Research Article

New molecular and field evidences for the implication of mycotoxins but not aristolochic acid in human nephropathy and urinary tract tumor

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To find out whether ochratoxin A (OTA), citrinin (CIT), aristolochic acids (AA) are etiologic agents of Balkan endemic nephropathy (BEN) or Chinese herbal nephrotoxicity, and associated urinary tract tumor (UTT), we have compared (i) in human kidney cell culture, the DNA adduct formation and persistence of OTA/CIT and AA adducts (ii) analyzed DNA adduct in several tumors from human kidney suspected to be exposed to either OTA and CIT, or AAs (iii) analyzed OTA, CIT, and AA in food. In kidney cell cultures, formation of specific OTA-DNA adduct and AA-DNA adduct were detected in the same range (around 10 adducts/10^9 nucleotides) and were time- and dose-dependent. After 2 days all disappeared. DNA adduct related to OTA and CIT are found in human kidney tissues from Balkans, France, and Belgium whereas no DNA adducts related to AA could be found in any tumors of BEN patients from Croatia, Bulgaria, or Serbia. No DNA adduct was found in kidney biopsy or necropsy of the French women suspected to be exposed to AA. OTA and CIT are more frequently found in rural area. AA was never detected. All these plead for implication of mycotoxins, especially OTA, in BEN and UTT.

Keywords: Aristolochic acid / BEN / Ochratoxin A / Slimming regimen / Urinary tract tumor

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1 Introduction

Balkan endemic nephropathy (BEN) is a familial chronic tubulointerstitial disease with insidious onset and slow progression to terminal renal failure. It was first described in Serbia and in Bulgaria. It affects people living in the alluvial plains along the tributaries of the Danube river in Serbia, Bosnia, Croatia, Bulgaria, and Romania. The disease usually affects adults in their fourth/fifth decade with eventual end-stage renal failure in their sixth decade. An association between BEN and urinary tract tumors (UTT) was recognized in the three affected countries (for reviews see [1–4]). In 1972, on the basis of a series of epidemiological observations, Akhmeteli [5] suggested that fungal toxins could be involved in the etiology of BEN. Nikolic *et al.* [6] demonstrated that the very intimate link between BEN and UTT can be explained by insult from an environmental contaminant. The intake of the agent at high doses causes nephropathy and early appearance of renal failure (BEN) during the third and the fourth decade of the patient's life. However, at low doses of the potential causative agent the nephropathy is not observed, 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. 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

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Abbreviations: AA, aristolochic acids; **BEN,** Balkan endemic nephropathy; **CIT,** citrinin; **OTA,** ochratoxin A; **UTT,** urinary tract tumor

of one family, while neighboring 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 molds, while neighbors may not be exposed to this factor.

A study of food contamination conducted in Bulgaria has demonstrated that a higher percentage of the staple food (maize and bean) was contaminated by ochratoxin A (OTA) in the endemic area than in the nonendemic area [7, 8]. The reanalysis of the data [2] 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 amounts of both toxins. The amount of CIT was often ten times higher in bean or maize from affected families compared to those of nonaffected ones. Vrabcheva *et al.* [9] found that in the BEN endemic area, wheat samples could also be contaminated by OTA and CIT, 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 [10]. The carcinogenicity of OTA is enhanced by simultaneous presence of CIT [11, 12].

More recently, the hypothesis that aristolochic acid (AA), alkaloids produced by *Aristolochia*, may contribute to the aetiology of BEN has been proposed [3, 13, 14]. This hypothesis has been based on the fact that in Belgium some women have developed a similar nephropathy after being treated with slimming regimen suspected to contain AA [15]. *Aristolochia fangchi* used by inadvertence in slimming regimen may contain different types of AA (Fig. 1): 30% of aristolochic acid I (AA I); 70% of aristolochic acid II (AA II) are produced by *A. fangchi*. The detection of specific AA-DNA adduct several months after cessation of treatment [16] even though that no AAs could be found in the pills [17, 18], is considered as a relevant biomarker.

As so long persistence of DNA adduct is questionable, in this manuscript, we compare DNA adduct formation and persistence of DNA adducts in human kidney cells treated by OTA, CIT, or AAs. Analysis of human biopsies (from women having followed a slimming regimen suspected to be contaminated by AA) and renal tumors (from Balkan region and other European countries) for DNA adducts related to OTA and/or AA are discussed.

2 Materials and methods

2.1 Materials

OTA (benzene free, CAS# 303-47-9), mix AA I (38%), and AA II (62%) were purchased from Sigma (Saint Quentin Fallavier, France). Pure AA I and pure AA II were purchased from Applichem (Biochemica, Chemica Synthesis services) Darmstatdt, Germany. The authentic OTA standard, carbon-bounded deoxyguanosine-C8-OTA monophosphate (C-C8 dGMP OTA) (Fig. 2A), was synthetized at the University of Guelph as described in Faucet *et al.* [19]. The 7-(deoxyadenosine-N6-yl) aristolocham I and II; and 7-(deoxyguanosine-N2-yl) aristolactam I and II used for comigration were prepared by incubation of calf thymus DNA in presence of microsomes and either pure AA I or pure AA II (Figs. 2B and C) The following enzymes: (proteinase K (used as received), RNase A, RNase T1 (boiled 10 min at 100°C to destroy DNases), and micrococcal nuclease (dialyzed against deionized water)) were purchased from Sigma; spleen phosphodiesterase (centrifuged before use) was from Calbiochem (VWR, France), nuclease P1 (NP1), and T4 polynucleotide kinase were from Roche diagnostics (Meylan, France). [γ^{32}P-ATP] (444 Tbq/mmol, 6000 Ci/mmol) was from Amersham (Les Ullis, France); Dubelco's Eagle's minimum essential media (D-EMEM) were prepared with Gibco products (Cergy Pontoise, France); phosphate saline buffer, trypsine, fetal calf serum, streptomycin, and penicillin were from Life-Technologies (Cergy-Pontoise, France); rotiphenol (phenol saturated with TRIS-HCl at pH 8) was from Rothsichel (Lauterbourg, France); salmon testis DNA was from Sigma and was purified before use; cellulose MN 301 was from Macherey Nagel (Düren, Germany); polyethyleneimine (PEI) was from Corcat (Virginia Chemicals, Portsmouth, VA); Whatman No 1 paper (ref. 6130932) was from VWR (France) and PEI/cellulose TLC plates used for ^{32}P postlabeling analyses were prepared in the laboratory at Toulouse, France. All reagents (potassium chloride, sodium hydrogen carbonate, sulfuric acid, phosphoric acid, hydrochloric acid, acetic acid, and sodium dihydrogen phosphate) were of normal grade.

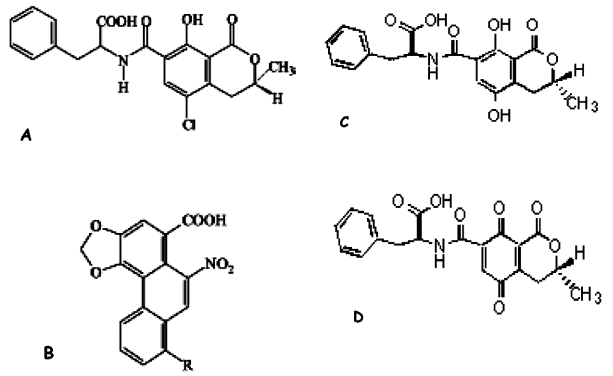


Figure 1. Chemical structure of ochratoxin derivatives and AA derivatives.(A) ochratoxin A (OTA); (B) AAs, R=OCH3 (AA I); R=H (AA II); (C) ochratoxin hydroxyquinone (OTHQ); (D) ochratoxin quinone (OTQ).

