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Evidence of a new dechlorinated ochratoxin A derivative formed in opossum kidney cell cultures after pretreatment by modulators of glutathione pathways: Correlation with DNA-adduct formation

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Ochratoxin A (OTA), a nephrotoxic mycotoxin probably implicated in human Balkan endemic nephropathy and associated urothelial tumors, induces renal carcinomas in rodents and nephrotoxicity in pigs. OTA induces DNA-adduct formation, but the structure of the adducts and their role in nephrotoxicity and carcinogenicity have only partly been elucidated. *In vivo*, 2-mercaptoethane sulfonate (MESNA) protects rats against OTA-induced nephrotoxicity but not against carcinogenicity, indicating two different mechanisms leading to nephrotoxicity or carcinogenicity. To better understand how DNA-adduct could be generated, opossum kidney cells (OK) have been treated by OTA alone or in presence of several compounds such as MESNA or *N*-acetylcysteine (another agent that, like MESNA, reduces oxidative stress by increasing of free thiols in kidney), buthionine sulfoximine (BSO) (an inhibitor of glutathione-synthase), and alpha amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid (ACIVICIN) (an inhibitor of gamma glutamyl transpeptidase). Cytotoxicity of OTA on OK cells was evaluated by applying the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. None of the listed agents diminished OTA cytotoxicity significantly; ACIVICIN even increases OTA cytotoxicity. In contrast, analysis of the HPLC profiles of OTA metabolites produced during these incubations indicated that the pattern, the quantity of metabolites, and the nature of the derivatives were modulated by these agents. Ochratoxin B (OTB), open-ring ochratoxin A (OP-OA), 4 hydroxylated OTA, 10 hydroxylated OTA, OTA without phenylalanine, OTB without phenylalanine, and a dechlorinated OTA metabolite could be identified by nano-ESI-IT-MS.

Keywords: Biotransformation / DNA-adduct / Mass spectrometry / Ochratoxin A / Opossum kidney cells

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1 Introduction

Ochratoxin A (OTA) is a potent mycotoxin produced by *Penicillium* and *Aspergillus* species [1, 2]. It is a common contaminant of foodstuffs [3, 4]. OTA is toxic to numerous animal species, the kidney being the main target organ (for review see [5]). OTA has been associated with Balkan endemic nephropathy (BEN), a chronic kidney disease characterized by renal failure and a high incidence of urinary tract tumors. Occurrence of BEN is restricted to areas of the Balkans where high levels of OTA have been found in food in the past. OTA causes kidney and liver tumors in mice and rats [6 – 10]. The International Agency for Research on

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Abbreviations: ACIVICIN, alpha amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid; **BEN,** Balkan endemic nephropathy; **BSO,** buthionine sulfoximine; **COX,** cycloxygenase; **GGT,** gamma glutamyl transpeptidase; **GSH,** glutathione; **HQ,** hydroquinone; **LOX,** lipoxygenase; **MESNA,** 2-mercaptoethane sulfonate; **NAC,** *N*-acetylcystein; **OK,** opossum kidney cells; **OTB,** ochratoxin B; **OTA,** ochratoxin A; **OP-OA,** open-ring ochratoxin A; **4-OH-OA,** 4 hydroxylated OTA; **OTHQ,** quinone OTA; **ROS,** reactive oxygen species

Cancer (IARC) classified OTA as a possible carcinogen (group 2B), based on sufficient evidence for carcinogenicity in animal studies and inadequate evidence in humans [8]. The IARC working group considered that the epidemiological data from endemic areas were not convincing as BEN and UTT patients had not been separated. Furthermore, the two diseases affect very often the same patients. The Joint FAO/WHO Committee on Food Additives and Contaminants (JECFA) has set the provisional tolerable weekly intake (PTWI) for OTA at 100 ng/kg body weight [3]. This assessment used nephrotoxic effects in pigs as the most toxicological endpoint. The acceptable intake levels of OTA are controversially discussed because of the evidence suggesting genotoxicity of OTA and the fact that OTA is a rodent carcinogen. The mechanisms by which OTA is carcinogenic are not entirely elucidated and two hypotheses are still discussed: (i) carcinogenicity of OTA is due to indirect mechanism that would result in a classification as epigenetic carcinogen [11, 12] or (ii) due to direct covalent binding of OTA on DNA, suggesting genotoxic mechanisms to be involved in the carcinogenicity [13 – 15].

DNA-xenobiotic binding is considered to be a critical step in the initiation of mutagenesis and carcinogenesis [16]. The process of chemical carcinogenesis is initiated by the covalent binding of carcinogens or their reactive metabolites to DNA, thus forming DNA adducts [16]. In all animal studies a good correlation between DNA-adducts formation, frequency of mutations (for a review see [17]), and the incidence of tumors has been found [18]. Most chemical carcinogens require metabolic activation to interact with cellular macromolecules including DNA [16]. The OTA molecule comprises a *para*-chlorophenolic moiety and contains a dihydroisocoumarin group that is linked to L-phenylalanine *via* an amine bond. OTA thus exhibits chemical similarity with chlorophenol [19]. Dai *et al.* [20] demonstrated that after biotransformation *via* peroxidase, pentachlorophenol (PCP) reacts with guanine [20], and more recently authors described similar results with OTA [21].

As 2-mercaptoethane sulfonate (MESNA) protects 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 in a long-term rat study [22]. In this study, MESNA suppressed the kidney karyomegalies but an increase in the incidence of kidney tumors was observed [22]. These findings suggest different mechanisms for OTA-induced karyomegalies and renal carcinogenicity. With the aim to confirm the hypothesis of a quinone pathway in the metabolism of OTA [20, 21, 23], we subsequently tested [22] the effect of *N*-acetylcysteine (NAC), a precursor of intracellular cysteine and glutathione (GSH), and a reactive oxygen species (ROS) scavenger [24, 25], BSO (buthionine sulfoximine oxide), an inhibitor of GSH synthase known to protect rats against *p*-aminophenol toxicity [26], and alpha amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid (ACIVICIN), an inhibitor of gamma glutamyl transpeptidase (GGT) that blocks the cytotoxicity of hydroquinone (HQ)-S-conjugates [27]. Analysis of DNA-adduct formation indicated that following these treatments, the pattern of DNA-adduct changes as some disappeared whereas others corresponding to DNA-adducts no. 1, 2, 3, found in kidneys of animals having developed a kidney tumor [22], remained. These adducts are similar to those formed in human kidney or urinary bladder tumors in patient suffering from nephropathy in endemic areas of BEN [28] and European subjects suffering from kidney or urinary bladder tumors and having OTA in blood and tissues (unpublished result). When rats were treated with ACIVICIN prior to OTA administration, only one adduct persisted, which has the same chromatographic properties as that of adduct no. 3 [22]. This adduct has the same chromatographic properties as the synthetic C-C8 dG OTA [29]. In consideration of these findings, it was the aim of the present study to determine in opossum kidney cells (OK), the pattern of OTA metabolites and to modulate biotransformation by pretreatment with MESNA, NAC, BSO, and ACIVICIN, respectively.

