) shown in Fig. 4 [407, 408]. The use of these adduct standards in postlabelling analysis shows that both adducts comigrate with OTA-DNA adducts formed in kidney of rats developing tumours [408]. The C-C8-dG-OTA adduct is also formed in kidney of pig fed OTA [249, 408]. *In vitro* incubation of OTA with polydG-dC, polydA, polydC and polyT has also demonstrated that DNA adducts are formed mainly on guanine, but also on adenine [227, 395]. Preliminary results obtained by MS indicate that OTA induces breaks in the oligomer (8-mer). The base excised corresponds to OTA-modified guanines [348]. These data are in line of those obtained by Obrecht-Pflumio and Dirheimer [409, 410]. In polydG-dC in addition to individual adducts, we also observed crosslink formation [227, 395]. Indication of simultaneous types of modification induced by OTA in DNA was revealed in an *in vivo* study [223]. The main dG adduct is also formed when COX and LOX enzymes are highly expressed [301, 227, 395].

More recently the DNA adduction properties of the hydroquinone metabolite OTHQ have been compared to the parent OTA [411]. Initial experiments were carried out to determine whether OTHQ can react covalently with DNA in the absence of metabolic activation. Like other hydroquinones, OTHQ undergoes autoxidation ($t_{1/2}$ = 11 h at pH 7.4, 37°C) to generate superoxide and the quinone electrophile OTQ [314, 404]. Thus, OTHQ is more reactive than OTA,

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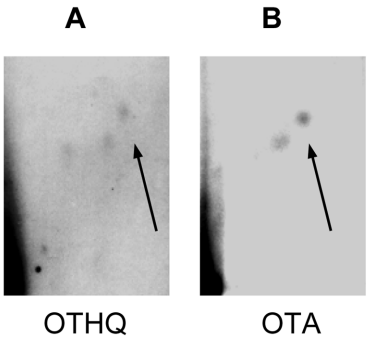

Fig. 5. 32P-postlabeling analysis of salmon sperm DNA following incubation for 24 h at 37°C in 20 mM TRIS-HCl pH 7.4, 0.1 M KCl with (A) OTHQ (1 mM) in the absence of added cofactors and (B) OTA (1 mM) in the presence of pig kidney microsomes and NADPH.

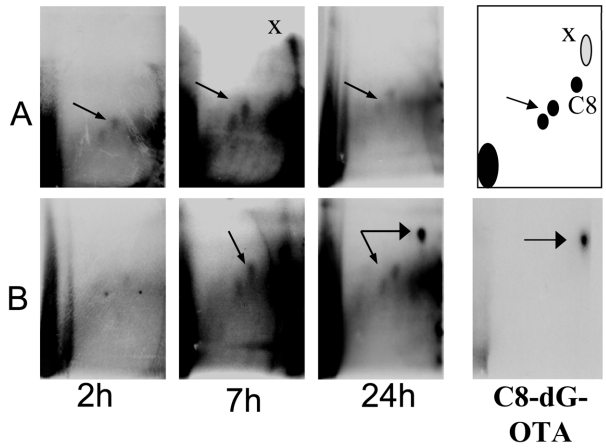

Fig. 6. TLC maps of 32P-labelled DNA adducts obtained following treatment of human kidney cells (HK2) with OTHQ (1 mM) [A] or OTA (1 mM) [B] for 2, 7 and 24 h. Postlabeling of C-C8 dG-OTA standard

which is not oxidised by O_2 alone, and OTHQ may be capable of interacting covalently with DNA through an autoxidative process. Treatment of salmon sperm DNA with OTHQ or OTA (1 μM) yielded three faint adduct spots for OTHQ, while no discernable adduct spots were detected for OTA. To draw comparison between DNA adduction mediated by OTHQ with OTA *in vitro*, pig kidney microsomes in the presence of NADPH was employed to activate OTA. Figure 5 shows the adduct pattern for OTHQ by autoxidation *versus* the adduct pattern generated by OTA following treatment with the pig kidney microsomes. With microsomal activation OTA yields two clear adduct spots (indicated by the arrow) that possess the same migration properties as two of the adduct spots detected from the autoxidation of OTHQ (also indicated by the arrow). This result shows a direct correlation between DNA adduction mediated by the autoxidation of OTHQ with DNA adduction by OTA following microsomal activation. This result also correlates with our earlier findings that OTHQ reacts with GSH directly to form a conjugate that is also produced by OTA and GSH following activation by rat liver microsomes and NADPH [404]. It is also important to point out that the adduct spots detected in Fig. 5 do not comigrate with the C-C8 OTA adduct standard (Fig. 4). This finding is consistent with the known tendency of quinone electrophiles to react with nucleophilic nitrogen atoms on the DNA bases [412]; C8-dG adduct formation has not been reported for a quinone electrophile.

