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MESNA PROTECTS RATS AGAINST NEPHROTOXICITY BUT NOT CARCINOGENICITY INDUCED BY OCHRATOXIN A, IMPLICATING TWO SEPARATE PATHWAYS

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Summary: *Ochratoxin A (OTA) a nephrotoxic mycotoxin, probably implicated in human Balkan Endemic Nephropathy, induces renal carcinomas in rats, the males being more affected than the females. OTA induces DNA adduct formation, but the structure of these adducts and their role in nephrotoxicity, genotoxicity and carcinogenicity has only partly been elucidated. Earlier studies suggested that lipid peroxidation (LPO) is involved in the carcinogenic and genotoxic effects of OTA and that enzymes implicated in arachidonic acid metabolism participate in the biotransformation of OTA. Since 2-mercaptoethane sulfonate (MESNA) protect rats against nephrotoxicity and carcinogenicity induced by oxidative stress by increasing free thiol groups in kidney, the potential protective effect of MESNA on renal toxicity and carcinogenicity induced by OTA was examined of in a long term rat study. MESNA decreased significantly OTA-induced karyomegalies (p=0.018) in the kidney of male Dark Agouty and Lewis rats and also the number of individual DNA adduct, but did not protect against renal adenocarcinomas in male Dark Agouty, even the incidence is increased. Some kidney OTA-DNA adducts persisted. Therefore other agents involved in glutathion peroxidase activity have been tested in an acute study. Pretreatment of male rats by N-acetylcysteine (NAC), (another agent which, like MESNA, reduces oxidative stress by increase of free thiol in kidney), buthionine sulfoximine (BSO) (an inhibitor of glutathionesyntase) and acivicin (an inhibitor of gamma glutamyl transpeptidase (GGT)) modified the repartition of individual spots as detected by 32P-postlabelling. Altogether these results demontrate i) separate mechanisms for the induction of karyomegalies and tumours by OTA in rat kidneys, ii) that OTA induced OTA-bound DNA adducts in addition of putative LPO-derived exocyclic DNA adducts iii) implication of glutathion pathway in the formation of DNA-reactive OTA metabolites and iv) allow to pinpoint that a quinone pathway is probably involved in renal genotoxicity and carcinogenicity by OTA.*

Key words: *Ochratoxin A, carcinogenesis, nephrotoxicity, DNA adducts, 2-mercaptoethane sulfonate (MESNA), oxidative pathway, glutathione*

Introduction

Ochratoxin A (OTA) is a mycotoxin probably implicated in Balkan Endemic Nephropathy (BEN) (1-6). OTA was found to be nephrotoxic to all animal species tested including birds and mammals. It induces kidney carcinomas in rats, the male being more affected than the female (7, 8).

Omar *et al*. (9) had first demonstrated that lipid oxidation (LPO) was involved in the mechanism of action of OTA. The implication of oxidative pathways in the OTA-genotoxicity was further derived from the observation that superoxide dismutase and catalase given to mice before OTA administration inhibited DNA adduct formation in kidneys (10). DNA adducts were formed *in vitro*, after incubation of OTA only in the presence of rat kidney microsomes, containing high amounts of peroxidases, whereas no DNA-adducts were detected after incubation with liver microsomes (10). Protection from the OTA-genotoxicity by indomethacin and aspirin (inhibitors of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes) in the urinary bladder and kidney of mice has been observed (11), DNA adduct formation was also prevented by antioxidant vitamins, all these findings support the implication of a peroxidase pathway in OTA-biotransformation (12). Earlier studies

have shown that the carcinogenic and genotoxic effects of OTA are related to its biotransformation by enzymes implicated in arachidonic acid metabolism, notably COX, LOX-related glutathione-S-transferase (GST), and the CYP 2C11-related epoxygenase (13,14). Recently we have demonstrated that these two latter enzymes are both required for the expression of OTA-genotoxicity (15). Reactive oxygen species (ROS) generated by peroxidase catalysis can initiate free radical reactions which lead to the oxidation of xenobiotics (reviewied in 16) and/or of polyunsaturated fatty acids. LPO of the latter leads to the formation of aldehydes, such as malondialdehyde and *trans*-4-hydroxy-2-nonenal, which react with DNA to form exocyclic adducts (17). Although, there is good evidences that OTA can induce DNA adducts, their structure and role in the nephrotoxicity, genotoxicity and carcinogenicity of OTA remains to be elucidated (8, 10-15, 18, 19). Are they resulting from ROS production or from direct covalent binding of OTA metabolites and how to relate this to nephrotoxicity and carcinogenicity of OTA?