2 Materials and methods

2.1 Chemicals

OTA (benzene free), DTT, spermidine, bicine, NAC, ACIVICIN, BSO, DMSO, Proteinase K, RNaseA, RNase T1, and micrococcal nuclease were from Sigma (Saint-Quentin Fallavier, France); spleen phosphodiesterase was from Calbiochem (VWR, France); nuclease P1 and T4 polynucleotide kinase were from Roche diagnostics (Meylan, France). [γ^{32}P-ATP] (444 Tbq/mmol, 6000 Ci/mmol, Amersham, France); Eagle's minimum essential medium (EMEM) was prepared with Gibco products (Cergy Pontoise, France); phosphate saline buffer, trypsin, fetal calf serum, streptomycine, and penicillin were from Life-Technologies (Cergy pontoise, France); rotiphenol (phenol saturated with Tris-HCl, pH 8) was from Rothsichel (Lauterbourg, France); cellulose MN 301 was from Macherey Nagel (Düren, Germany); polyethyleneimine (PEI) was from Corcat (Virginia Chemicals, Portsmouth, VA); Whatman no. 1 paper was from VWR; and PEI/cellulose TLC plates used for ^{32}P-post-labeling analyses were prepared in the laboratory of Toulouse. All reagents (potassium chloride, sodium hydrogen carbonate, sulfuric acid, phosphoric acid, hydrochloric acid, acetic acid, sodium dihydrogen phosphate) were of normapur grade. All solvents (methanol, chloroform, ACN, propan2-ol, *n*-hexane) were of HPLC grade from ICS (Lapeyrouse-Fossat, France). Deionized water from a Milli-Q sys-

tem (Millipore, France) was used for HPLC analysis and preparation of all aqueous solutions. 4-S-OH-OA, 4-R-OH-OA, and 10 hydroxylated OTA (10–OH-OA) were prepared by Dr Størmer [30]. Quinone OTA (OTHQ) was gifted by Professor Richard Manderville, Guelph University, Canada.

2.2 DNA-adduct analysis

DNA-adducts were analyzed by postlabeling method after purification of DNA as described in details in [15].

2.3 Cell culture

Opposum kidney cells (OK) provided by ATCC (American Type Culture Collection, Manassas, Virginia, USA), were cultured in 200 mL flasks in EMEM containing 44 mM $NaHCO_3$, 5% fetal bovine serum (FBS), 2% vitamins, 2% of nonessential amino acids, 1% streptomycin, penicillin for 48 h at 37°C under 5% CO_2. After trypsin digestion, the cells were resuspended in the medium to obtain 1×10^6 cells *per* mL. Cells were preincubated or not for 1 h with several modulators including ACIVICIN (1 μM), BSO (100 μM), MESNA (100 μM), and NAC (100 μM) in absence of FBS. Thereafter the cells were incubated for 30 min to 7 h in the presence of 0.05–100 μM OTA. For DNA-adduct detection, OK cells were treated for 24 h in the presence of 10 μM OTA and the modulators as described above. All experiments were performed three times and determination was performed in triplicate.

2.4 Cytotoxicity evaluation

Cellular viability was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, using the Cell titer 96® nonradioactive cell proliferation assay (Promega, Charbonnières, France). The intensity of color formed was directly proportional to the number of viable cells and was detected using a 96-plate reader at 490 nm.

2.5 Analysis of OTA metabolites

2.5.1 OTA and metabolites extraction from cell supernatant

Cell culture medium was extracted as described by Petkova-Bocharova *et al.* [31] with minor modifications as described below. In brief, the cell culture medium (30 mL) was mixed with 20 mL $MgCl_2$ 0.1 M/HCl 0.05 M, pH 1.5, and extracted three times with chloroform (25 mL). Combined chloroform extracts, obtained under centrifugation (10 min, 5000 rpm, 4°C) were dried under vacuum, dissolved in 1 mL methanol, filtered, dried under nitrogen, and finally dissolved in 500 μL methanol.

2.5.2 HPLC analyses

In order to obtain a separation to base line with a solvent compatible with use of MS and in a short time (less than 60 min), we developed new HPLC conditions. The metabolites were separated on prontosil 250 mm × 4 mm, 3 μ using the following gradient: solvent A: MeOH/ACN/6.5 mM ammonium formate (200/200/600) adjusted to pH 3 with formic acid; solvent B: MeOH/ACN/6.5 mM ammonium formate (350/350/300) adjusted to pH 3 with formic acid. Program: T0 100% A; T10 100% A; T25 30% A; T30 30% A; T45 0% A; T55 0% A; T58.

2.5.3 Identification of metabolites

The nature of metabolite was first analyzed by comigration with synthetic standard. Each peak was collected, and analyzed by nano-ESI-IT-MS on a spectrometer LCQ (Finnigan MAT, San Jose, USA). Nano-ESI-IT-MS analysis was performed with a commercial nanospray ESI source (The Protein Analysis, Odense, Denmark) using glass capillaries (The Protein Analysis), which were positioned using a stereomicroscope, directly at a distance of about 1 mm from the entrance hole of the heated transfer capillary. The capillaries were palladium and gold coated for electrical contact. The glass capillaries were filled with 5 μL of analyte solution. Nebulizer gas was not necessary in this spray mode. The nanospray needle voltage was set to 700 V. The heated transfer capillary was kept at a temperature of 150°C. Open-ring ochratoxin A (OP-OA) was analyzed in the negative ion mode. The full-scan mass range was *m/z* 100–800, and the product mass range was *m/z* 115–300 for MS^2 experiments and 90–350 for MS^3 experiments. Parent ion isolation width was 1 *m/z* for the synthetic and the natural compounds (collision energy: 55% (MS^2) and 60% (MS^3)). Ochratoxin B (OTB) was analyzed in the negative ion mode. The full-scan mass range was *m/z* 100–700, and the product mass range was *m/z* 100–400 for MS^2 experiments and 85–330 for MS^3 experiments. Parent ion isolation width was 1 *m/z* for the synthetic and the natural compound (collision energy: 65% (MS^2) and 60% (MS^3)). Derivative 7 (dechlorinated OTA) was analyzed in the negative ion mode. The full-scan mass range was *m/z* 105–1000, and the product mass range was *m/z* 105–400 for MS^2 experiments and 75–300 for MS^3 experiments. Parent ion isolation width was 1 *m/z* for the synthetic and the natural compound (collision energy: 45% (MS^2) and 50% (MS^3)). For each compound, activation Qz was 0.25 and activation time was 30 ms. All experiments were performed using the "Automatic Gain Control" (AGC) whereby the accumulation time is automatically adjusted by monitoring

the ion abundance (maximum 800 ms). The software used was Xcalibur 1.2 from ThermoQuest (San Jose, USA).