2.2 Cell culture

Human kidney cells (HK2; CRL-2190) were provided by ATCC (American Type Culture Collection, Manassas, Vir-

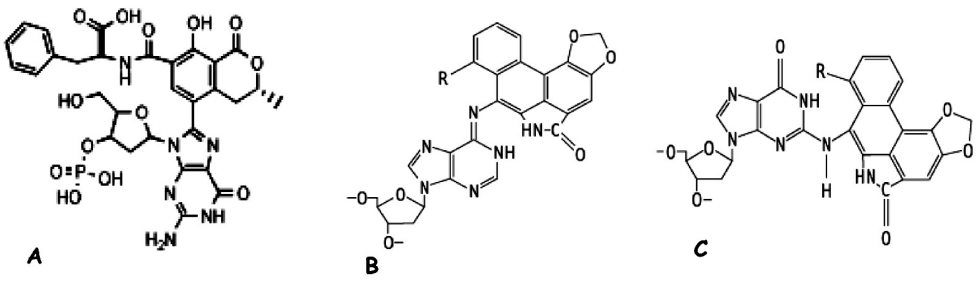


Figure 2. Chemical structure of putative DNA adducts. (A) Carbon bound deoxyguanosine-C8-OTA adduct (C-C8dGMP-OTA) (B) 7-(deoxyadenosine-N6-yl) aristolocham I (R=OCH3) and II (R=H); (C) 7-(deoxyguanosine-N2-yl) aristolactam I (R=OCH3) and II (R=H).

ginia, USA). HK2 cells were cultured in D-EMEM medium containing 44 mM $NaHCO_3$, 5% fetal bovine serum (FBS), 2% vitamins, 2% of nonessential amino acids, 1% streptomycin, 1% penicillin, for 48 h at 37°C under 5% CO_2. After trypsin digestion, the cells were resuspended in this medium to obtain 1×10^6 cells *per* mL and treated as follows. For DNA adduction, the cells were incubated for 2–48 h in presence of either 10 nM; 0.5, 1, 2.5 μM OTA, for 2–72 h in presence of 1, 10, or 50 μM CIT, for 2–48 h in presence of 0.1–5 μM pure AA I or mix of AA I and AA II, for 2–48 h in presence of 0.1–2.5 μM pure AA II. All experiments were performed in triplicate. The results are expressed as average value ± SD [20].

2.3 Cytotoxicity test

The cell cytotoxicity was tested using the celltiter 96® non-radioactive cell kit proliferation assay from Promega (Charbonnières, France) containing the DYE solution and the STOP solution. The assay was performed as recommended by manufacturer's instructions. It is based on the capacity of living cells to convert the tetrazolium salt into formazan which absorbs at 570 nm. The absorbance value (A) is positively correlated with the cellular survival. Cell cover on the bottom of the flask is removed with trypsin digest (2 min at 37°C). The action of trypsin is stopped with medium culture cell and harvested cells are centrifuged at 1400 rpm (Sigma 3K-15), 10 min at 4°C. Cells were resuspended in 1 mL of medium and counted with a 'Mallassez cell'. After dilution, each well of the plate is seed with 5555 cells. After one night for the cell adhesion at 37°C, 10 μL of each tested compounds (OTA, CIT, AA I, AA II, mix AA I and AA II) is applied for 24 h at 37°C. At the end of the incubation, 15 μL of DYE solution containing the tetrazolium salt were added to the 96 well plates for 4 h at 37°C. The reaction was stopped by the stop solution overnight at 37°C. Then, the absorbance was measured with a 96 well plate reader at 570 nm. The test doses ranged from 100 nM to 100 μM, whatever the compounds. Values correspond to the average of percentage of cell survival measured in ten assays. Statistical analysis was done using the software SPSS 13.

2.4 Human kidney tissues

A total of 60 renal tissues from patients suffering from nephropathy and urothelial cancer in different countries have been analyzed: 10 from an endemic region of Serbia; 16 from endemic and nonendemic region of Croatia; 8 from endemic region of Bulgaria; 18 from French patients (provided by Professor Plante, Rangueil Hospital, Toulouse, France); 7 from French women (5 provided by Professor Pourrat, details in [21]), Purpan Hospital, Toulouse, France; the two samples from Nice were provided as purified DNA by Professor Schmeiser (details in [16]), Deutsche Krebs Forschung, Heidelberg, Germany, and 1 sample from Belgian woman (provided by Dr. Arlt (details in [16]) suspected to be exposed to AA *via* slimming regimen. Three different parts of the same kidneys from French patients have been analyzed (healthy part, peritumoral part, tumoral part).

Isolation of DNA and ^{32}P-postlabeling analysis of DNA adducts are described in details in Pfohl-Leszkowicz & Castegnaro *et al.* [22].

2.4.1 *In vitro* incubation

Human microsomes preparation and conditions of incubation were described in details in Tozlovanu *et al.* [20].

2.5 Analysis of OTA and CIT

OTA was extracted from tissue, blood, and urine as follows. Tissue (0.2 g) was potterized with 8 mL $MgCl_2$ 0.1 M/HCl 0.05 M, pH 1.5 and extracted three times with chloroform (8, 4, 4 mL). Combined chloroform extracts, obtained under centrifugation (10 min, 5000 rpm, 4°C) were portioned twice against sodium hydrogen carbonate 0.1 M (16 mL). Aqueous phase was acidified to pH 1.5 and

extracted three times with chloroform (16 mL). The combined chloroform extracts were dried under vacuum, dissolved in 1 mL methanol, filtered, dried under nitrogen, and finally dissolved in 200 μL methanol. The same protocol was applied to 2 mL urine, 1 mL blood, and 500 μL plasma.

OTA and CIT in food were extracted as described by Molinié *et al.* [23]. OTA and CIT were analyzed on RP HPLC using C18 column PRONTOSIL 120 (250×4.0 mm^2) with inner porosity of 3 μm, under isocratic condition (mobile phase: orthophosphoric acid at 0.33 M/ACN/propan-2-ol (600:400:55), flow rate 0.8 mL/min). Detection was performed with a programmable Merck HITACHI FL Detector L-7485 (excitation 340 nm, emission 465 nm for OTA; 331 and 500 nm for CIT).

2.5.1 Analysis of AA in blood, tissues, fluids, and food

The extraction and the HPLC separation conditions are identical to that described above for OTA and CIT. Since AA derivatives do not respond under the fluorescence conditions, they were detected by UV at 260 nm. Under these conditions, neither OTA nor CIT could be detected.

2.6 Food samples collection

2.6.1 Wheat sampling

During the year 2000, 35 wheat samples were collected in the north-east of France. Nineteen were collected in the farms and 16 in cooperatives.

From July 2001 to June 2002, 83 wheat samples were collected from 12 different sites of storage belonging to cooperative from Midi-Pyrénées (west-south of France). Some samples (28) were collected immediately after harvest, without storage; 41 were collected at different date of the storage; 14 were stored in farm several months before storage in cooperative.

2.6.2 Total diet during 1 month

Fifteen families (ten with high incidence and five without any BEN record) were chosen to participate in the sample collection. The selection was done with the help of a local medical team under special criteria.

Sampling was done applying the duplicate diet as described in Castegnaro *et al.*, [24]. During the period of 27 days, sampling of foodstuffs was conducted. Also, samples of blood serum and urine were collected for further analyses. Foodstuffs were sampled on regular daily base from each family in form of a duplicate portion as for the additional family member. Total daily meal was weighted. All the meals of single family for each day were mixed, homogenized and stored at −20°C. During whole sampling period, for each examinee all data on eating habits, meal content, quantity and ingredients, way of cooking, and other relevant information were enregistered on regular daily bases. Questionnaire was conducted in order to collect medical and other personal informations relevant for the study.

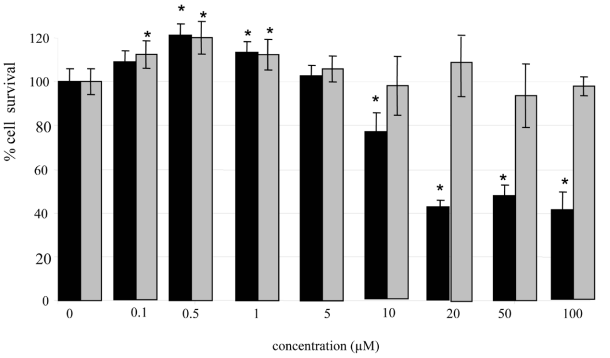


Figure 3. Cell viability of Human kidney cells (HK2) treated by mycotoxins. Cells were treated by increasing amount (0.1 – 100 μM) of CIT (grey) or OTA (black). Viability is expressed as percentages ± SD (ten analyses/point). *Significantly different from the control.

3 Results

3.1 Effect of OTA, CIT, or AAs on cell viability

Human kidney cells were treated with increasing amount of either OTA (10 nM – 100 μM) or CIT (0.1 – 100 μM), or AA I (1 nM – 100 μM) or AA II (1 nM – 100 μM), or the mix of AA I (38%) and AA II (62%).