Primary function of glutathione peroxidase is to counteract oxidative attack (20) using glutathione (GSH) as electron donor. Some substances act as antioxidant because they modulate the GSH-recycling. For example, MESNA and N-acetylcysteine (NAC) efficiently decreased, *in vitro*, the formation of exocyclic DNA adducts (21) arising from the reaction of *trans*-4-hydroxy-2-nonenal, a major LPO product, with deoxyguanosine (22) and protected rats from renal oxidative damage induced by ferric nitrilotriacetate (23). Similarly, MESNA can protect against the nephrotoxicity from some antineoplastic agents such as *cis*-platin (24) or ifosfamide (25), by increasing the level of free thiol in renal epithelia (26). Thus, the potential protective effect of MESNA and NAC against OTA induced DNA-adduct formation were explored. The role of other substances such as buthionine sulfoximine (BSO) and Acivicin which interferes also with the recycling of GSH and are efficient to reduce nephrotoxicity of hydroquinone and p-aminophenol, have been tested (27,28).

Materials and methods

Chemicals

Long-term rat study: Ochratoxin A (OTA, benzene free) was purchased from Food Science and Technology Division, Pretoria, South Africa), MESNA from Asta Medica (Frankfort, Germany), sodium bicarbonate, from Merck (Darmstadt, Germany) was of analytical quality grade. N-acetylcystein (NAC), acivicin and buthionine sulfoximine (BSO) were from Sigma (L'Isle d'Abeau, France).

^{32}P-postlabelling assay for DNA-adducts: Proteinase K, RNase A and T1, and micrococcal nuclease were purchased from Sigma (L'Isle d'Abeau, France); T4 polynucleotide kinase and [γ ^{32}P-ATP], 222 Tbq /mmol (3000 Ci/mmol) were from Amersham (Les Ullis, France); spleen phosphodiesterase from Worthington Biochemicals (Freehold, NJ, USA); nuclease P1 from Boehringer (Manheim, Germany); rotiphenol from Rothsichel (Lauterbourg, France); cellulose MN 301 was from Macherey Nagel (Düren, Germany); polyethyleneimine (PEI) was from Corcat (Virginia Chemicals, Portsmouth, VA, USA). The PEI/cellulose TLC plates were prepared in our laboratory.

Treatment of animals for the carcinogenic study

Male Dark Agouty (DA) and Lewis rats were purchased from the Zentral Institut für Versuchstierzucht (Hannover, Germany).

The animals, 12-weeks old, were divided into 4 groups of 20 animals each and treated as follows. Group 1 received, three times a week (Monday, Wednesday, Friday), intragastric intubation of 0.4 mg per kg body weight of OTA as a solution in 0.1 M sodium bicarbonate ($NaHCO_3$),. Group 2 received the same treatment as group 1 but in addition, MESNA was administered at the concentration of 1 mg/ml in drinking water, every day. Group 3 was treated by the MESNA solution in drinking water alone and group 4, as control, received solution of sodium bicarbonate alone, used as the vehicle of OTA, in the same manner as in groups 1 and 2. All animals were kept under standard laboratory conditions (room temperature 21 ± 2 ^{o}C; relative humidity 60 ± 15%; air exchange rate 15 times/hour; 12h-12h light-dark cycle) in the animal house of the Hannover Medical School. A maximum of 2 animals of the same sex were housed in Macrolon type 3 cages (810 cm3) on absorbent softwood (H3/4, Hahn & Co., Kronsberg, Germany). The animals received a standard diet 1324 (Altromin Gmbh & Co KG, Lage, Germany) and tap water or the solution of MESNA in water, *ad libitum*. Each diet charge was examined by the Institut für Tiergeshundheit und Lebensmittelqualität Kiel (ITL) of the Landwirtshaflishen Untersuchung- und Forschungsantalt (LUFA) to ensure that it was OTA free. Rat were sacrificed at the end of the long term study (2 years) by CO_2 and autopsied completely. The Half of them were frozen at -80°C until DNA adduct analysis, the others were preserved in 10% buffered formalin until histological examination.