3 Results

3.1 DNA-adduct formation in opossum cells treated by OTA in the presence or absence of GSH modulators

Figure 1 shows the DNA-adduct pattern of OK cells treated with OTA in the presence or absence of the tested modulators. To be able to compare these findings with previous studies, the numbering was the same as in our previous paper [15]. When OK cells were treated with OTA alone, three adducts (no. 1 – 3) could be detected. When cells were treated with MESNA prior to OTA exposure, these three adducts did occur together with three other adducts (no. 4 – 6). On the contrary, when OK cells were treated with NAC prior OTA exposure, adduct no. 1 and 3 decreased dramatically, whereas adduct 4 increased. When OK cells were simultaneously treated with OTA and BSO only adduct no. 1 persisted but was decreased, compared to treatment with OTA alone. Simultaneous treatment of cells by OTA and ACIVICIN inhibits almost formation of all adducts, except adduct no. 4.

3.2 Analysis of OTA metabolites formed in OK cells

The metabolites formed were first analyzed by HPLC. At least 20 different fluorescent-responsive compounds appeared using the described method optimized for HPLC MS/MS of OTA metabolites (Fig. 2A). Some of these compounds coeluted with pure standard, notably OTA without phenyalanine (OT alpha), 4S-OH-OA, 4R-OH-OA, OTB, OTHQ, OTA methyl ester (OA-Met). In contrast no peak corresponds to 10-OH-OA or OTB without phenyalanine (OT beta). The amount of metabolites formed correlated with the concentration of OTA during treatment of cells (Fig. 2B).

Each peak has been collected individually and the structures of some of them were identified by nano-ESI-IT-MS. As demonstrated by Fig. 3, synthetic OTB and synthetic OP-OA have spectra similar to those of the compounds from OK cells with OTA, which eluted at the same retention time. The pattern of metabolites formed depended on the pretreatment of OK cells prior to OTA treatment. We compared the evolution of ten metabolites (Fig. 4), five of known structure (OTB, 4-OHOA, OPOA, OAMet, and OTHQ) and five of unknown structure. Pretreatment of OK cells by MESNA, NAC, BSO, ACIVICIN increases the global biotransformation of OTA, respectively, by a factor of 3, 1.5, 5, and 2. Formation of OP-OA and 4-R-OH-OA is increased by a factor of 16 and 10, respectively, after BSO pretreatment. BSO pretreatment increased also the formation of OTB (five-fold), OAMet (four-fold), and the dechlorinated derivative 7 (nine-fold). The formation of these derivatives was also stimulated after ACIVICIN pretreatment, but to different extent than by BSO. The respective increases after ACIVICIN pretreatment are four-fold for OP-OA; eight times for 4 hydroxylated OTA (4-OH-OA); two times for OTB; two-fold for OAMet, and ten-fold for dechlorinated derivative 7. MESNA pretreatment did

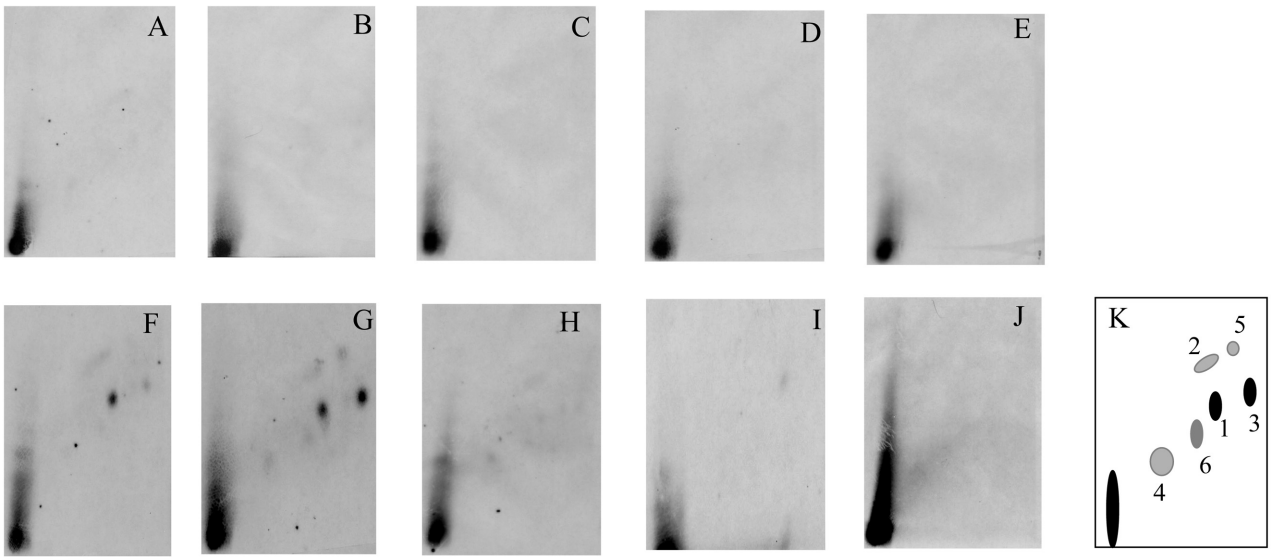


Figure 1. DNA-adduct in opposum kidney cells treated for 24 h (A): untreated controls (B) in presence of MESNA (C) NAC (D) BSO (E) ACIVICIN. In the second series, OTA (10 μM) was tested alone, (F) or in combination (G) OTA + MESNA (H) OTA + NAC (I) OTA + BSO (J) OTA + ACIVICIN; (K) numbering of DNA in OK cells.