Further insight into the role of the OTQ/OTHQ redox couple in OTA-mediated DNA adduction was derived from study of the dose- and time-dependence of adduct spot formation in human cell lines. Figure 6 shows the adduct pattern obtained following OTHQ (1.0 μM) or OTA (1.0 μM) treatment of human kidney (HK2) cells. For OTHQ, two adduct spots noted in Fig. 5 from the autoxidation of OTHQ were visible following 2 h incubation time (spots indicated by the arrow in Fig. 6). After 7 h these adduct spots grow in intensity and then decline after 24 h, which may be due to adduct repair. In the case of OTA, no discernable adduct spots were detected following 2 h incubation. However, after 7 h the two adduct spots noted in the TLC maps for OTHQ were visible. After 24 h OTA shows a very clear and new adduct spot (indicated by the large-headed arrow) that comigrates with the C-C8 OTA adduct standard (Figs. 4 and 6). The amount of adduct ascribed to C-C8 OTA-dG present in Fig. 6 was ~18 adducts/10^9 nucleotides. This adduct forms at a slower rate than adducts attributed to OTQ and it is not generated by the OTHQ metabolite. The regioselectivity for C8 attachment at dG by OTA suggests the intermediacy of a radical species that may involve reductive dehalogenation of OTA to form a carbon-centred radical, as discussed previously [313].

These experiments provide new ^{32}P-postlabelling evidence for DNA adduction by the hydroquinone metabolite (OTHQ) of the chlorophenolic mycotoxin OTA. OTHQ reacts directly with DNA to form adduct spots that are ascribed to covalent attachment by the quinone electrophile (OTQ) generated by OTHQ autoxidation. OTHQ is the first OTA derivative to react covalently with DNA in the absence of metabolism. The adduct spots generated by OTHQ treatment are also produced by the parent OTA following *in vitro* activation with pig kidney microsomes or in human cell lines. In human kidney cells, clear kinetic differences in adduct formation were visible that highlight the more facile OTQ formation from the OTHQ precursor; OTQ-mediated adducts form slower from OTA. These experiments support the hypothesis that the OTQ electrophile participates in OTA-mediated DNA adduction and that the observed adducts may contribute to OTA carcinogenesis.

DNA adduct deriving from OTHQ are mainly formed after biotransformation of OTA by CYP 2C9, enzyme exhibiting epoxygenase activity, whereas C-C8 dG-OTA adduct

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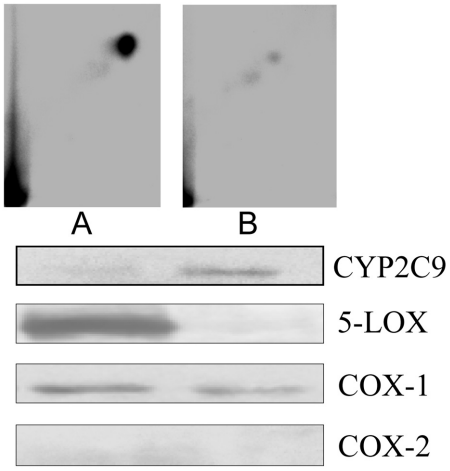

Fig. 7: TLC maps of 32P-labelled DNA adducts obtained following in vitro incubation of salmon testes DNA in presence of human microsomes expressing mainly 5-LOX [A] or mainly CYP2C9 [B].

is formed after biotransformation by 5-LOX which is a microsomal GSH (Fig. 7).

Interestingly, recently the DNA-adducts related to OTHQ, in addition to that related to C-C8dG OTA, have been found in tumoural tissues of Croatian and Serbian suffering EN (Fig. 8). C8-C dG OTA adduct was already observed in tumoural kidney of Bulgarian and French patients and for which OTA was detected in blood and tissue.