Treatment with low doses of OTA or CIT (from 0.1 to 1 μM), increased the cell viability (Fig. 3). A significant increase up to 20% was observed after treatment with 0.5 μM of OTA or CIT. For higher doses OTA was cytotoxic whereas CIT did not decrease cell viability. Even with a

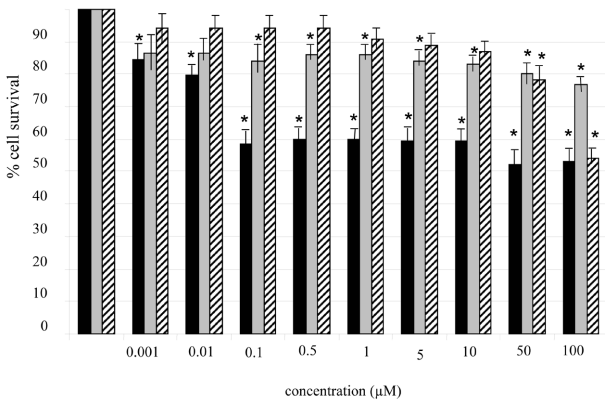


Figure 4. Cell viability of Human kidney cells (HK2) AA derivatives. Cells were treated by increasing amount (1 nM – 100 μM) of AA I (black); aristolochic II (grey); mix of AA I (38%) and AA II (62%) (Hatched). Viability is expressed as percentages ± SD (ten analyses/point). *Significantly different from the control.

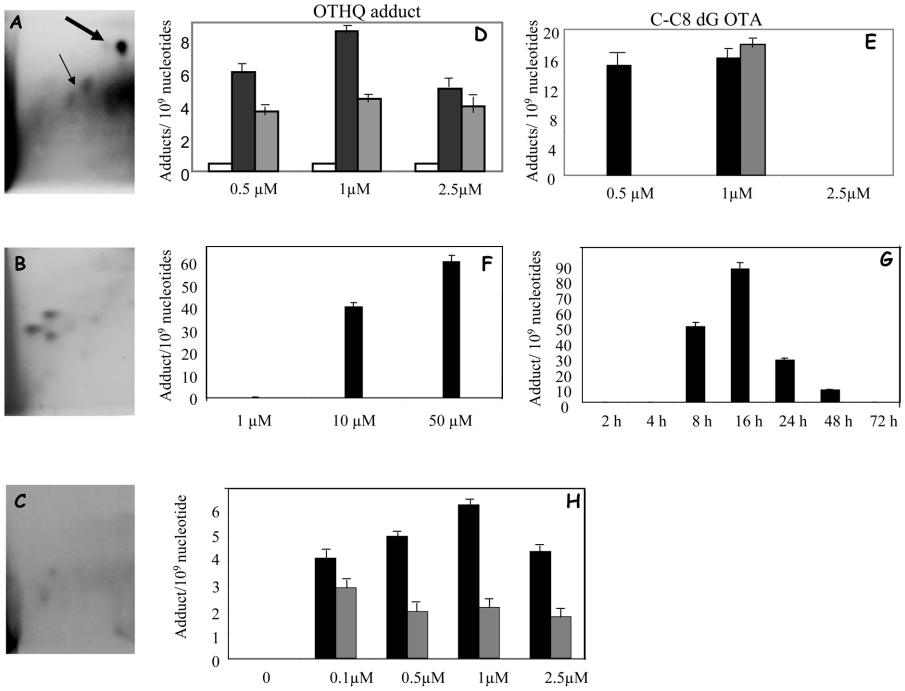


Figure 5. Time- and dose-dependent DNA adduct formation in human kidney cells treated by OTA; CIT or AA II. Example of DNA adduct pattern in human kidney cells treated by (A) OTA; (B) CIT; (C) AA II; (D) amount of OTHQ-specific DNA adduct (faint arrow) detected after treatment with 0.5, 1, 2.5 µM OTA for 2 h (white); 7 h (black); 24 h (gray). (E) Amount of C-C8 dGMP-OTA (bold arrow). (F) Total DNA adduct formed after treatment of cells for 8 h with increasing amount of CIT. (G) Kinetic of DNA adduct formation after treatment of cell with 50 µM of CIT. (H) Total DNA adduct (dA-AA II + dG-AA II) formed after treatment of cells with increasing amount of pure AA II for 7 h (black), 24 h (gray). DNA adduct amounts are expressed as number of adduct/10^9 nucleotides ± SD (three analyses).

dose of CIT as high as 100 µM, no decrease of cell viability occurred. In contrast, 10 µM of OTA leads to a reduction of cell viability above 25%. Neither AA I, nor AA II increased cell viability (Fig. 4). AA I is more cytotoxic than AA II as a treatment with 0.1 µM AA I decreased cell viability to 60%, whereas only a slight decrease of cell viability (around 25%) is observed with 100 µM of AA II. Nevertheless, increasing amount of AA I did not significantly decrease the cell viability. Only for the highest dose, the mixture of the both AAs decreased cell viability to 50%, in the same range as AA I alone.

3.2 DNA adduct formation in treated human kidney cells

We have compared the DNA adduct formed in human kidney cells treated with different amounts of OTA, CIT, or AAs for various time (Figs. 5 and 6). Example of DNA adduct patterns obtained after treatment of cells with OTA, CIT, or AA II are shown in Figs. 5A–C respectively.

OTA induced two adducts related to formation of ochratoxin hydroxyquinone (OTHQ) (faint arrow, [20]), and the adduct comigrating with C-C8 dGMP-OTA [19] (Fig. 5A). Figures 5D and E have showed dose and time dependence of the two adducts related to OTHQ (Fig. 5D) and of C-C8 dGMP-OTA (Fig. 5E). Whatever the concentrations of OTA, DNA adduct formation of these two adducts has reached a maximum after 7 h, decreased after that, and disappeared after 48 h. The formation of C-C8dGMP-OTA was detected after 7 h with the two lowest doses. With 1 µM, the formation increased till 24 h. This adduct has completely disappeared after 48 h. Figure 6A shows the kinetic of DNA adduct formation with low dose (10 nM) of OTA. OTHQ-related DNA adducts appeared after 12 h of treatment and increase till 24 h. C-C8dGMP-OTA adduct was observed after 18 h of treatment and decreased after that.

CIT induced mainly three adducts (Fig. 5B). The formation of these adducts was dose-dependent (Fig. 5F). Figure 5G shows the time-dependent and persistence of these adducts when HK2 cells were treated with 50 µM of CIT. After 3 days no more adducts were detected.

Two to four adducts were formed when cells were treated either by extract of *A. fangchi* containing 38% of AA I and 62% of AA II, or pure AA I, or pure AA II. Treatment of human kidney cell with increasing amount of pure AA II (0.1–2.5 µM) induced mainly two adducts (Fig. 5C) comigrating with 7-(deoxyadenosin-N6-yl) aristolactam II (dA-

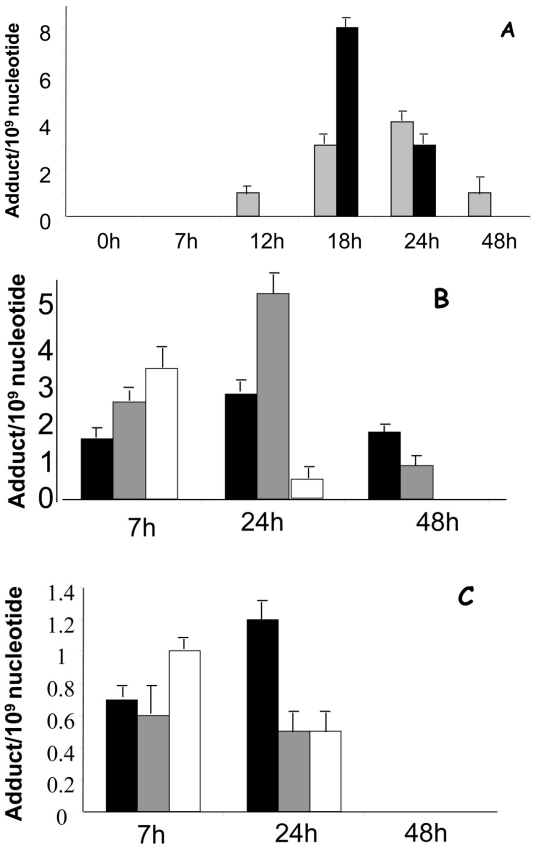


Figure 6. Time- and dose-dependant DNA adduct formation in human kidney cell (HK2) treated OTA [A] or AAs [B,C]. [A] OTHQ related DNA adduct (grey), C-C8dGMP-OTA (black) after treatment by 10 nM OTA. [B,C] Total DNA adduct formed after treatment of cells for 7, 24, 48 h with increasing amount (0.1 μM, black; 0.5 μM grey; 5 μM white) of (B) pure AA I (C) the mix of AA I (38%) and AA II (62%).