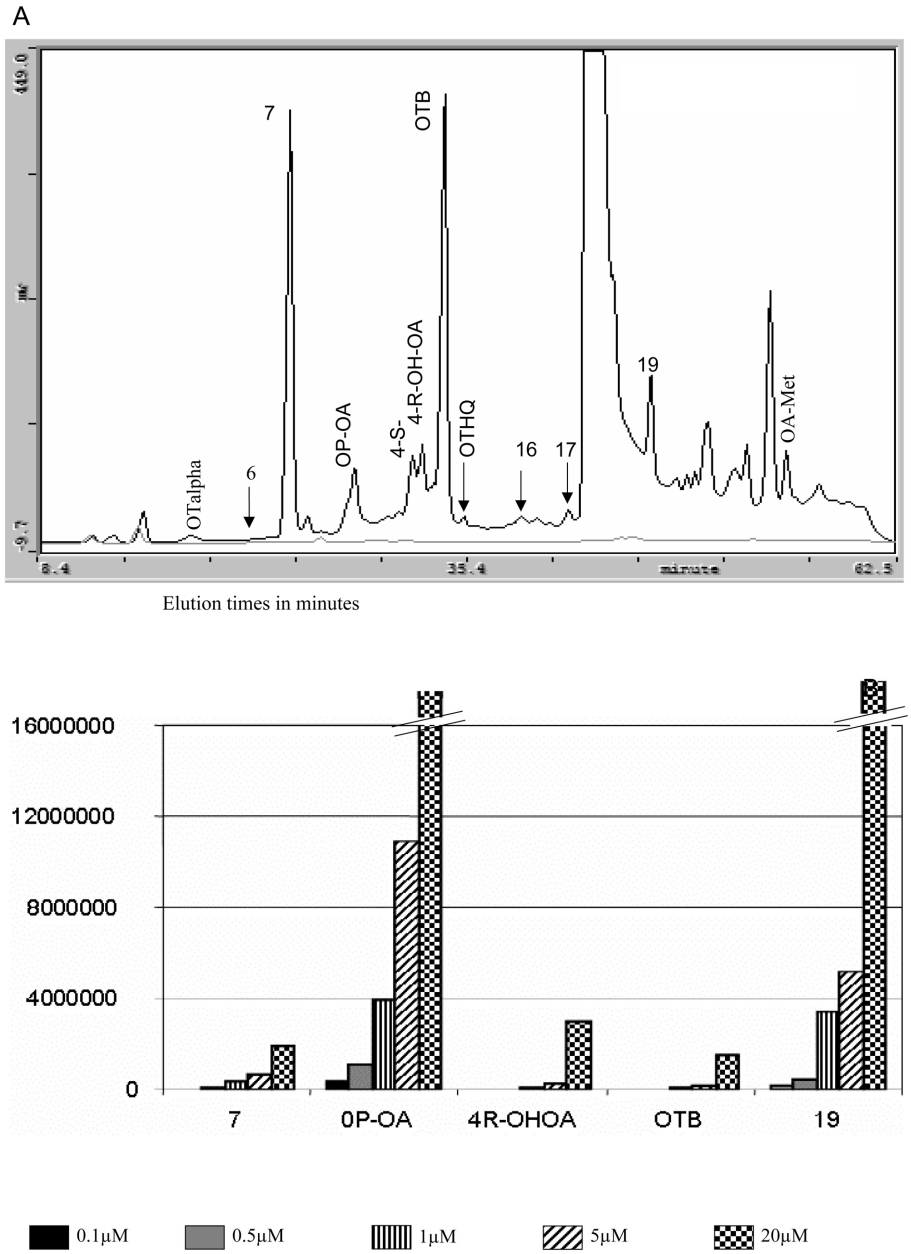


Figure 2. (A) Example of HPLC separation of OTA derivatives detected by fluorimetry that were formed in OK. For details of HPLC method see Section 2. (B) Dose-related formation of OTA metabolites in OK after 8 h of OTA treatment. Derivative 7 (dechlorinated OTA); OTB, ochratoxin B; OP-OA, open ring OTA; 4R-OH-OA, hydroxylated OTA, derivative 19.

not induce the formation of OP-OA, OTB, or 4-OH-OA, whereas it induces slightly the formation of the dechlorinated derivative 7 (two-fold) (Fig. 4A). In addition, MESNA pretreatment induced also the formation of two OTA derivatives (6 and 16) of unknown structure, three and five-fold, respectively (Fig. 4B). On the contrary, pretreatment by ACIVICIN or BSO decreases partially or totally the formation of these latter metabolites, and also the formation of derivative 19 that is more lipohilic than OTA (Fig. 4B). MESNA or NAC did not modify the amount of derivative19. Interestingly the formation of OTHQ is decreased by all treatment by a factor of 1.5, 3, 2, and 10 after treatment with MESNA, NAC, BSO, and ACIVICIN, respectively. BSO and ACIVICIN have opposite effects on the formation of derivative 17, which is increased by BSO (four-fold) whereas its formation is decreased by ACIVICIN (four times). NAC and MESNA both increase the formation of derivative 17 by a factor of 2.