Despite evidence for the genotoxicity of OTA [219, 220, 223, 304, 307, 383–385, 388, 389, 409, 410, 413], other researchers have been unable to detect direct DNA damage by OTA [315] and favour an indirect mechanism that involves oxidative stress [350, 396] and OTA-mediated cytotoxicity [391]. A recent perspective by Turesky [414] summarises the argument against direct genotoxicity by OTA.

One reason that an interlaboratory analysis of DNA samples from OTA-treated rats has failed to reach a consensus regarding the nature of adduct spots mediated by OTA is due to differences in methodology. For example, ^{32}P-postlabelling analyses carried out by Mally *et al.* [315] used phosphodiesterase (SPD) in a ratio of 1 mU/μg DNA, which will lead to incomplete DNA hydrolysis. Indeed, the activity of Calbiochem SPD is such that ~10–15 times more should have been added for complete hydrolysis of the OTA-treated DNA. The authors also used Nucleobond columns for DNA clean-up, which leads to DNA of variable quality with contamination by protein and RNA; DNA of high purity is critical for reliable DNA adduction results [415–417]. The chromatographic pH conditions for ^{32}P-postlabelling analyses, with D1 at pH 6.8 and D3 at pH 3.5, also do not conform to standard published procedures, where D1 is run at pH below 6.0 and D3 is run at ~pH 6.4. For generation of OTA-DNA adduct standards (Fig. 4) the photoreaction was carried out with OTA at 500 mM and dG at 20 mM; conditions that are at an exceedingly high concentration and do not match the conditions reported by Dai *et al.* [407] for isolation and characterisation of the adduct standards. Also, it appears that no purification of the photoreaction was carried out and at best the solution would have been a mixture of C-C8 and O-C8 adducts. Overall, the work performed by Mally *et al.* [315] and the evidence presented to show that OTA fails to generate DNA adducts was not convincing.

In the work described by Gautier *et al.* [391] Qiagen tip-2500 columns to isolate DNA and commercial plates from Macherey Nagel (MN) were used to separate adducts using conditions described by us, but the solvent concentrations were not adjusted to the specific plates, probably due to a lack of reference adduct material for proper control. In fact, we have shown that migration of OTA-DNA adducts on MN plates is completely different from that on home-made plates (manuscript in preparation). As specified earlier [415] the quality of the plates is vital for the analysis of OTA-DNA adducts by the sensitive ^{32}P-postlabelling technique.

The argument that adduct spots mediated by OTA may be due to OTA-mediated cytotoxicity [391] is also flawed. The ^{32}P-postlabelling analysis for OTA-mediated DNA adduction shown in Fig. 6 was carried out using 1 μM OTA, conditions in which OTA shows no cytotoxicity in H2K cells. In fact, OTA is weakly cytotoxic and typically shows no cytotoxicity in cells treated with =10 μM OTA even after 48 h incubation time [418]. Concentrations ranging from 25–100 μM OTA are frequently employed to study OTA-mediated cytotoxicity.

6.3 Modulation of gene expression-relation with cell signalling

Development of tumour is a multifactorial process, implicating alteration of DNA after metabolic activation of carci-

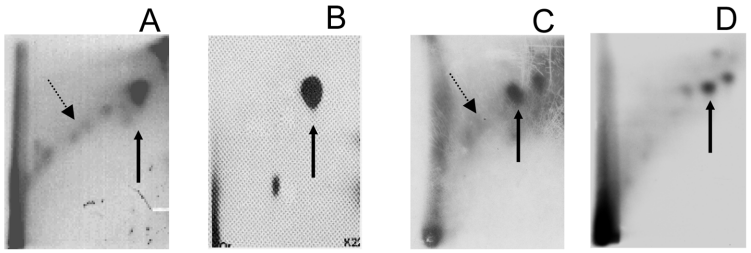

Figure 8: TLC maps of 32P-labelled DNA adducts of human kidney from Croatia [A], Bulgaria [B], Serbia [C], France [D]. Bold arrow shows C-C8dG-OTA; faint arrow shows specific OTHQ adduct

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nogens and subsequent mutations that can alter proto-oncogen function or tumour suppressor [419–421]. In general, the influence of toxics on gene expression is linked to mediator of inflammation such prostaglandins and leucotriens. Metabolites of AA have been implicated in the pathogenesis of several cancers [422]. One primary target for environmental factors includes cellular regulatory proteins which are essential for control of cell growth, DNA repair and programmed cell death. Eicosanoids derived from AA are important lipid messengers playing a substantial role in cell growth and in tumour promotion, progression and metastasis. The modulation of transcription and activation of the enzymes involved in signal transduction is related to the activation of mitogen-activated protein kinases (MAPKs). Two MAPK cascades (Raf/MEK/ERK and MEKK/MKK4/JNK) have been implicated in eicosanoid biosynthesis, and activation of transcription factors such c-fos and c-jun.