Treatment of rat by NAC, BSO, Acivicin before acute OTA-treatment

Male Lewis rats (IFFA CREDO, Les Arbresle France) weighing 125 ± 10 g, aged seven weeks, were given OTA (in 0.1 M aqueous $NaHCO_3$ pH 7.4) at a single dose of 2 mg/kg body weight by gastric intubation. Two hours before OTA administration, 3 groups of three animals received by gastric intubation either NAC (500 mg/kg b.w. in water), acivicin (10 mg/kg b.w. in NaCl 9 g/l) or BSO (1g/kg b.w. in NaOH 0.1 M pH 8.5). Control rats (n = 3) received the vehicle only. One group (n = 3) received OTA alone.

All animals were kept under standard laboratory

conditions (room temperature 21 ± 2 °C; relative humidity 60 ± 15%; air exchange rate 15 times/hour; 12h-12h light-dark cycle) and housed individually. All animals received the same diet *ad libitum*. Rats were sacrificed by decapitation 48h after OTA dosing or vehicle administration. Kidney were excised and frozen at −80°C until further processing

DNA adducts analysis

DNA was extracted from kidney and purified as described previously in Pfohl-Leszkowicz *et al*, 1991 (18). The method used for 32P-postlabelling was that previously described by Reddy and Randerath, 1986 (29) with minor modifications. In brief, DNA (7 µg) was digested at 37°C for 4h with micrococcal nuclease (183 mU) and spleen phosphodiesterase (12 mU) in a reaction mixture (total volume 10 µl) containing 20 mM sodium succinate and 10 mM $CaCl_2$, pH 6. Digested DNA was treated with nuclease P1 (6µg) at 37°C for 45 min before 32P-postlabelling. Normal nucleotides, pyrophosphate and excess ATP were removed by chromatography on polyethyleneimine cellulose plates in 2.3 M NaH_2PO_4, pH 5.7 (D1) overnight. Origin areas containing labelled adducted nucleotides were cut out and transferred onto another polyethyleimine-cellulose plate, which was run in 4.77 M lithium formate and 7.65 M urea, pH 3.5 for 4.5 h (D2). Two further migrations (D3 and D4) were performed perpendicularly to D2. The solvent for D3 was 0.6 M NaH_2PO_4 and 5.95 M urea, pH 6.4 for 3h, and the solvent D4 was 1.7 M NaH_2PO_4, pH 6 for 2 h. Autoradiography was carried out at −80°C for 24 or 48 h in the presence of an intensifying screen. Spots were scraped off and their radioactivity counted by the Cerenkov procedure.

Statistical analysis

Comparisons of the incidence of tumours were carried out with the Fisher exact test. The statistics for the analysis of DNA adducts were performed using the Wilcoxon Rank Sum Test.

Results

Effect of MESNA on long-term genotoxicity and carcinogenicity of OTA treated rats:

The results of histological analysis performed in the urinary tract are presented table 1. After OTA treatment alone, 19/20 male DA rats and 16/20 male Lewis rats had karyomegalies in kidneys. Malignant basophilic adenocarcinoma in kidney were found in 6/20 male DA rats and in 2/20 male Lewis. For the urinary bladder, 2/19 male DA presented malignant transitional cell carcinoma.

In the groups of animal receiving both OTA and MESNA, the number of karyomegalies decreased significantly ($p = 0.018$) to respectively 12/20 and 11/20 for male DA and male Lewis. The number of renal tumours in male DA rats increased significantly in group of animal receiving OTA and MESNA as compared to those receiving OTA alone (8/20 animals are affected *versus* 6/20) but no more bladder tumour were detected. In the male Lewis rat groups treated by OTA and MESNA, only one animal developed a renal tumour. Representative patterns of DNA-adducts in kidneys from rats treated by OTA for 2 years are shown in Figure 1. In general, MESNA decreased both the number of individual DNA adducts and the total adduct levels resulting in lower inter-individual variability. In male DA rats, the strain most susceptible to cancer, 4 to 9 individual adducts instead of 3 to 19 were detectable in kidney.