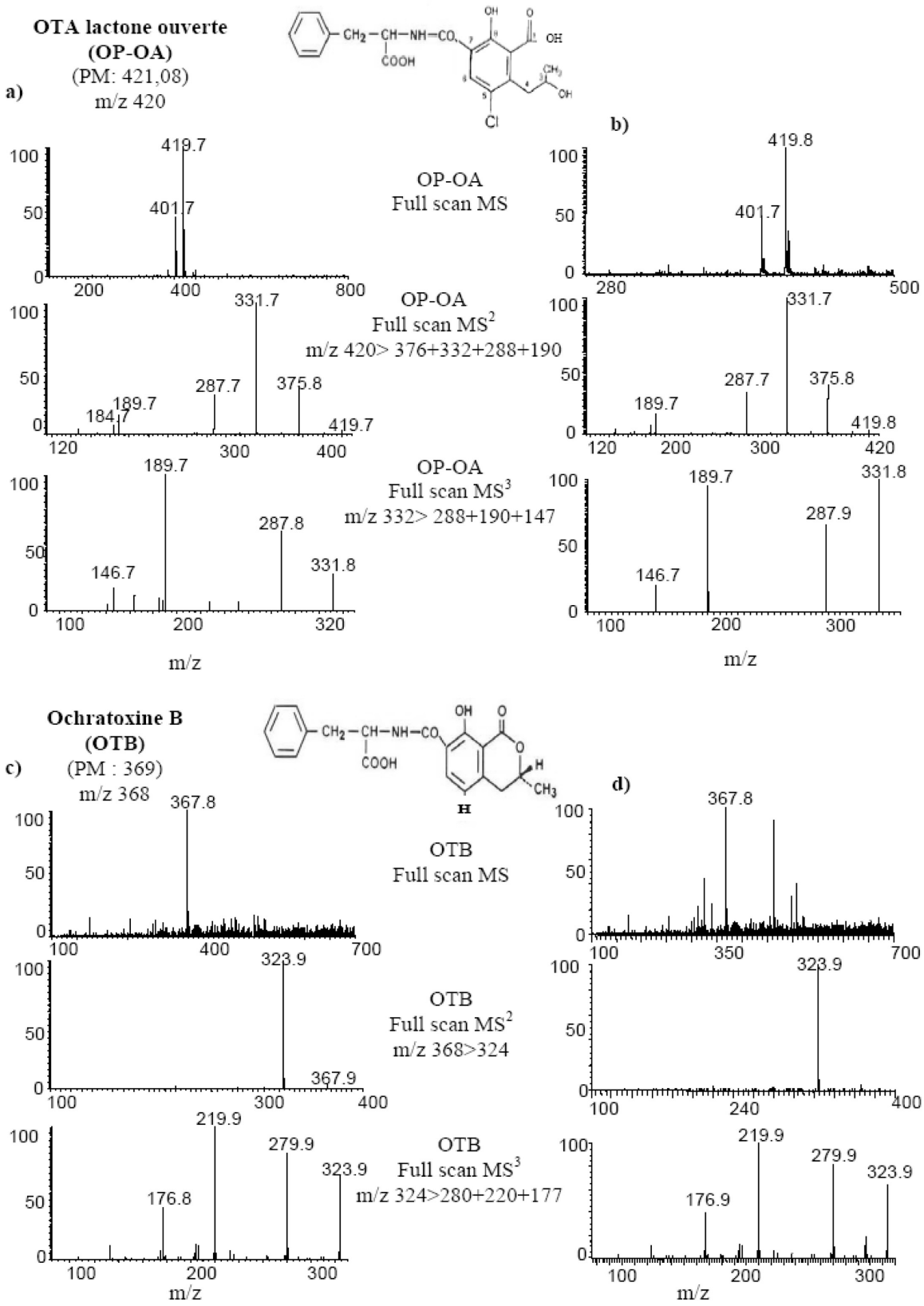


Figure 3. Comparison of mass spectrum of synthetic OP-OA (a) and OTB (c) with isolated corresponding metabolites obtained from OK by nano-ESI-IT-MS (b, d).

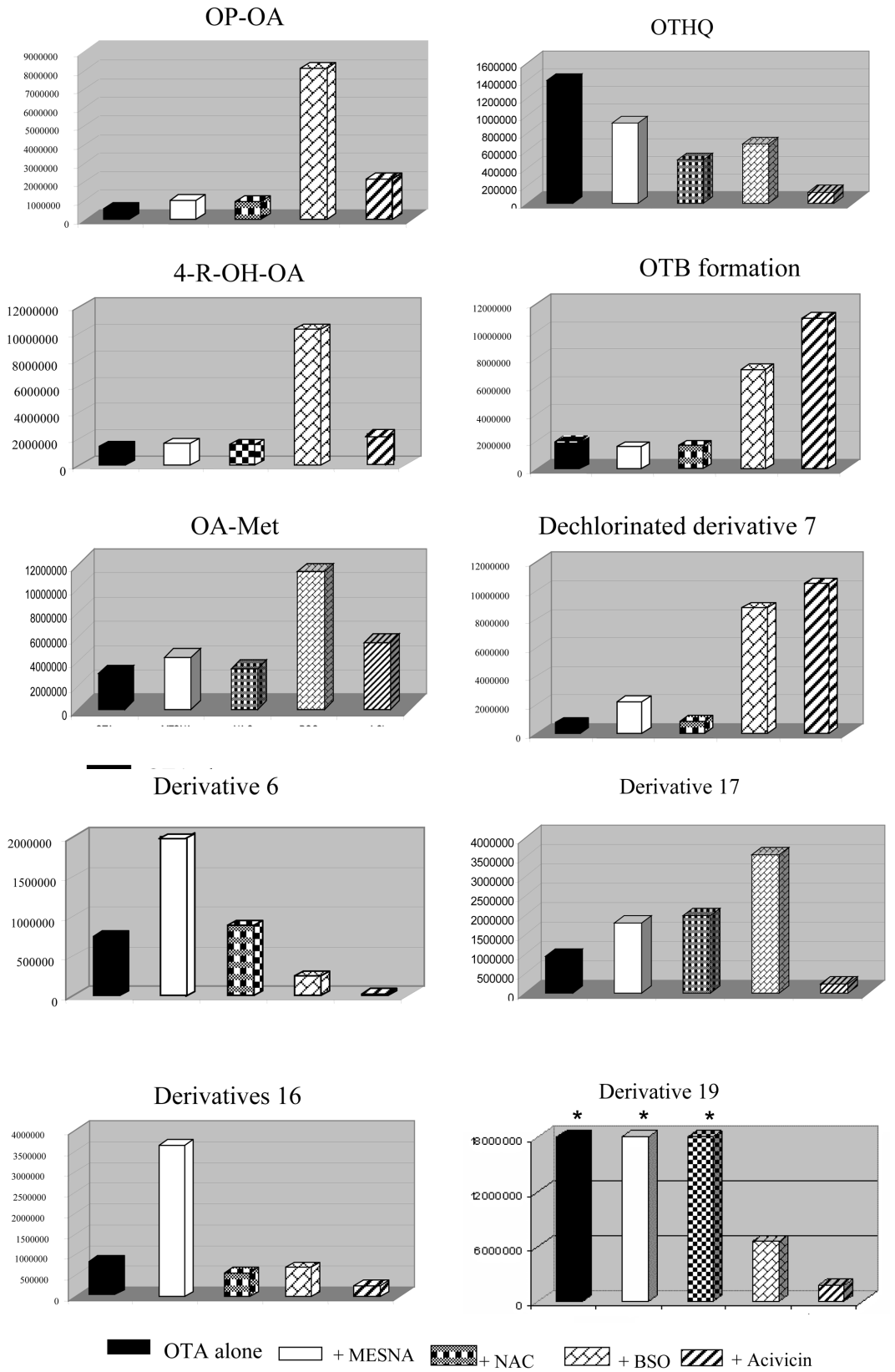


Figure 4. Differences in OTA biotransformation in presence of OK cells pretreated by several modulators of GSH metabolism. Ordinate represent arbitrary units.