AA II) and 7-(deoxyguanosin-N2-yl) aristolactam II (dG-AA II) reaching the maximum after 16 h of treatment with 1 μM AA II (Fig. 5H). These adducts disappeared after 48 h. Treatment of cells with pure AA I induced formation of three adducts (data not shown). Two of them comigrated with dA-AA I and dG-AA I, the third comigrated with dA-AA II (data not shown). After 7 h of treatment, total DNA adduct formed by pure AA I increased with the dose (Fig. 6B). With the lowest doses of AA I (0.1 and 0.5 μM) the DNA adduct reached a maximum after 24 h but decreased at 48 h. The highest amount of DNA adduct (5.5 adduct/10^9 nucleotides) was reached after 24 h of cell treatment with 0.5 μM of AA I. For the high dose of AA I (5 μM), almost all DNA adduct disappeared after 24 h and were completely repaired after 48 h. Treatment of cells with AA mix lead to the formation of much less DNA adducts (around ten times less) (Fig. 6C). After 24 h the total DNA adducts whatever the doses were dramatically reduced. All DNA adduct have disappeared after 48 h.

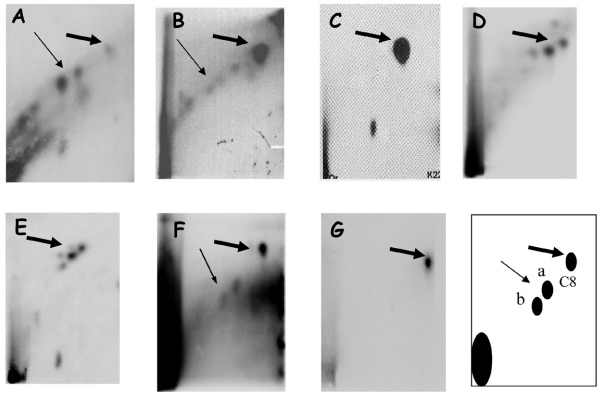


Figure 7. Example of DNA adduct pattern of some human kidneys related to mycotoxins. Kidney of Human suffering nephropathy and urothelial tract tumors from (A) Serbia; (B) Croatia; (C) Bulgaria; (D) France; (E) Belgium; (F) human kidney cell treated by OTA; (G) C-C8 dG OTA and drawing of OTHQ adducts (a, b) and C-C8 dGMP OTA (C8).

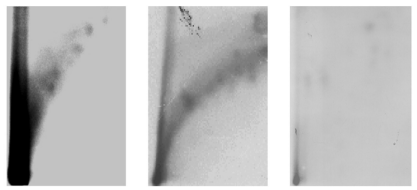


Figure 8. Example of other DNA adduct pattern of human kidneys. (A) DNA adduct pattern of kidney from a French patient (B) DNA adduct pattern specific of PAH contamination, Croatian patient K (C) DNA adduct pattern from a Croatian patient L suffering inflammation.

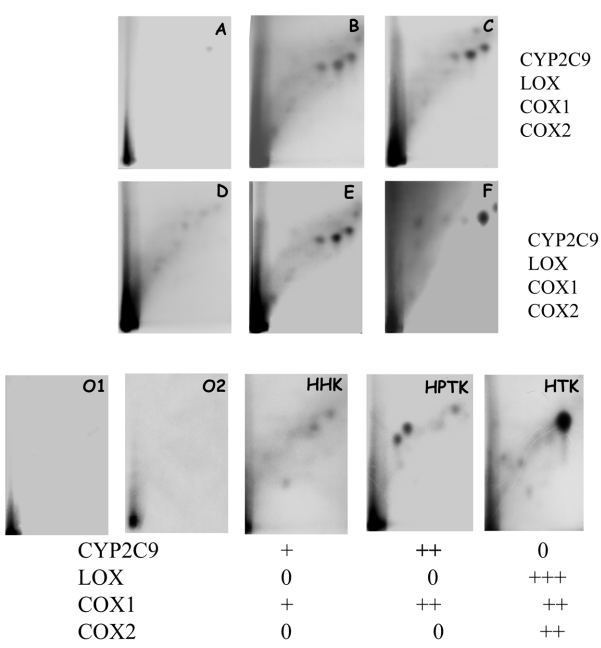
3.3 DNA adduct analysis in human kidney tumors from Balkan region and other EU countries

A total of 60 human kidney tumors from Croatia, Bulgaria, Serbia, France, and Belgium have been analyzed. Thirty percent of samples exhibited DNA adducts related to OTA (some examples are presented in Fig. 7). Interestingly when samples contain OTA-related DNA adducts, the C-C8 dGMP-OTA is always observed (bold arrow). In Croatian and Serbian patients, DNA adducts due to the quinone-form of OTA (OTHQ) were also clearly observed (faint arrow). In some samples, CIT-related adducts were observed. Figure 8 shows examples of DNA adduct patterns not related to mycotoxin. Some were related to polycyclic aromatic hydrocarbons (PAH). DNA adducts related to AA were never observed. Table 1 summarizes the characteristic of the Croatian patient. The four patients living outside of the BEN region and who are not rural exhibited a DNA adduct pattern not related to mycotoxins. All the seven rural patients from the BEN area had OTA-related DNA adduct, four of them had in addition CIT-related adduct. Two rural patients outside the BEN region had also OTA and CIT-

Table 1. Characteristic of Croatian patient

Patient	Age	Job[a)]	Geographic area[b)]	Pathology	Type of adduct[c)]
A Male	70	R	NE	Tumor of ureter	Other
B Male	54	R	E	Tumor of pyelon	CIT+++
C Female	46	R	NE	Tumor of kidney	Other
D Female	58	R	E	Tumor of ureter	CIT++; C-C8 dGMP OTA++ OHTQ+
E Male	45	R	NE	Tumor of pyelon	CIT + C-C8 dGMP OTA+
F Male	51	R	NE	Stenosis, hydronephrosis ureter	CIT ++; C-C8 dGMP OTA++
G Female	38	NR	NE	Calculosis, pyelonephrosis, inflammation	Other
H Female	52	NR	NE	Severe renal dysfunction, stenosis ureter	Other
I Female	61	R	NE	Chronic inflammation	Other
J Female	58	R	E	Tumor of pyelon	CIT, OTA (OTHQ)
K Male	51	NR	NE	Hydronephroma	PAH
L Female	51	NR	NE	Calculosis, hydronephrosis	NO ADDUCT
M Female	60	R	E	Tumor of ureter	CIT ++; C-C8 dGMP OTA+++; OTHQ ++
N Female	68	R	E	Tumor of pyelon	C-C8 dGMP OTA++
O Female	60	R	E	Tumor of pyelon	C-C8 dGMP OTA+; OTHQ +
P Female	66	R	E	Stenosis ureter	C-C8 dGMP OTA +

a) R = rural; NR = not rural.'
b) E = endemic region; NE = nonendemic region.
c) CIT-specific adduct of CIT; C-C8dGMP OTA-specific adduct of OTA; OTHQ-specific adduct of quinone form of OTA; PAH-specific adduct of polycyclic aromatic hydrocarbons.



	A	B	C
CYP2C9	+	++	++
LOX	0	0	0
COX1	0	++	++
COX2	0	0	++

	D	E	F
CYP2C9	+	++	0
LOX	0	0	+++
COX1	+	++	++
COX2	0	0	++

CYP2C9	+	++	0
LOX	0	0	+++
COX1	+	++	++
COX2	0	0	++

Figure 9. Comparison of DNA adduct in several part of kidney of two patients and *in vitro* formation. (A, D) Healthy part of patient 1 and 2 respectively; (B, E) peritumoral part of patient 1 and 2 respectively; (C, F) tumoral part of patient 1 and 2 respectively; [O1] *in vitro* incubation of OTA + DNA without any microsome; [O2] *in vitro* incubation of DNA + microsome without OTA; [HHK] *in vitro* incubation of DNA + OTA + healthy human kidney microsomes; [HPTK] *in vitro* incubation of DNA + OTA + human peritumoral kidney microsome; HTK *in vitro* incubation of DNA + OTA + human tumoral kidney microsomes. Tables indicate the expression of cytochrome (CYP) and peroxidases (LOX, COX) in the different parts of the tissue.

related adduct. The three other rural patients outside of the BEN region had other type of DNA adduct or no adducts.

Figure 9 shows DNA adduct pattern of three part of the same kidney. In general, more individual DNA adducts were observed in peritumoral (Figs. 9B and E) compared to tumoral part (Figs. 9C and F). No adduct or only a few were observed in healthy part (Figs. 9A and D). DNA adduct pattern was dependent of the expression of biotransforming enzymes (Cytochrome P 450 (CYP) and peroxidases such cyclooxygenase (COX) or lipooxygenase (LOX)). Induction of LOX and COX_2 favored the formation of C-C8dGMP-OTA (HTK), whereas CYP2C9 and COX_1 favored formation of OTHQ-related adduct (HPTK).