The total adduct levels (expressed as adducts/109 nucleotides) observed in kidney from male DA varied from 2.6 to 114 (in OTA-treated rats) to 10 to 20 (in OTA + MESNA treated rats). The changes in number and levels of adducts were due to the disappearance of several spots (# 6, 10-12) and the reduction in intensity of all remaining spots (except spots # 4 and 5). In male Lewis rats after MESNA treatment, adducts spots # 2, 10, 15-17 disappeared and adduct spot # 4 became less intense. Adducts # 5 and 7 were not modified but two adducts, notably the adduct # 8, appeared. As compared to male DA rats, the total level and the number of different adducts were lower.

Table 1

Lesions observed	Male Dark Agouty				Male Lewis			
	OTA	OTA+ MESNA	MESNA	NONE	OTA	OTA+ MESNA	MESNA	NONE
Basophilic cell adenocarcinoma (malignant)	30	40	0	0	10	5	0	0
Small tubular epithelial adenoma (benign)	5	0	0	0	0	0	0	0
Multifocal tubular epithelial karyomegaly	95	60	0	0	80	55	0	0
Multifocal simple tubular epithelial hyperplasia	80	80	7	0	95	70	73	70
Chronic multifocal interstitial pyelonephritis	35	40	27	30	75	35	47	40
Multifocal nephrocalcinosis	70	45	73	60	50	35	53	30
Multifocal Yellow brown pigment deposits	90	93	67	40	20	10	0	0
Proteinaceous intracellular casts	15	10	0	0	75	25	20	90
Cortical Cyst(s)	0	0	0	0	50	30	27	20
Chronic progressive nephrosis	0	0	0	0	0	30	13	0
Focal hemorrhage	0	0	0	0	0	5	0	0
Bladder carcinomas	10	0	0	0	0	0	0	0

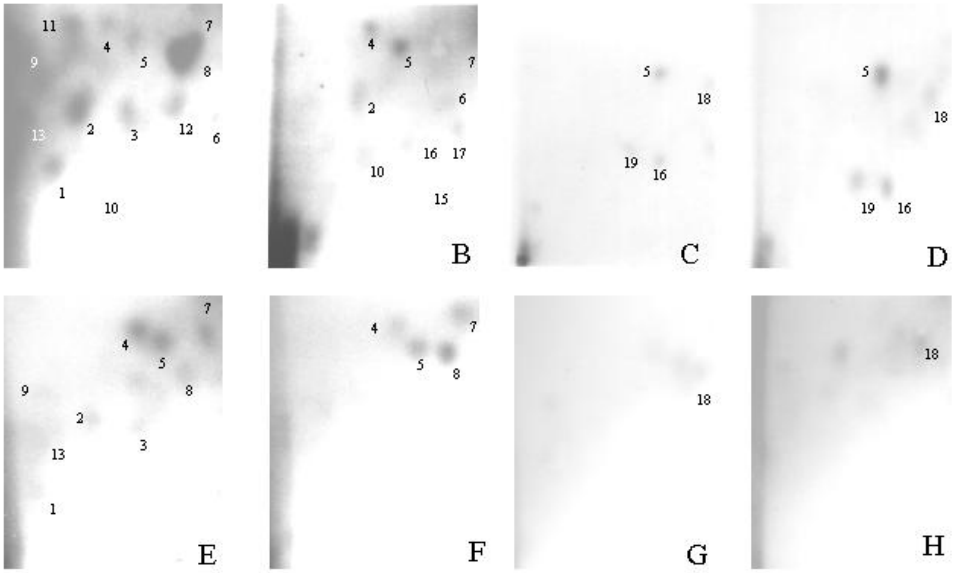

Fig. 1. Autoradiograms of DNA adduct spots in the kidney of rat treated for 2 years by ochratoxin A , [A, B] OTA alone, [E, F] in presence of MESNA treatment. [A, E] correspond to male DA rats, [B, F] to male Lewis rat. Adduct fingerprints were obtained after 48h of exposure.