OTA modulates not only the expressions of CYP 450 enzymes, but also of COX and lipox. A clear induction of CYP 2C9, LIPOX and COX2 was observed in kidney tumoural tissue of human with high intake of OTA. In OK cells, a time and concentration dependent induction of CYP 1B1 was also observed [140]. These inductions are also observed in liver and kidney of rats [223] and in pig kidney fed OTA [301]. The expression of several of these enzymes is sex dependent, notably CYP 1B1 [423] and CYP 2C which are induced by sex hormone [424–426]. It has been demonstrated that CYP 1B1 is overexpressed in a range of human malignancies, but not in normal tissues [427–430]. In pigs, OTA-modulation of the expression of COX-1, COX-2 and 5-LIPOX is different in genders. COX1 was inhibited in male cortex whereas an overexpression is observed in females. COX-1 plays a crucial role in the preservation of renal function and is constitutively expressed in all tissues, whereas COX-2 is less constitutively apparent but can readily be induced [431]. Its induction has been related to tumour cell growth and progressive renal injury [432–438]. Chronic inflammation is a recognised risk factor for epithelial carcinogenesis. COX-2 is known to be involved in inflammation process by production of prostaglandins and thromboxane. Expression of COX-2 appears to provide a survival advantage to transformed cells. COX2 was found to be differentially expressed in RCC and normal tissue. COX2 expression was significantly enhanced in RCC suggesting that COX-2 plays a role in renal tumourigenesis [439, 440]. A relationship has been established between COX-2 expression and tumour size [438]. Metabolites of AA produced by LIPOX or COX have been implicated in the pathogenesis of several cancers. During AA oxidation, free radicals that can activate xenobiotics into biologically reactive intermediates are produced [441]. In the extrahepatic tissues such as kidney, lung, and brain, several xenobiotics are co-oxidised by either prostaglandin-synthase (PGHS), LOXs [442, 443] or microsomal AA epoxygenases, carried by CYP 2C and 2B [444, 445]. We demonstrated that OTA is substrate for such co-oxidation [306, 392, 395].

The eicosanoids produced regulate induction of several oncogene such as c-fos, c-jun. We demonstrated that OTA-induced phopholipase A2, COX-2 and LIPOX, by ERK 1 & 2 pathway. This subsequently leads to induction of c-jun (JNK) [395, 446, 447]. JNK signalling pathway cause activation of the transcription factor AP-1, a process implicated in oncogenic transformation. It may play a role in both, tumour growth and tumour suppression [448]. In MDCK-C7, induces MAPK [449] and JNK [450]. OTA-induced long-term activation of ERK1/2 in MDCK-C7 cells is associated with epithelial dedifferentiation and transformation [451]. More recently, a dose-dependent activation of ERK1/2 and also of p38 in proximal tubular cells (OK cells) has been observed [452]. OTA-induced phenomena typical for chronic interstitial nephropathy, like loss of cells and epithelial tightness, necrosis and apoptosis as well as markers of inflammation, fibrosis and epithelial-to-mesenchymal transition in proximal tubular cells [452]. Thus, OTA decreases protection of kidney function due to inhibition of COX-1, and increases risk a cancer link to overexpression of COX2 in male. An increase of COX-2 expression counteracts apoptosis and increase proliferation.