Table 2. Amount of DNA adduct, OTA blood concentration, and OTA kidney concentration of French patients with kidney carcinoma

Patient	Age[a)]	pattern[b)]	Total DNA-adduct level[f)]		OTA blood concentration (mg/L)	OTA organ concentration (ng/g)
			T	PT[c)]		
1 Male	70	OTA	5	20	0.13	0.44
2 Male	85	OTA	3	2	2.49	0
3 Male	53	OTA	3	2	0.12	0
4 Male	66	OTA	28[d)]		LOD	0.89
16 Female	75	OTA	15	41	LOD	1.16
17 Female	58	OTA	10	43	LOD	0.84
13 Male	60	OTA + PAH	8	115	0.12	0
25 Male	63	?[e)]		?	0.15	1.76
10 Male	71	One spot	2	2	0.18	0
12 Male	65	Other	1	3	0	0
18 Male	77	Other	16[d)]		0.17	0
19 Male	66	Other	17	65	0	0
20 Male	75	Other	5	21	0	0
21 Male	72	Other	10	14	0	0
22 Male	64	Other	17[d)]		0	0
23 Female	73	Other	4	33	0.14	0
24 Female	71	Other	0	6	0	0
27 Female	83	No adduct	0	0	0	0

a) Age of the patient the day of surgery.
b) OTA-specific adduct of ochratoxin A; PAH-specific adduct of polycyclic aromatic hydrocarbons; other = unknown pattern.
c) T, tumoral part; PT, peritumoral part.
d) Only tumoral part was available.
e) Tissue was to much necrosed to allow recovery of enough DNA.
f) Expressed as number of DNA-adducts/10^9 nucleotide.

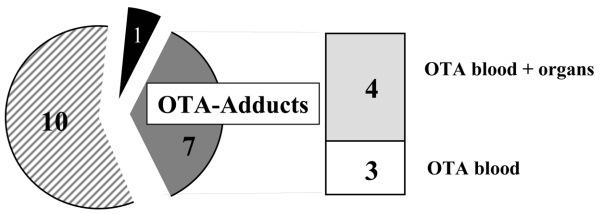


Figure 10. Correlation between OTA-DNA adduct and presence of OTA in blood and kidney. OTA-related DNA adduct pattern (gray); other DNA adduct pattern (hatched); unknown DNA adduct pattern because kidney was necrosed avoiding DNA extraction but presence of large amount of OTA in blood and kidney (black).

Table 2 summarizes French data. One-third of patient have OTA-related DNA adduct in their kidney. Interestingly, OTA was found in blood and kidney of all patients having OTA-related DNA adducts (Fig. 10).

3.4 DNA adduct detection in women having followed slimming regimen suspected to contain A. fangchi

In order to understand the potential implication of AAs in the nephrotoxicity of some women treated with a slimming regimen suspected to contain *A. fangchi* [21], we have analyzed blindly five biopsies from French women (case described in Stengel *et al.* [21]) and one kidney from a Belgian women (case described in Arlt *et al.* [16]). No DNA adduct was observed from the French women (Figs. 11A–E), whereas, DNA adduct related to OTA are observed almost exclusively from the Belgian patient (Fig. 11F). No adduct corresponded to those of AAs. In addition we have also analyzed the two kidneys and two ureters from the French women from Nice (case described in Arlt *et al.*, 2004 [16]). The first woman has developed a transitional cell carcinoma in the right urinary tract with invasive metastases. She received kidney transplantation in 1998, and died in 2000. She took herbal mixture 12 months in 1992. The second patient did not develop any cancer. Hemodialysis was initiated in 1999. She died in 2001. Several DNA adducts are detected in the kidney and the ureter of the first patient (Figs. 12A and B). None of these adducts were related to AA (Fig. 12A). The main adduct has the same chromatographic properties than C-C8dGMP-OTA. No DNA adduct was detected in the kidney or in the ureter of the second patient (Figs.12C and D).

3.5 Evaluation of the presence of OTA or CIT, or AAs in food

3.5.1 Wheat analysis

Wheat samples collected in farm were significantly more frequently contaminated by OTA and to higher level than wheat samples collected in cooperative. Occurrence of CIT was lower in farm samples, but the highest level (512 μg/kg) was found in farm sample (Table 3). Thirty-seven farm

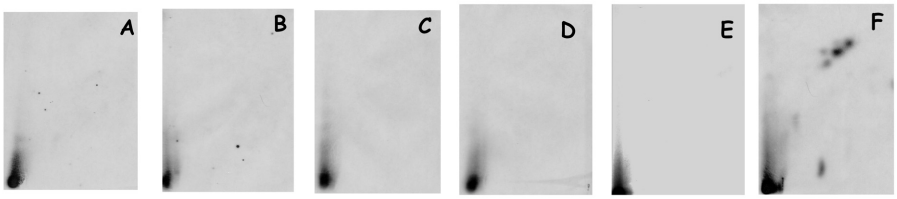


Figure 11. Blind analysis of DNA adduct patterns of kidney biopsies of: (A – E) French and (F) Belgian women that had been treated with slimming regimen containing AAs.

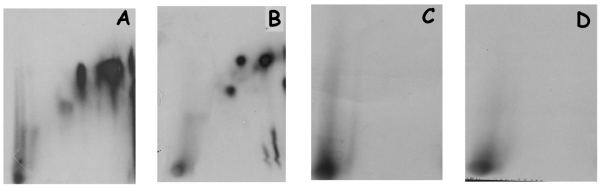


Figure 12. DNA adducts patterns of French patient having followed a sliming regimen. Kidney necropsies (A, C) and ureter (B, D). French patient 1 (A, B); French patient 2 (C, D).

Table 3. Occurrence of OTA and CIT in French wheat

	Farm samples			Coop samples	
	OTA[a)]	CIT		OTA	CIT
F1	5.6	<LOD	S1	<LOD	<LOD
F2	6.5	<LOD	S2	<LOD	44.6
F3	<LOD	<LOD	S3	<LOD	<LOD
F4	3.15	<LOD	S4	<LOD	0.86
F6	0.84	<LOD	S5	<LOD	<LOD
F7	0.91	<LOD	S6	<LOD	0.88
F8	<LOD	<LOD	S7	<LOD	<LOD
F9	<LOD	LOQ	S8	<LOD	1.1
F10	6.26	512	S9	<LOD	7.46
F1	<LOD	0.84	S10	<LOD	1.04
F12	<LOD	<LOD	S11	5.3	<LOD
F13	<LOD	<LOD	S12	2.4	0.95
F14	1.07	<LOD	S13	<LOD	1.04
F15	6.73	0.86	S14	6.6	3.8
F16	<LOD	0.86	S15	<LOD	<LOD
F17	<LOD	<LOD	S16	<LOD	15
F18	30.77	24.57			
F19	<LOD	7.24			
F20	5.2	<LOD			
	11/19	7/19		3/16	10/16
	(58%)	(37%)		(19%)	(62.5%)

a) Concentration expressed in μg/kg.

samples were simultaneously contaminated by OTA and CIT, whereas 12.5% of coop samples contained both toxins.

More than a half of the wheat samples collected in south-east of France contain trace of OTA and/or CIT ranging from the LOQ (<0.2 μg/kg) to 11.6 μg/kg of OTA; and from LOQ to 6 μg/kg of CIT (Fig. 13). OTA was the main contaminant (78% of the contaminated samples). Only 12% of the contaminated samples contain OTA over 5 μg/kg

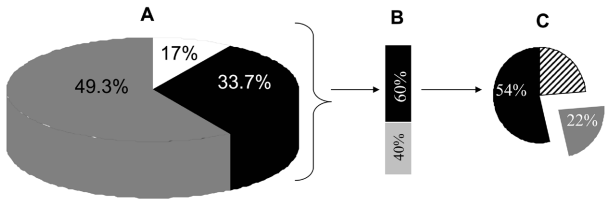


Figure 13. OTA or CIT analyses of wheat samples from south-east of France. (A) Type of samples analyzed (black) unstored sample; (gray) stored in grain storage cooperative (white) stored in farm before cooperative; (B) (black) percent of samples contaminated by either OTA or CIT; (gray) not contaminated; (C) mycotoxin repartition (black) only contaminated by OTA; (gray) only by CIT (hatched) OTA + CIT.

(Table 4). Figure 14 shows the respective concentration of OTA and CIT. The samples cocontaminated have been more often stored in farm (7/12). Harvesting after a rainy period increased also the risk of cocontamination; and the level of contamination. In some cases the amounts of the both toxins were similar; in other case the amount of CIT was higher (two to six-fold); sometime the amount of OTA was higher than that of CIT (up to three times). AA was never detected.