Effect of NAC, BSO and acivicin on OTA-induced DNA-adducts formation in rat kidney acutely treated by OTA

Figure 2 shows representative example of DNA adduct patterns obtained in kidneys from male Lewis rats (n = 3) that were pretreated, two hours before OTA-treatment, by several modulators of the glutathione pathway. Twelve distinct adduct spots were observed 48h after OTA administration, reaching a total DNA adduct level of 70 ± 5 adduct/10^9 nucleotides. Pre-treatment by NAC (a compound which like MESNA, reduces oxidative stress by increase of free thiol in kidney) decreased the total adduct level by a half, some adducts (# 6, 12) completely disappeared while others were less intense and adduct # 8 newly appeared. Pre-treatment by BSO (inhibitor of gamma-glutamylcysteinesyntase, depleting cellular GSH) two hours before OTA treatment, reduced considerably the number of individual adduct spots. Four of them persisted (adducts # 1, 7, 8, 12) yielding a total level of 17 ± 3 adduct/10^9 nucleotides. When rats are pre-treated by acivicin, only one adduct persisted (adduct # 7) reducing the total adduct level to 8 ± 1 adduct/10^9 nucleotides.

Discussion

The first aim of this study was to determine whether co-administration of MESNA, an antioxidant, involved in the recycling of free thiol in kidney (30) affords a protective effect against renal genotoxicity and carcinogenicity induced by OTA.

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 tumor formation was observed in male DA rats. In contrast, MESNA prevented bladder tumor formation. MESNA modified also 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 the DNA adduct spots. This suggests different mechanisms for OTA-induced karyomegalies, renal carcinogenicity and DNA adduction. Changes in nucleus, notably karyomegalies, occurring during necrosis have been attributed to LPO (31). DNA adducts which are suppressed by MESNA could be secondary LPO-derived exocyclic adducts, whereas the persistent ones may be adducted OTA metabolites.

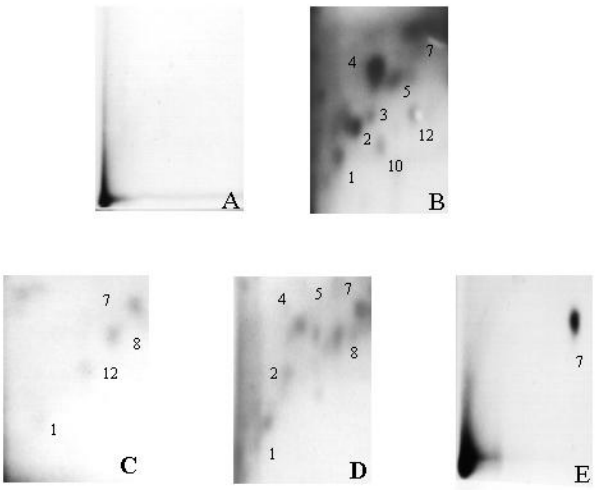

Fig. 2. Autoradiogram of DNA adduct spots in kidney from Wistar rats treated by a single OTA-dose and pre-treated by glutathione modulators: [A] control rats (without any treatment), [B] OTA alone [C] pre-treated by buthionine-sulfoximine [D] pre-treated by N-acetyl-cysteine [E] pre-treated by acivicin. Adduct fingerprints were obtained 48h after exposure.