6.4 Other mechanisms

OTA induces Ca^{2+} increase into the cells [453, 454]. This could lead to apoptosis, but also could explain cell signalling. Indeed, cytosolic Ca^{2+} is a modulator of numerous cellular events, like metabolism, transport and gene expression. Both Ca^{2+} elevation and MAPK-based phosphorylation are necessary for full activation of $cPLA_2$ [455], which in turn allow liberation of AA. Increase of intracellular pH *via* disruption in membrane anion conductance, inhibition of mitochondrial transport and prevention of heat-shock protein 70 induction has been recently reviewed by Gekle *et al.* [456]. OTA induces oxidative stress, which may then lead either to subsequent damage or to initiation of apoptotic process. Increased apoptosis rates in kidney may lead to polycystic kidney disease, glomerular sclerosis or interstitial fibrosis [457–459]. These damages were found in porcine nephropathy and BEN [457, 458, 460, 461]. In rat kidney apoptosis in the proximal tubule as well as in distal parts occur after OTA administration together with oxidative stress [462]. Evidence for oxidative stress being relevant for the biological effects of OTA is derived from animal studies, describing the attenuation of OTA-dependent toxic effects and reduced formation of DNA lesions in kidney and other organs by coadministration of various antioxidants [393]. Recently, OTA oral dosing to Fischer rat with doses inducing tumours lead to oxidative damage [463]. With the same dose, we observed covalent binding of OTA [53]. Oxidative stress could arise during metabolic transfor-

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mation of OTA, and thus could be a consequence of biotransformation.

7 Coexposure to OTA and other mycotoxins

7.1 Presence of ochratoxin C (OTC)

OTC, the ethylester of OTA, is often found simultaneously to OTA in food and feedstuffs including various crops and animal tissues, and even human blood [464]. OTC has been found in considerable concentration in red and white wine also containing OTA [465]. OTC is a more hydrophobic form of the toxin. OTC is rapidly converted into OTA in the body [291, 367]. The very low distribution half-life of OTC (6 min) compared to that of OTA (160 min) suggests that esterification of OTA facilitated its penetration in cell, which in turn affects maximal tissue concentrations and overall toxicity. OTC is extremely toxic to animals, such as pig, poultry [464] and dairy cattle [467]. In cell culture it has been recently demonstrated that OTC is more toxic than OTA [468, 469]. OTC in THP-1 cell (human monocyte/macrophage line) showed a stronger suppressive effect on most of the functions (inhibition of mitochondrial activity, inhibition of phagocytic capacity of macrophages). In the same way, membrane integrity, cell proliferation and metabolic activity of human kidney cell lines were reduced by OTA and OTC. Surface markers of human kidney cells (CD 46, CD 55, CD 59) are modulated. OTC is more toxic than OTA, between four and ten times depending on the end point. Simultaneous presence of OTA and OTC increases these toxic effects. These effects appeared at low concentrations in the subtoxic range. Living cells can be damaged both by long-lasting oxidative stress and by inhibition of the intracellular formation of radicals, which is responsible for microbicial activity. Oxidative metabolism is known to play an important role in the toxicity and genotoxicity of OTA. Dose and type-dependent effects of OTA and OTC on radical formation of porcine phagocytes at concentration below toxicological significance have been observed. At low concentrations, radical formation of monocytes is stimulated. OTC is more toxic than OTA. A synergy between OTA and OTC is also observed in animals [470].

7.2 presence of CIT

CIT is a polyketide mycotoxin isolated from *Penicillium citrinum* [471]. CIT is a yellow compound and its chemical formula is 4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-(3R)-3H-2-benzopyran-7-carboxylic acid (Fig. 9) that may exist as *para*- and *ortho*-tautomers. Similar to OTA, CIT is nephrotoxic to animals. In dogs, two doses of 10 mg/kg bw of CIT-induced renal lesions in the proximal tubules [472]. Damages were also reported in the distal and proximal tubules of beagle dogs exposed to 10 mg/kg bw CIT [473].

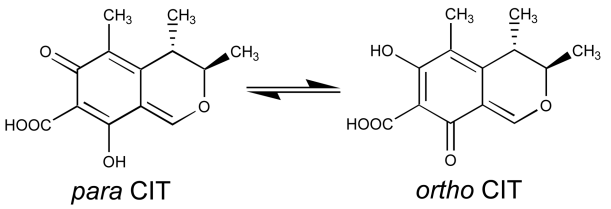

Figure 9. Tautomeric structures of CIT.

In the rat, i.p. doses of 50 mg/kg bw of CIT-induced damage to the epithelial cells of the proximal tubules [474]. Similar results have been reported by Jordan *et al.* [475, 476] and Phillips *et al.* [477]. Damage of the proximal tubules (brush border disruption, cytoplasmic rarefaction, swelling of interdigitating processes, condensed mitochondria, azotemia, metabolic acidosis and hypokaliema, glycosuria, increased blood urea nitrogen and serum creatine but decrease of creatinine clearance) were obtained at CIT doses of 67 and 130 mg/kg bw in rabbits [478–480]. CIT-induced damages of proximal tubules were also obtained in various birds (turkey, ducklings, white leghorns, chicks [48, 482]). Renal toxicity of CIT in swine was characterised by lesions and desquamation of renal epithelial cells of the convoluted tubules, dilatation of tubules, and thickening of glomerular interstitium; the excreted urine volume can also be 2.5-fold higher than normal [483, 484].