3.5.2 Total diet

OTA content of food samples collected every day during 1 month in 15 families from Balkan region have been analyzed week-by-week on pooled food. The average OTA contaminations *per* day have been calculated (Fig. 15). As DNA adduct analyses shown CIT-related DNA adducts in addition to OTA-related DNA adduct, we have analyzed in details the food day-by-day of three families. The first family (BEN 1) has four members of different ages (between 48 and 19 years old) and has direct progenitor who died from BEN. The second family also has four members (age range of 42 – 14 years old), and had two direct progenitors who died from BEN. For the third family (also four members, age range of 44 – 19 years old) there was no record of BEN cases in the ancestry. In the food from three families OTA could be found (Table 5), but the prevalence was higher in both BEN families; 39, 28.5, 12.5% for BEN 1, BEN 2, non-BEN respectively (Fig. 16). CIT is only found in the food from BEN families (12.8%, 21.4% for BEN 1 and

Table 4. Range of OTA and CIT contamination

OTA range	Number of sample	% Contaminated (n = 39)	% Total (n = 83)
LOQ < OTA < 3 μg/kg	27	70	32
3 < OTA < 5 μg/kg	7	17.9	8.4
>5 μg/kg	5	12	6
CIT range	Number of sample	% Contaminated (n = 25)	% Total (n = 83)
LOQ < CIT < 5 μg/kg	19	82.6	22.9
>5 μg/kg	4	17.4	4

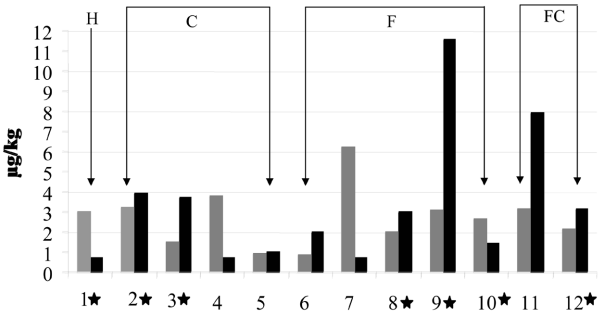


Figure 14. Amount of OTA (black) and CIT (gray) in sample cocontaminated; H sample collected during harvest; C samples stored in cooperative; F samples stored in farm; FC samples stored first in farm and then in cooperative; *samples harvest after a rainy period.

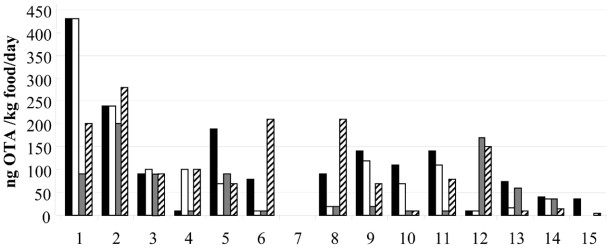


Figure 15. Daily average of concentration of OTA in food collected in 15 famillies week 1 (black); week 2 (white); week 3 (grey); week 4 (hatched).

BEN 2 respectively). On the basis of the weekly OTA concentration in food samples, the food intake of each member of the families and their weight, the individual exposures have been calculated (Fig. 17). The OTA intake of BEN 1 family varies between 0.65 and 6.64 ng/kg bw/day every week. The intake of BEN family 2 is regular over the month around 1.8 ng/kg bw. On the contrary, Non-BEN family has low or no intake, except 1 wk reaching almost 1.5 ng/kg bw/day due to a very high intake 1 day in the week.

3.6 OTA and CIT in urine

OTA and CIT have been analyzed in the urine of each member of the three families on day 7; day 13; day 20; day 27 of the study. An example of HPLC separation with fluorimetric detection is given Fig. 18. OTA and some of its metabolites have been detected whatever the week of urine collection in the urine of the father, the boy, and the girl of the BEN 1, and in the father and the two boys of BEN 2 families. Urine OTA concentrations are given in Table 6. OTA was neither detected in the urine of mothers nor in the urine of members of the non-BEN family. CIT has been detected in three samples of urine: (i) urine of the father of BEN 1 family, collected at the end of the first week; (ii) urine of the girl of BEN 1 family, collected at the end of the third week; (iii) urine of the boy of the BEN-2 family collected at the end of the first collection. Never OTA or CIT have been detected in urine of non-BEN family.

Table 5. OTA and CIT amount in daily pooled food

OTA (ng/kg) Day of collection	BEN 1 family	BEN 2 family	Non-BEN
1	49 (CIT+)[a)]	56	252
2	58	nd	nd
3	nd	79	nd
4	186	nd	nd
5	nd	nd (CIT+)	nd
6	nd	nd	nd
7	225	>LOD OTA	LOQ
8	nd	119	nd
9	nd	nd	nd
10	115	nd	nd
11	nd	nd	LOQ
12	nd	nd	nd
13	nd	nd	nd
14	nd	123 (CIT+)	nd
15	nd	nd	nd
16	56	nd	nd
17	nd	152 (CIT+)	nd
18	145	nd	nd
19	29 (CIT+)	nd	nd
20	nd (LOD CIT)	nd (LOD CIT)	nd
21	133	100	nd
22	nd	nd	nd
23	nd	99 (CIT+)	29
24	nd	nd	nd
25	LOD	Trace (CIT+)	nd
26	LOD	nd	nd
27	nd	nd	nd
28	nd	nd	nd
OTA occurrence	11/28	8/28	4/28
CIT occurrence	3/28	6/28	0/28

a) Number correspond to amount of OTA expressed as ng/kg, present of CIT is mentioned in bracket.

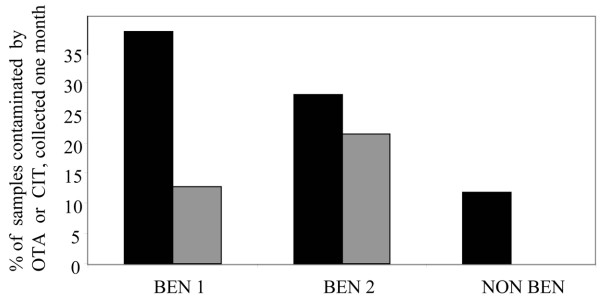


Figure 16. Prevalence of food contaminated by OTA (black) and CIT (grey) from two families affected by BEN (BEN 1 & 2) *versus* on family not affected by BEN (Non-BEN).

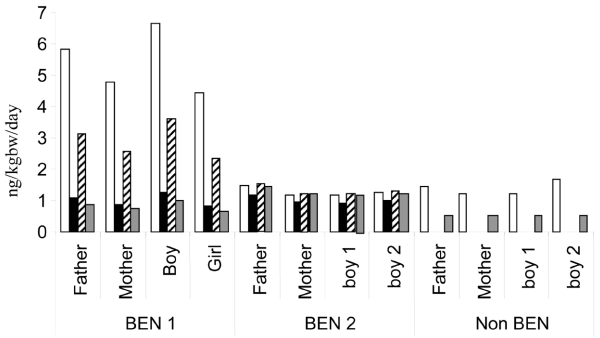


Figure 17. Average daily OTA intake week-*per*-week for each member of three families expressed as ng/kg bw/day, week 1 (white); week 2 (black); week 3 (hatched); week 4 (grey).

3.7 Aristolochic acids content in food and urine

In the same samples (food and urine), the presence of AAs (AA I or AA II) has been analyzed by HPLC with UV detection. In a few food samples, one peak is detected at 260 nm. In urines of all members of the Non-Ben family collected at the end of the third week, two peaks have been detected. Nevertheless, as shown in Fig. 19, none of these compounds corresponds to either AA I or AA II.

4 Discussion

The aim of our study was to compare the implication of two classes of potential etiological agents (mycotoxins including

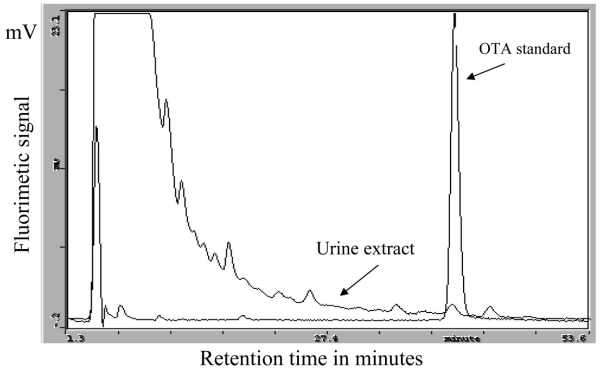


Figure 18. Example of HPLC separation of OTA in urine extract; comparison with OTA standard eluted under the same conditions of elution: Column C18, 3 μm; mobile phase: orthophosphoric acid at 0.33 M/ACN/propan-2-ol (600:400:55), flow rate 0.7 mL/min. Detection by fluorimetry: excitation 340 nm, emission 465 nm.