In case of halogenated alkenes, hydroquinone, bromobenzene and p-aminophenol, renal toxicity and carcinogenicity is caused in the proximal tubular cells, by some glutathione S-conjugates formed in the liver, transported to the kidneys where they accumulated in a GGT-dependant manner (32). After transport across the plasma membrane, GSH-conjugates are subject to an initial cleavage by GGT and subsequently to cleavage by a dipeptidase to yield the S-cysteinyl derivative of the compound. GGT which is essential for recycling GSH promotes the toxicity of some xenobiotics by facilitating their re-absorption (33). Inhibition of GGT activity, which is abundant in the proximal convoluted tubules of kidney (33,34), was shown to block in renal proximal tubular cells the cytotoxicity of hydroquinone-S-conjugates arising from bromobenzene, para-aminophenol and menadione (27,35). Lau *et al*., 1990 (36) have found that the renal proximal tubular necrosis induced by paraminophenol or bromobenzene is a consequence of oxidation of a quinol conjugate to the quinone followed by covalent binding to tissue macromolecules. The presence of a quinone pathway in the metabolism of OTA, which is a chlorinated compound, was recently shown (37). To confirm this hypothesis, we have therefore tested in an acute OTA treatment, the effect of (i) NAC a precursor of intracellular cysteine and glutathione, an a ROS scavenger (21,23), (ii) BSO, shown to protect rat against p-aminophenol (28) and (iii) acivicin, an inhibitor of GGT which blocks the cytotoxicity of hydroquinone-S-conjugates (27). NAC decreased only partially DNA adduct formation. After treatment with MESNA or NAC, the same adduct spots (# 4,5,7,8) persisted in the cancer susceptible male DA and Lewis rats. After BSO treatment, which deplete glutathione in kidney, only four adducts persisted (# 1,7,8,12). Disappearance of adducts # 4 and 5 indicated that these adducts were formed in the kidney via a mechanism involving glutathione. The adduct # 7 was

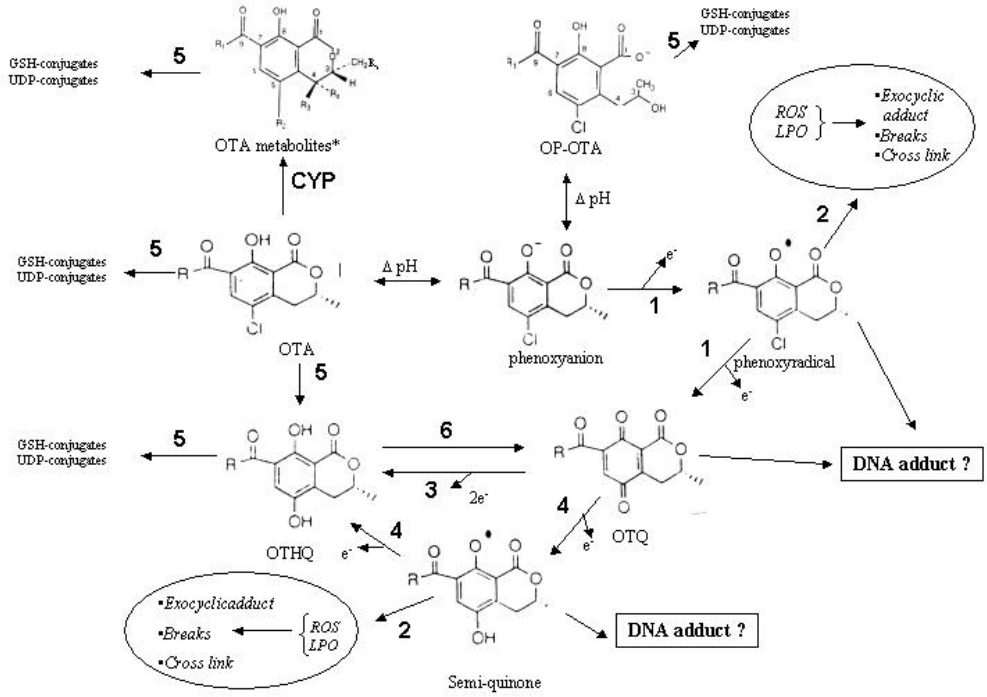

Fig. 3. Scheme of some possible biotransformation reactions of OTA and putative structure of some reactive intermediates
Structure of metabolites include: OTA, ochratoxin A; OTB, dechlorinated OTA; OTC ethylester OTA; 4R, S,-OH-OTA, hydroxylated OH in position 4, OP-OTA open-lactone; OTHQ, hydroquinone OTA; OTQ, quinone OTA.
Abbreviations: ROS, reactive oxygen species; LPO, lipoperoxidation; GSH, glutathion; UDP, uridin diphosphate.