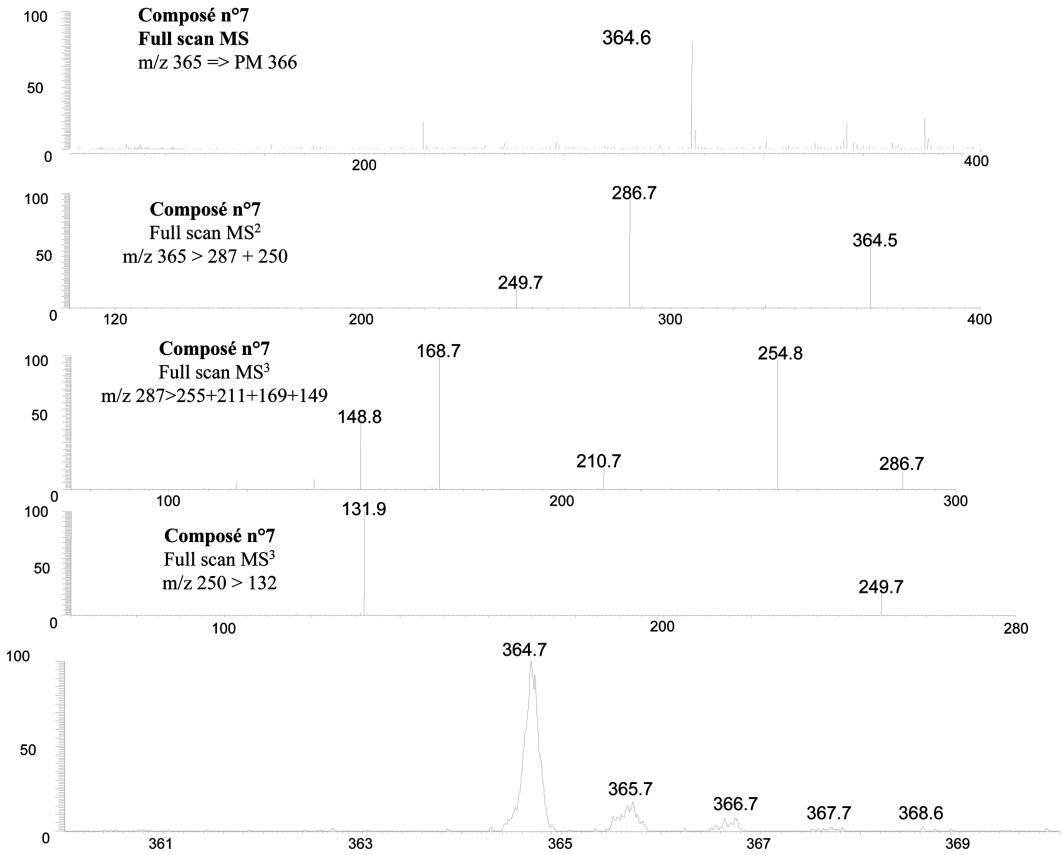


Figure 5. Mass spectrum of a dechlorinated OTA metabolite (compound 7).

3.3 Identification of derivative 7

Analysis of compound 7 by nano-ESI-IT-MS revealed that this compound is a dechlorinated OTA derivative of 366 molecular mass (mw) (Fig. 5).

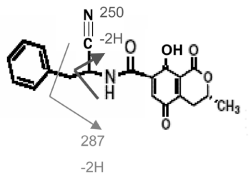
The parent ion was isolated before CID, leading to a specific fragmentation of this ion. The pseudomolecular ion peak $[M-H]^-$ of this metabolite in negative mode has a value of *m/z* 365. The parity of the pseudomolecular ion indicate that this compound contains zero or an even number of nitrogen atoms. The fragmentation of this compound in negative mode exhibit two major peaks: (a) *m/z* 287 corresponding to the elimination of the aromatic cycle (*m/z* 77), and (b) *m/z* 250 corresponding to cleavage of the benzene group with CH_2 (*m/z* 91) followed by the cleavage of the cyanide group (*m/z* 26). The parity of this last ion indicates that this compound contains at least two nitrogen atoms. Moreover, the CO_2 (*m/z* 44) loss observed in OTA fragmentation, was not detected in this metabolite. This indicates that the –COOH residue may have been lost during the metabolization process and has been replaced by CN. The derivative 7 does not contain any chlorine. In the spectrum of the OTA-derivative 7 the abundance of the M + 2 ion (*m/z* 368) is around 3% eliminating the possibility of chloride presence in the molecule.

Hence, we propose the following structure for the OTA metabolite, which needs to be verified: derivative 7 has two nitrogen atoms. The carboxylic group (COOH) and the chlorine atom were removed to concord with the lack of CO_2 loss in the fragmentation and the isotopic pattern of the pseudomolecular ion. The molecular formula of compound 7 is $C_{20}H_{18}N_2O_5$. (Fig. 6).

3.4 Viability of opossum cells treated by OTA in presence or absence of GSH modulators

OK cells were treated with increasing amount of OTA (0.05 – 100 μM). The survival rate was measured after 24 (Fig. 7A) and 48 h of exposure to OTA in presence or absence of the modulators. The cell survival decreases as a function of OTA doses. After 24 h of treatment with 0.5 μM of OTA, the OK cells survival was about 80%, and remains above 40% with 20 μM of OTA. The same experiment was

nanoESI-IT-MS



PM 366

$C_{20}H_{18}N_2O_5$

Fragmentation negative mode

MS^2 365> 287 / 250

MS^3 365> 287> 255 / 169 / 149

MS^3 365> 250> 218 - 132

Fragmentation positive mode

MS^2 367> 335 / 160

MS^3 367> 335> 317 / 220 / 217

MS^4 367> 335> 317> 217 / 156

Figure 6. Putative structure of the compound 7.