OTA and CIT; and AA derivatives) in some nephropathies and UTTs. Although DNA adduct detection has been considered as a relevant biomarker in case of AA contamination [16], possibility to persistence of DNA adduct several months or years after cessation of treatment is highly questionable. DNA adducts are frequently used as biomarkers of exposure to chemicals that are either electrophilic or are metabolized to electrophiles. This is readily possible in experimental systems with known exposure and duration, but it is a much more complex issue in molecular epidemiological studies. Experimental studies have shown that with constant dosing, a steady-state concentration of DNA adduct will occur, where the number of new adducts formed each day equals the number of adducts that are lost due to repair. Thus if the exposure is relatively constant in a molecular epidemiological study, it can be reasonably assumed that adducts are at a steady-state concentration. In contrast, if exposures are intermittent and of unkown and variable amount, little inference can be made other than that exposure occurred. Many different DNA adducts are now being measured, but a major issue in interpretation relates to their meaning. The presence of a chemical-specific DNA adduct in

Table 6: Concentration of OTA and CIT in 24 h urine collected every week expressed in μg/L

Members of family	Week 1	Week 2	Week 3	Week 4
Mother BEN1	ND[a)]	ND	ND	ND
Father BEN 1	0.1 μg/L (OTA) 0.37 μg/L (CIT)	ND	0.375 μg/L (OTA)	ND
Son BEN 1	0.221 μg/L (OTA)	0.3 μg/L (OTA)	0.25 μg/L (OTA)	0.103 μg/L (OTA)
Girl BEN 1	0.1 μg/L (OTA)	0.25 μg/L (OTA)	1 μg/L (OTA) 2 μg/L (CIT)	0.102 (OTA)
Mother BEN 2	ND	ND	ND	ND
Father BEN 2	ND	0.375 μg/L (OTA)	0.375 μg/L (OTA)	ND
Son 1 BEN 2	ND 0.43 μg/L (CIT)	ND	0.25 μg/L (OTA)	0.25 μg/L (OTA)
Son 2 BEN 2	0.255 μg/L (OTA)	0.573 μg/L (OTA)	0.37 μg/L (OTA)	0.125 μg/L (OTA)

a) ND, not detectable.

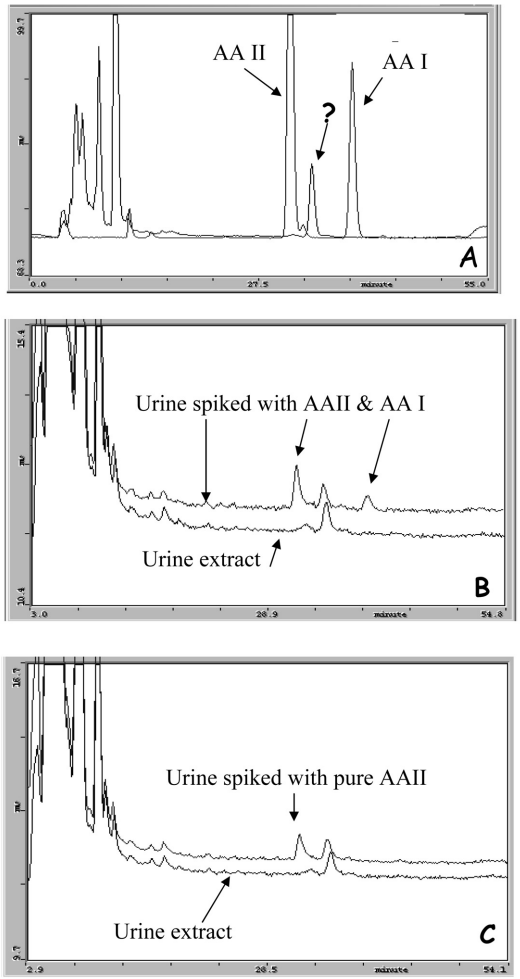


Figure 19. HPLC analysis of food (A) and urine extract (B). Separation conditions are similar to OTA separation. AAs are detected by UV at 260 nm (optimum for AAs). (A) Comparison with AA I and II mix (B) urine has been spiked with AA I and II mix (C) urine has been spiked pure AA II.

human DNA is a good indication that exposure to that chemical occurred [25]. Carcinogen-DNA adducts in target tissues are a more relevant marker than internal dose, because the former reflect not only individual differences in absorption and distribution but also difference in the metabolism (activation *versus* detoxification) and the extent of repair of DNA damage. DNA adduct data are useful to clarify carcinogenicity results, to elucidate the mechanism of carcinogenesis or to evaluate low dose range effects.

For this reason we have analyzed the formation and the persistence of DNA adducts, in human kidney cells treated with increasing amount of either AA I, or AA II or both of them, or in presence of OTA or CIT for 7, 24, and 48 h. In cell culture whatever the doses and the time of exposure, only a small amount of AA-related DNA adducts were formed in kidney cell culture whereas a large amount of DNA adduct were formed in these cells after OTA or CIT treatment. All DNA adducts (related to AA or to OTA) have disappeared after 2 days. This indicates a fast repair of these adducts, whatever the kind of DNA adducts, ruling out the possibility to find DNA adduct in kidney of woman several years after cessation of contamination. This is in line with the fact that no adduct was found in the kidney tissues of the women treated with slimming regimen which we were provided.

Interestingly, DNA adducts related to OTA were found in human kidney tumors from patient from BEN region. Several DNA adduct have the same chromatographic properties than OTHQ, C-C8dG-OTA. The concentration of adducts measured in a DNA sample is the result of a balance between rates of formation, removal by repair and cell death, and dilution by DNA replication as the newly synthesized strand does not contain adducts [26]. The life time of DNA adducts depends on their structure (*i. e.*, the carcinogen from which they are derived) and generally it lies in the range of days to weeks (occasionally a few months) [27]. DNA adducts are believed to be markers of exposure over such a period. DNA adduct generally reflect recent exposure rather that in the distant past. A sporadic DNA adduct measurement is, therefore, rather noninformative if not related to consideration of the timing and duration of exposure [28 – 31]. For DNA adducts that are rapidly repaired, steady-state levels are dominated by repair; for adducts that are refractory to repair, the cell turnover will be the rate limiting factor. In a previous study, we have demonstrated that the preferential formation of either C-C8dGMP OTA or OTHQ-DNA adduct depends of expression of some biotransforming enzymes. Indeed, OTHQ-related DNA adduct were formed after *in vitro* incubation in presence of kidney microsomes of untreated pig or healthy human expressing mainly cyclooxygenase COX_1 and CYP $2C_9$, whereas C-C8dGMP OTA was formed mainly after incubation in presence of kidney microsomes from pig fed OTA or from human tumor, expressing mainly COX_2 and lipoxygenase [20]. This is noteworthy that induction of COX_2 often occurred during cancer process, notably in kidney [32 – 34]. Incubation in presence of microsome from peritumoral part of human kidney has lead to the formation of the two OTHQ adducts in addition to C-C8 dGMP OTA ([22], for a review see [4]). Comparison of DNA adduct in tumoral part *versus* peritumoral part confirmed that difference in metabolic capacity of the tissue induce formation of different adduct. More different DNA adducts are measured in peritumoral part and reached a higher level. In carcinogenic study we observed mainly C-C8dGMP OTA in tumor, and demonstrated that DNA adduct pattern was dependent of the metabolic capacity of the tissue [35, 36]. Lower DNA adduct level observed in tumoral part could be explained by the rapid proliferation of the tumoral cells inducing a dilution, but also by the disappearance of the DNA adduct due to accurate or not repair. Interestingly, OTA and CIT-related DNA adducts are formed in range of nanomolar concentrations where the proliferation of kidney cells was observed.

In contrast, treatment of cell with high dose of OTA decreased cell viability and lead to the formation of less DNA adduct. These data are in line of those of Gekle *et al.* [37] who demonstrated that low dose of OTA (in the range of every-day exposure) affect proximal tubule and collecting duct functions in the way of proliferation, cell differentiation, and activation of extracellular signal-regulated kinases, whereas high doses (>1 μM) decreased cell viability and increase necrosis [37]. This is particularly important, as it has been demonstrated that mice which had the fastest tumor induction had the highest rates of cell division [38]. In our survey, kidney tissues in which OTA-related DNA adducts were observed contained also OTA. Moreover, OTA was found in the blood of the patients confirming exposure.