compounds	R1	R2	R3	R4	R5
OTA	phenylalanine	Cl	H	H	H
OTB	phenylalanine	H	H	H	H
OTC	Esterified phenylalanine	Cl	H	H	H
4R-OH-OTA	phenylalanine	Cl	H	OH	H
4S-OH-OTA	phenylalanine	Cl	OH	H	H
4R-OH-OTB	phenylalanine	H	H	OH	H
10-OH-OTB	phenylalanine	Cl	H	H	OH
OTα	OH	Cl	H	H	H
OTβ	OH	H	H	H	H

the only one that persisted after acivicin pre-treatment implying that it is not generated by ROS, nor by GSH conjugation. Its formation seems to involve biotransformation by lipoxygenase as this adduct appeared as the major adduct in OTA-treated cells when this LOX-pathway was enhanced (14,15), and in kidney of mice pretreated by Vitamin A, a known inducer of LOX (12). Adduct # 8 which appeared in kidney of male Lewis rats treated by MESNA and in Wistar rats pre-treated by NAC seems to be formed by a ROS-independent pathway as this adduct was found in cells expressing specifically human CYP 2C9 (14). Interestingly, the DNA adducts which persisted (# 1,4,5,7) or appeared (# 8) in kidney of OTA-treated rats had similar chromatographic properties than those found in renal tumours from Bulgarians patients suffering from BEN (38). These adducts were also detected in pigs which had developed OTA-related nephropathy (39) and were those that persisted in kidney of OTA-treated mice and rats (18,40). Most importantly, in the present study, these DNA adducts (# 1,4,5,7) were commonly observed in both male rats strains with OTA induced renal tumours a finding that would explain the higher susceptibility of male rats**.** These data support the notion that only some specific OTA related adducts are relevant to renal carcinogenesis, while the total DNA adduct level may be related to an overall (geno)toxic potency of OTA.

The biotransformation of OTA, a chlorinated compound, is complex and involves several biotransforming enzymes such as cytochrome P450s (33), but also glutathione transferases and lipoxygenase (13-15). The metabolites conjugated to GSH and/or UDP are excreted in bile and in kidney (34). At least 20 different metabolites of OTA including OTB, OTC, OH-OTA, OP-OTA, Otα, Otβ and metabolites of unknown structure were detected (34, 35). Based on our results from this study we summarise known and hypothetical pathways for OTA metabolism in Figure 3.

Conversion of OTA into quinone (OTQ) by redox cycling generates ROS that can lead to DNA breaks and LPO-derived exocyclic adducts (pathway 2). The quinone can undergo either a two-electron reduction by action of the NAD(P)H:quinone reductase to form hydroquinone (pathway 3), or a one electron-reduction to yield a semiquinone (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^{*}) (38-40) 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 as MESNA and NAC and explains OTA-induced karyomegalies which have been observed and its reduction which we have observed after MESNA treatment. OTHQ could be formed directly by CYP and/or GST (pathway 5) and be oxidized into OTQ (pathway 6).

One of the important enzymes in the genotoxicity pathway of OTA is leukotriene C4 synthase (LTC4). This enzyme is a member of the group of non heme Fe containing enzymes, capable of oxidising glutathione (GSH) to the oxidised form GSSG and simultaneously generating superoxide anion radicals, which may contribute to oxidative stress in cells but also participate to glutathione conjugation of xenobiotics (47) (pathway 5). In general glutathione-S-transferases are involved in detoxifying pathways, but in some cases they contributes to the reactivity and toxicity of xenobiotics, notably by formation of the thiyl radical which reacts with macromolecules and is favoured by the formation of peroxyl radicals (reviewed in 48) (pathway 2).

In conclusion, our present study demonstrated (i) that two different mechanisms are implicated in the nephrotoxicity and carcinogenicity induced by OTA, since MESNA only prevented karyomegalies in rat kidney but not renal tumors, (ii) OTA induced OTA-bound DNA adducts in addition of putative LPO-derived exocyclic DNA adducts (iii) allow to pinpoint critical DNA-adducts, biotransformation enzymes and a quinone pathway that are probably involved in renal genotoxicity and carcinogenicity by OTA.

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