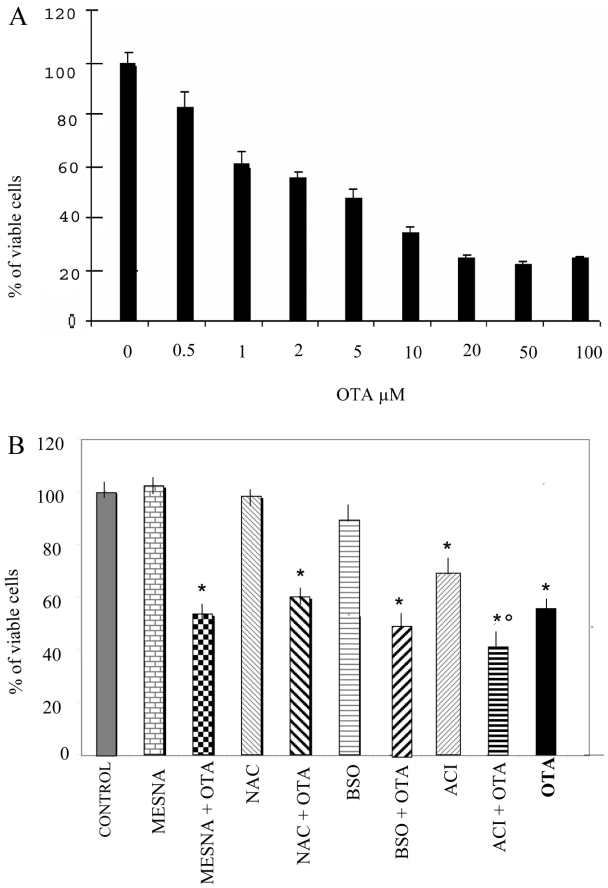


Figure 7. Cytotoxicity of OTA in OK (A), and after pretreatment of the cells with either MESNA; NAC; BSO, or ACIVICIN (B) * statistically different from the control; ° statistically different from OTA treated samples.

conducted after pretreatment of cells by MESNA (100 µM); BSO (100 µM); NAC (100 µM); and ACIVICIN (1 µM). As shown in Fig. 7B, exposure of cell to MESNA, BSO, and NAC alone did not induce any significant cytotoxicity. In contrast, ACIVICIN reduced cell survival. None of these compounds decreased OTA cytotoxicity. When OK cells were pretreated with ACIVICIN and thereafter exposed to OTA, the survival rate is lower than following treatment with OTA alone, indicating that ACIVICIN increases OTA cytotoxicity.

3.5 Cytotoxicity induced by two metabolites

As large amounts of OTB and OP-OA are formed after BSO and ACIVICIN treatment, the cytotoxicity of these two metabolites on OK cells were tested as well (Fig. 8). At low concentration, both metabolites are less cytotoxic than OTA. A decrease of OK cells viability by a factor of two is observed after treatment of cells with 2 µM of OTA and with 7 µM of OTB. We observed less than 45% of survival when the cells were treated with 10 µM of OTB, whereas the survival after OTA treatment at the same dose was 35%. OTB is more cytotoxic than OP-OA, and OP-OA is less toxic than OTA or OTB. A slight decrease of survival after OP-OA treatment is observed with 10 µM (90%), and drop to 40% with 100 µM. Coexposure to OTB and OP-OA has an additional effect on cytotoxicity (data not shown).

4 Discussion

In order to better understand how DNA-adduct could be generated and if biotransformation *via* GSH pathway is involved, OK have been treated by OTA alone or in presence of MESNA or NAC, two agents that reduce oxidative stress by increasing free thiols in the kidney [24, 25]. Moreover BSO, an inhibitor of GSH-synthase known to protect ras against *p*-aminophenol toxicity [26] and ACIVICIN, an inhibitor of GGT, which blocks the cytotoxicity of HQ-S-conjugates, were tested [27].

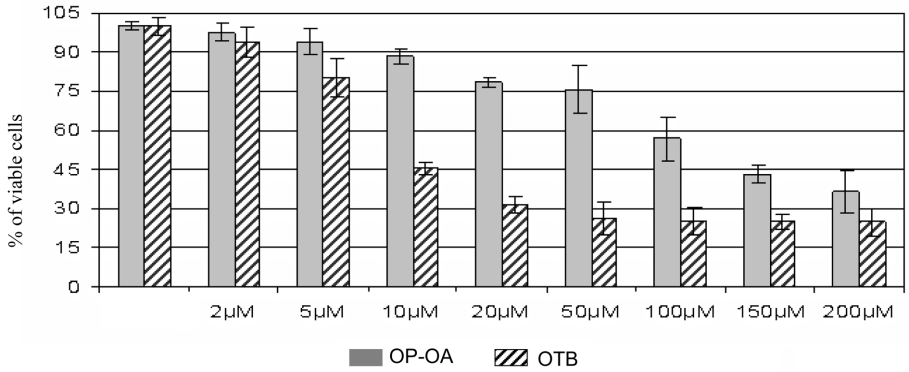


Figure 8. Cytotoxicity of increasing concentrations of OP-OA and OTB on OK.

The OK cell is a continuous cell line derived from the proximal tubule of American opossum kidneys [32]. When grown to confluence, these cells express many characteristics of proximal tubular cells, including albumin transport, and hormonal receptor [33] and thus they have been widely used as a model system to study renal tubular protein transport [34–37].

In OK cells, OTA induces the formation of three DNA-adducts (no. 1–3). Simultaneous treatment of cells with OTA and MESNA increases the intensity of adducts (no. 1–3), and three new adducts appeared (no. 4–6). Even though the mechanism of MESNA and NAC was presumed to be similar, *i.e.*, antioxidant action due to increase of intracellular thiol [25, 30], significant difference was observed. Indeed, when cells were simultaneously treated with OTA and NAC, adducts no. 1 and 3 were detected but to a lower extent than when cells were treated with OTA alone or in presence of both OTA and MESNA. NAC induced also the formation of adduct no. 4. Induction and increase of OTA-DNA-adduct formation following treatment with MESNA or NAC was already observed in the *in vivo* study with rats [22, 38]. The only difference in DNA-adduct pattern formed by OK cells and *in vivo* in rats is the presence of an additional adduct called no. a in Fig. 9. As already shown *in vivo*, depletion of GSH recycling by BSO or ACIVICIN considerably reduced DNA-adduct formation. In both cases, only one adduct is detected, but with different characteristics in BSO- and ACIVICIN- treated cells. The persisting adduct in BSO-treated cells is adduct (no. 1), whereas ACIVICIN treatment induces the formation of adduct no. 4. This adduct is not similar to the sole adduct persisting in kidney of rat treated by ACIVICIN and OTA [22]. Indeed, *in vivo*, the persisting adduct was adduct no. 3 (Fig. 9), which has the same chromatographic properties than the *C*-C8 dG OTA [29]. In OK cells the formation of the adduct no. 3, is inhibited by BSO and ACIVICIN whereas it is enhanced by MESNA and NAC treatment. Taken together, these findings suggest that the mechanism