In contrast, none of DNA adduct observed in human kidney correspond to AA adducts. In kidney of six French women treated with slimming regimen containing AA no DNA adduct was observed. More interestingly, the kidney tumors from a Belgian women also treated by slimming regimen contain OTA-related DNA adduct but no AA-related DNA adduct. This confirm the data from Nortier *et al.* [15] who reported OTA-related DNA adducts, but no AA-DNA adduct in four out of 22 renal tissues from patients treated with Chinese herb containing AAs and suffering end stage renal failure. This was also the case for one of the French woman from Nice. Thus, we do not confirm the data obtained by Arlt *et al.* [16] with the same samples and the same purified DNA. Absence of AA-related DNA adduct in patient of Balkan region is in line with the results from Wiessler [39] who did not find any AA-related DNA adduct. He has reported ,no DNA adduct related to AA can be observed in Human kidney in Balkan region. Although AA has been used therapeutically for decades, no case of human poisoning has been reported. It has not been possible to detect adducts in humans by ^{32}P postlabeling'.

DNA adducts have vastly different potentials for causing mutations. Some adduct are highly mutagenic, whereas other are not mutagenic and do not lead to heritable effects [40]. In the latter case, the presence of such adducts is simply a measure of exposure. OTA induces different type of DNA adduct as the result of its metabolic transformation. The repair of the OTA-related adducts (OTHQ-related adduct and C-C8dGMP-OTA adduct) was differential. Under conditions of steady-state exposure, the levels of chemical-specific DNA adducts may be expected to reflect the biologically significant individual exposure to specific agents which in turn could be expected to correlate with risk as exemplified by the adducts of aflatoxin B1 [41]. Thus the measurement of DNA adduct in target tissue has the potential to be not only an exposure marker but an individual cancer risk marker (for a review see [26]). Unfortunately, we do not know which of the OTA-related DNA adducts are mutagenic. In most cases, the DNA adducts that lead to initiating events in inducing a tumor are removed from the tissue many years in advance of tumor growth and detection. This explain the lower level of DNA adduct in tumoral part. So the adducts in the tumor have perhaps nothing to do with the adducts that induced the tumor. Nevertheless, they presence indicate clearly an exposure. It should be keep in mind that once OTA reaches the bloodstream, it is bound to serum proteins (>99%). 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, and thus new DNA adduct will be formed. In 50% of the tumors of Croatian farmers (6/12), OTA and CIT-related DNA adduct were observed. All farmers from the endemic region have OTA-related DNA adducts. In French tumor 30% of tumor exhibited also OTA-related DNA adduct. Analysis of wheat and food confirmed the exposure to OTA and CIT but not to AA. Indeed, wheat samples stored in farm are significantly more contaminated by OTA than wheat stored in cooperatives. About 20% of samples are simultaneously contaminated by CIT. Analysis of 1 month food of three Serbia families indicates that in both BEN families, OTA was found every week, whereas in the non-BEN family, OTA in the food is scattered. Although we have analyzed in depth only the daily intake of three families, our data has showed highest food contamination by OTA and CIT in BEN families. This is in line with the data obtained previously in Bulgaria [9, 24]. Based on nephrotoxicity of OTA in pig, the JECFA (Joint Expert Committee on Food Additives) proposed for OTA a PTWI (Provisional Tolerable Daily Intake) of 100 ng/kg of body weight *per* week, which corresponds to 14 ng/kg of body weight *per* day. On the basis of tumor formation by OTA as an endpoint, Kuiper-Goodman and Scott [42] proposed a Virtually Safe Dose (VSD) of 1.8 ng/kg body weight/day. Thus, using these informations for risk assessment on these three families, the VSD is often over passed, at least half of the time for all members of the BEN 1 family, while in the BEN 2 family the father has regular intake around this value and other members, including children have an intake just below this value. For the non-BEN family, this value is exceeded only once in the month. In addition, both BEN families have food contaminated by CIT in contrast to the non-BEN family. This is of the most importance, as it has been proven in animal studies that CIT increase the kidney tumors formation induces by OTA [11, 12]. This is also in line of the data of Stoev *et al.* [43] who have demonstrated that nephrotoxicity in pig is related to simultaneous exposure to several mycotoxins in feed. In contrast neither AA I nor AA II is found in food or urine of these families. Thus for these BEN affected families, the exposure to OTA and CIT is proven while there is no such indication for the AA I and AA II which have not been detected in all samples analyzed so far. The non-BEN affected family is exposed to only OTA at lower frequency and lower, but not to CIT and not to AA I or AA II.

The relationship between OTA excreted in urine with OTA intake is complex. In general, the elimination for human is low (average value comprised between 20 and 80 ng/day) independently of the dose ingested, till the

Table 7. Comparison between BEN and AA nephropathy[a)]

Features	Balkan endemic nephropathy	AA nephropathy
Age of patient	50 – 60 nephropathy >65 cancer	35 – 45
Rural incidence	High	No
Familial clustering	Important	No
Rate of progression	Very slow >20 years	Rapid 6 – 24 months
Kidney morphology: Atrophy outline	Symmetric smooth	Asymmetric irregular
Tubular atrophy	Extensive	Extensive
Interstitial fibrosis	At the late stage (diffuse)	Extensive
Hypertension	No	Yes
Animal study	Same symptoms in pig treated by OTA [4, 43] with low doses Kidney cancer in rodent low repeated doses OTA (reviewed by [4]) Increased by CIT [11, 12]	Kidney damage (rabbit/rat) only with very high dose (>5 mg/kg/bw) after ip administration [54, 55] Stomach cancer in rodent [54, 56]
Marker of exposure in BEN area	First data 1970	
Food	Higher OTA and CIT prevalence in BEN region	No detection of AA in food
Urine	Higher OTA and CIT prevalence in BEN region	No detection of AA in urine
DNA adduct	OTA related in BEN human tumors [53] OTA related in some patient treated by slimming herbs ([15], this study)	In 1994, Wiessler [39] reported no DNA adduct related to AA can be observed in Human kidney in Balkan region; although AA has been used therapeutically for decades, no case of human poisoning has been reported. It has not been possible to detect adducts in humans by ^{32}P postlabeling
Mapping of hypothesis to geographic distribution	Yes	unknown

a) Adapted from [2 – 4].

intake is below 100 ng/kg bw/week. The excretion for low intake is in the same range as for rat [24]. The OTA elimination increases dramatically and is multiplied by 10 – 50-fold for an average intake of 100 ng/kg bw/week [24, 44]. OTA has been found more often in the urine of people living in BEN-endemic villages than in those in nonendemic villages, and the highest amounts were seen in patients with BEN or UTT [45, 46]. In this study, we confirm the presence of OTA in urine of the males of the BEN families and the daughter whereas OTA was never detectable in either urine of the member of the non-Ben family. CIT was detected in urine when the members of the family have eaten food contaminated by CIT the day before urine collection. In contrast, OTA could be found in urine several days after OTA ingestion. OTA binding to serum proteins (>99%), facilitates its passive absorption in the nonionized form, but hinders its glomerular filtration [37]. OTA binds strongly to albumin (binding saturation above several hundred micrograms *per* millilitre of serum) [47], 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 [48]. The fraction of OTA bound to proteins 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. This was observed in Bulgarian study and in rat study [24, 46]. Moreover, it seems that contamination by CIT favored OTA elimination [49].

Altogether, our studies clearly demonstrate the exposure of Bulgarian and Serbian BEN/UTT affected populations to OTA and CIT, but no exposure to AA either from food analysis or urinary excretion. OTA-related DNA damages were also observed in the target tissues of Balkan patients but also in French patient and one Belgian patient. The contamination was more often observed in rural area, and farm. While extensive farming may use pesticides, the individual farms use them sporadically and here as in Bulgaria the levels of contamination in OTA and CIT can be very high. This is in line with a study that compared crops from ecological farms (farms in which no pesticides or fungicides were used) and conventional farms which show clearly higher OTA contamination in crops from ecological farms, about six times ([50]). Similar results were also reported in Denmark for the harvest years 1992 – 1999 [51]. In addition, inappropriate farm management practices were associated with higher OTA amounts [52].

No AA-related adducts was detected in these samples. Moreover, no DNA-adduct were either detected in the kidney tissues from women following slimming regimens indicating clearly that AAs could no be responsible of the disease. Dose and time dependant formation of DNA adducts induced by AA indicated that AA DNA-adducts are not persistent over 2 days in cell culture. Thus these DNA adducts are not more persistent than other DNA adduct. Table 7 summarizes features between Balkan endemic nephropathy

and AA nephropathy and highlights the implication of mycotoxins in BEN/UTT. All these plead for implication of OTA and CIT, in BEN and UTT, whereas AA derivatives are not implicated either in BEN or in slimming regimen.

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