The mycotoxin CIT is frequently found as a cocontaminant of OTA. Studies of food contamination conducted in Bulgaria demonstrated that a higher percentage of the staple food was contaminated by CIT in the endemic area than in the nonendemic area [45, 47]. In a recent re-analysis of the data obtained by Petkova-Bocharova *et al.* [45], the contamination of food consumed by affected families was compared with that of food consumed by unaffected families in the endemic and control areas. The results showed a striking difference, demonstrating that, in Bulgaria, affected families were much more frequently exposed to OTA and CIT [46].

It is very likely that humans and animals are always exposed to mixtures of mycotoxins rather than to individual compounds. Therefore, studies of the effects of mycotoxins in combination are very relevant in order to ascertain whether toxins interact with other toxins, which will provide for an improved and more realistic risk assessment. Table 2 summarises the combined effects of CIT and OTA where, in general, a synergistic effect has been demonstrated [125]. For example, *in vivo* studies have shown synergistic lethal effects of these two mycotoxins in mice [218, 485], dogs [473], guinea pigs [486] and neonatal rats [487]. Simultaneous administration of OTA (25 mg/kg) and CIT (200 mg/kg) enhanced the incidence of renal cell tumours in male DDD mice induced by OTA alone; CIT alone was not carcinogenic [216]. Carcinogenicity studies on CIT have given negative results for male Sprague–Daw-

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Table 2. Interaction of OTA with CIT[a)]

Test system/measurement	Effect	References
Hepatoma cells – RNA/DNA, protein synthesis	Synergistic	[495]
Renal cortical cells – organic ion transport	Additive or slightly synergistic	[496]
White leghorn pullets – kidney function	Antagonistic	[497]
Layer chick – renal ultrastructure	Additive	[481]
Mouse to hepato – renal carcinogenesis	Synergistic (renal only)	[216]
Chick embryo – morphology	Additive	[498]
Broiler chicks – growth depression, water consumption increase	Antagonistic	[499]
Foetal rat – malformation	Synergistic	[204]
Rat – renal Na^+-K^+ and Mg ATPase	Synergistic (Na^+-K^+ ATPase only)	[487]
Mouse – lethal effect	Synergistic	[485]
Guinea pig – lethal effect	Synergistic (females), additive (males)	[500]
Dog – nephrosis	Slightly synergistic	[473]
Miniature swine – renal-cortical cubes PAH ions and protein synthesis	Synergistic for protein synthesis; other effects additive	[501]

a) Adapted from [125].

ley rats fed 48 wk a diet containing up to 500 mg CIT [488]. However, benign clear cell adenomas of the kidneys were found in male F344 rats fed a diet containing 1 g CIT/kg for 80 wk [489]. It induces chromosomal abnormalities in the bone marrow cells [490] and induces sister chromatid exchange [491].

More recent studies have shown that OTA and CIT elicit a synergistic effect on mitogen-induced lymphocyte proliferation [492]. In this study, Bernhoft *et al*. [492] studied lymphocyte proliferation by *Penicillium* mycotoxins including CIT, cyclopiazonic acid (CPA), OTA, patulin (PAT), PIA, and roquefortine (RQC) using purified lymphocytes from six piglets. Only one of the 15 pairs of toxins elicited a statistically significant synergistic effect, OTA + CIT, indicating an interaction between the two toxins [492]. Combined cytotoxicity of OTA and other mycotoxins in renal cells [493] also shows interactive (synergistic) effects for OTA + CIT. Using a porcine renal cell line (LLC-PK1) and the MTT reduction test as a cytotoxicity end point, PAT, OTA, OTB and CIT were tested individually and in combination. The toxicity ranking for single toxin experiments was PAT > OTA ≥ OTB > CIT, with CIT yielding EC_{50} values of >400 μM. Potency orders for combinations was CIT + OTA > OTA + OTB > PAT + OTA and CIT + OTB > PAT + OTB > OTA + OTB, with CIT being more interactive than PAT with the ochratoxins, OTA and OTB [493]. Knasmüller *et al*. [494] also examined OTA, OTB and CIT for genotoxicity in human liver (HepG2) cells and have pointed out that the combined effects of these mycotoxins in food may have an impact on the overall cancer hazard to humans.