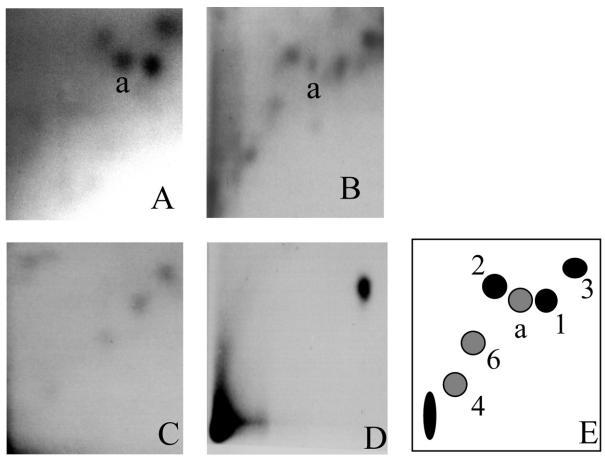


Figure 9. DNA-adduct pattern of kidney from Wistar rat treated by OTA in presence of several modulators (adapted from [22]): (A) with MESNA (B) with NAC (C) with BSO (D) with ACIVICIN (E) numbering using same number as for OK cells; no. a additional spot compared to pattern obtained in OK cells.

by which OTA induces genotoxicity is associated with its biotransformation. Several studies previously established a linked between lipoperoxidation and OTA metabolism [39–42] leading to HQ derivative able to react with guanine [21]. HQ are further metabolized by four pathways [43]: glucuronide conjugation, sulfoconjugation, oxidative conversion of HQ into benzoquinone (BQ), or cycloxygenase (COX)-mediated oxidation [44] followed by conjugation with GSH, which leads to metabolic activation, producing intermediates which are nephrotoxic [45]. Subsequent metabolism of the GSH conjugates *via* GGT and dipeptidase conjugate of HQ is an important determinant of the internal dose of these metabolites [46]. This last mechanism could be blocked by ACIVICIN. Sulfoconjugation and glucuronide conjugation of HQ interrupts the redox cycling of HQ to BQ. The main difference between the rat kidney and OK cells is that OK cells do not express sulfotransferase.

With the aim to confirm that OTA is biotransformed differently when OK cells were treated simultaneously by OTA and above-mentioned modulators of cellular GSH, the formation of several OTA derivatives was analyzed.

At least, 20 different compounds are formed by OK cells in presence of OTA, including OT alpha, OP-OA, 4-OH-OA, and OTB found previously by others in rat urine [47] and other systems (for a review see [14]). Their formation is dose-dependent. As described above, their formation was modulated by inhibitors of the GSH pathway and by antioxidants. In each case, pretreatment of OK cells increases the global biotransformation of OTA (1.5–5-folds) but the increase was not similar for each metabolite. NAC induces only a slight increase of OTA biotransformation and does not significantly modify the profile of OTA metabolite formed; this explain why DNA-adduct pattern was not modified. MESNA induces the formation of two unknown derivatives (no. 6, 16), which in contrast are inhibited by BSO and ACIVICIN, suggesting that these metabolites are GSH conjugates. BSO and ACIVICIN increase the formation of 4-OH-OA and OP-OA. These latter metabolites often are conjugated with GSH ([48], for a review see [14]). Thus their increase correlates with GSH depletion. The formation of derivative 17 of unknown structure is increased by MESNA, NAC, and BSO treatment whereas it is decreased by ACIVICIN treatment. Interestingly the formation of OTHQ is significantly decreased when OK cells were treated by OTA and ACIVICIN. The formation of OTB and of another dechlorinated OTA derivatives (mw 366, derivative 7) increased in the presence of ACIVICIN and BSO. Two mechanisms of biotransformation could be responsible for dehalogenation: one involving CYP 450 oxidation or lipoxygenase (LOX)/COX co-oxidation, and the second pathway involving GSH conjugation (for a review see [13, 14]). Depletion of intracellular GSH results in induction of LOX activity. Previously, we have observed that induction of LOX by phenobarbital in epithelial bronchial cells; increase the formation of OTB and other metabolite of unknown structure [49], whereas inhibition of LOX decreases the genotoxicity [50].

The putative structure of derivative 7 is given in Fig. 6. This derivative is a quinone derivative in which the carboxylic group is replaced by a cyanide group.

The biotransformation of OTA into several derivatives is important as not only the half-lives and the route of elimination of the metabolites but also the toxicity vary for individual [51]. For example, OP-OA (ring opening lactone) is more toxic [52]. Moreover, adduct formation depends on the biotransformation reactions. We confirm that OTB is less toxic than OTA, as previously described by O'Brien *et al.* [53] who reported an approximately ten-fold weaker effect of OTB. Interestingly, approximately 15% of each cell type (LLC-PK1, NRK-2) survived to high concentration (>100 μM) of OTA [53, 54]. Schaaf *et al.* [55] demonstrated also that in primary rat proximal tubule cells and LLC PK1 cells, OTA induces a concentration-dependent elevation of ROS particularly $OH^{\bullet}$ and an increase in the formation of 8-oxoguanine simultaneously to a depletion of cellular glutathion. These authors suggested that the oxidative metabolism could be explained by the formation of HQs of OTA (OTQ/OTHQ). The associated redox cycle would cause ROS generation and depletion of GSH. In this latter manuscript, the authors expressed that NAC completely prevent the OTA induces increase in ROS levels as well as the formation of 8-oxoguanine and completely protect against the cytotoxicity of OTA.

Our data are in line with the work of others research groups. The metabolism of OTA has been first extensively studied by the team of Størmer during the past 25 years (for a review see [56]), using *in vitro* and *in vivo* assays in liver of different animals. The 4(R)-OH OTA was mainly formed after incubation in presence of human and rat liver microsomes, whereas 4 (S)-OH OTA was essentially formed *via* pig microsomes [57–59] but also in hepatocytes [60]. Gross-Steinmeyer *et al.* [61] reported the formation of these metabolites in hepatocytes, and also the formation of six unknown OTA derivatives in the supernatant of the hepatocytes. Correlation between OTA biotransformation and toxicity has been observed using endpoint other than DNA-adducts detection. For example, in cells expressing CYP2C9, OTA exerts an increased cytotoxicity as measured by neutral red [62], and mutation frequency is increased [63]. In MDCK cells, OTA induce single-strand breaks in concentration-dependent manner. When an external metabolizing system (S9-mix from rat liver) was added, this genotoxic effect was significantly stronger, thus implying a role of metabolization [64]. OTA after biotransformation by mouse kidney microsome is mutagenic in *Salmonella typhimurium* assay [65]. Moreover, it has been demonstrated that in *Escherichia coli*, a cysteinyl-OTA conjugate is associated with OTA cytotoxicity [66].

Altogether, we confirm that DNA-adduct formation of OTA is linked to its biotransformation, mainly by a pathway involving quinone derivatives and GSH conjugation.

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