Recent studies from our laboratory have also determined that simultaneous administration of OTA and CIT enhances cytotoxicity, genotoxicity and the incidence of renal tumours in male mice [140, 502, 503, 504]. In these studies, several samples of wheat were collected from farms in various parts of France throughout the year. OTA and CIT levels in the wheat were analysed by HPLC with fluorimetric detection. More than 50% of the samples contained at least one of the mycotoxins with OTA levels between 0.1 and 68 μg/kg and CIT levels reaching 520 μg/kg. Of the contaminated wheat samples, 33% were cocontaminated with OTA + CIT. Cytotoxic effects of OTA, CIT, or OTA + CIT, were measured using BEAS-2B cells and the combined mixture consistently showed greater cytotoxicity than OTA alone. Genotoxicity was measured using ^{32}P-postlabelling for DNA adduct detection. In both the cases, specific DNA adduct patterns for each mycotoxin were observed. For OTA (1 μM), adduct levels were ~$64/10^9$ nucleotides, while levels for 10 μM CIT were ~$88/10^9$ nucleotides; no adducts for CIT were detected at 1 μM [140]. Interestingly, the combination of OTA (10 μM) + CIT (50 μM) generated mainly OTA-derived adduct spots (~$50/10^9$ nucleotides) with the levels of CIT-specific adducts being $2/10^9$ nucleotides. These studies showed that CIT is genotoxic at doses higher than OTA and that the mixture of the mycotoxins favours OTA-mediated DNA adduction. DNA adduct patterns of rat kidney after 3-wk feeding showed similar adduct spots to those observed in cell culture. Levels of the main OTA-DNA adduct, ascribed to the C-C8 adduct, were increased by the presence of CIT. These studies suggest a possible interaction between CIT and OTA that may involve electron-transfer processes. The quinone methide structure of CIT (Fig. 9) may be capable of oxidising OTA into the phenoxyl radical to promote C-C8 adduct formation. This would represent a nonenzymatic pathway for OTA bioactivation that could play a key role in the synergistic effects observed for OTA + CIT.

8 Conclusions

The chlorophenolic mycotoxin OTA is prevalent in food sources and represents a potential human health hazard.

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OTA is a potent renal carcinogen in rodents and it has been linked to the fatal human kidney disease, BEN. Understanding the mechanism of OTA-mediated carcinogenesis is vital for the risk assessment community to place restrictions on the OTA content in human foods. The literature on OTA-mediated toxicity and genotoxicity is dominated by controversial evidence, making it difficult to place realistic restrictions on OTA in human food samples because we do not yet understand its threat to human health. One group of researchers report negative findings for the genotoxicity of OTA and argue that OTA does not undergo biotransformation to form electrophilic intermediates capable of reacting with biopolymers. This group of researchers argues that OTA-mediated genotoxicity through DNA adduction cannot account for the carcinogenicity observed in rodent models, and that some other unknown mechanism of action must be responsible for the carcinogenicity by OTA observed in rodents. A second group of researchers that include the authors of this publication argue that OTA does undergo biotransformation to induce genotoxicity and that the adduct spots observed by ^{32}P-postlabelling represent attachment of an OTA-derived electrophile to DNA. Here an analogy to the established activity of other chlorophenol xenobiotics has been made and OTA-DNA adduct standards and synthetic metabolites, such as the hydroquinone OTHQ, have been prepared to support the OTA-mediated genotoxicity hypothesis. Because DNA adduction is strongly correlated with carcinogenesis, it is argued that DNA adduction coupled with oxidative DNA damage by OTA may play a key role in OTA-mediated carcinogenesis. These positive results for OTA-mediated genotoxicity provide convincing evidence that OTA should be viewed as a genotoxic carcinogen. Based on this classification we propose that the PTWI of OTA, currently set at 14 ng/kg bw/day based on nephrotoxicity in pigs, should be modified. The Virtually safe dose (VSD) of 1.8 ng/kg bw/day proposed by Kuiper-Goodman and Scott [202] that considers tumour formation by OTA as an endpoint would be a more prudent safety level to set for OTA intake.

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