



HAL
open science

Structures of Covalent Adducts between DNA and Ochratoxin A: A New Factor in Debate about Genotoxicity and Human Risk Assessment

Peter G. Mantle, Virginie Faucet-Marquis, Richard A. Manderville, Bianca Squillaci, Annie Pfohl-Leszkowicz

► **To cite this version:**

Peter G. Mantle, Virginie Faucet-Marquis, Richard A. Manderville, Bianca Squillaci, Annie Pfohl-Leszkowicz. Structures of Covalent Adducts between DNA and Ochratoxin A: A New Factor in Debate about Genotoxicity and Human Risk Assessment. *Chemical Research in Toxicology*, 2010, 23 (1), pp.89-98. 10.1021/TX900295a . hal-03474392

HAL Id: hal-03474392

<https://hal.science/hal-03474392>

Submitted on 10 Dec 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Open Archive Toulouse Archive Ouverte (OATAO)

OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible.

This is an author-deposited version published in: <http://oatao.univ-toulouse.fr/>
Eprints ID: 5719

To link to this article: DOI:10.1021/TX900295a
URL: <http://dx.doi.org/10.1021/TX900295a>

To cite this version: Mantle, Peter G. and Faucet-Marquis, Virginie and Manderville, Richard A. and Squillaci, Bianca and Pfohl-Leszkowicz, Annie (2010) Structures of Covalent Adducts between DNA and Ochratoxin A: A New Factor in Debate about Genotoxicity and Human Risk Assessment. *Chemical Research in Toxicology* pp. 89-98. ISSN 0893-228X

Any correspondence concerning this service should be sent to the repository administrator: staff-oatao@listes.diff.inp-toulouse.fr

Structures of Covalent Adducts between DNA and Ochratoxin A: A New Factor in Debate about Genotoxicity and Human Risk Assessment

Peter G. Mantle,^{*,†} Virginie Faucet-Marquis,[‡] Richard A. Manderville,[§] Bianca Squillaci,^{||} and Annie Pfohl-Leszkowicz[‡]

Centre for Environmental Policy, Imperial College London, London SW7 2AZ, U.K., Ecole Nationale Supérieure Agronomique, UMR CNRS/INPT/5503, Toulouse, France, Departments of Chemistry and Toxicology, University of Guelph, Guelph N1G 2W1, Canada, The Drug Metabolism and Pharmacokinetics Department, GlaxoSmithKline, Ware SG12 0DP, U.K.

The potent renal carcinogenicity of ochratoxin A (OTA) in rats, principally in the male, raises questions about mechanism. Chromatographic evidence of DNA adducts after ³²P-postlabeling analysis contrasts with experimental attempts to demonstrate the absence of OTA in such adducts. Proffered schemes for alternative epigenetic mechanisms in OTA carcinogenicity remain unsatisfying, while structural data substantiating DNA-OTA adducts has also been lacking. We report refined ³²P-postlabeling methodology revealing one principal adduct isolated in small amounts from the kidneys of all five Fischer and five Dark Agouti rats to which OTA had been given on four consecutive days. We also describe structural data for the principal adduct from OTA/DNA interaction *in vitro* and its subsequent preparative isolation by the postlabeling methodology (as C-C8 OTA 3'dGMP), essentially creating an ochratoxin B-guanine adduct. Reasoning for the unsuitability of experimental protocols in published evidence claiming nongenotoxicity of OTA is given. *In vivo* exposure of renal DNA to cycles of adduction with OTA, necessarily protracted for carcinogenesis to occur, can reasonably explain an occasional focal neoplasm from which metastasizing carcinoma could develop.

Introduction

Whether the nephrotoxic mycotoxin ochratoxin A (OTA) exerts its potent nephrocarcinogenicity in male rats by direct interaction with DNA has long been a matter of debate. First positive claims came from the application of ³²P-postlabeling methodology to DNA isolated from mice given OTA (1). Autoradiographic evidence of radiolabeled compounds separated after contact transfer on two-dimensional chromatograms was proposed as showing that OTA had made covalent bonds with nucleotides *in vivo*. Subsequently, postlabeling methodology has consistently detected adduct spots on chromatograms which were attributed to exposure of rats, mice, and pigs to OTA in laboratories in Strasbourg, Bordeaux, Lyon (IARC) and Toulouse. Little or no evidence for this conclusion was experienced in other laboratories (e.g., see ref 2), thus fueling the current reluctance to accept positive postlabeling evidence as an indicator of genotoxicity of OTA (3). Academic debate about whether or not OTA conforms to the current definition of a genotoxin might have rested as a minor unresolved esoteric controversy if it were not for concerns that experimental carcinogenicity in male rats and mice pointed to the possibility that OTA could be a human carcinogen (4–6). Clear demonstration that a chemical carcinogen is a genotoxin influences legislative attitude, whereby a 10-fold reduced human risk differential may be applied when assessing the extent of

exposure to a nongenotoxic carcinogen rather than to a carcinogen concerning which adducts of defined structure are known. Food and drinks industries with commercial interests in the significance of natural and largely unavoidable traces of OTA in some agricultural commodities, and a need to comply with statutory limits, will naturally prefer that OTA is shown not to be a genotoxin.

In recent years, several publications consistently claiming the nonexistence of direct OTA/DNA adducts have been noted (summarized in ref 7); concurrently, several other publications have claimed the opposite (summarized in refs 8 and 9). However, there has been little critical appraisal of the experimental material used in publications claiming that DNA/OTA covalent adducts do not occur. Meanwhile, we have addressed the difficult challenge of obtaining structural data relating to rat kidney DNA adducts. Demonstration of the structure of a principal DNA/OTA adduct, prepared *in vitro* from calf thymus DNA and isolated preparatively via postlabeling methodology, here extends proof of covalent binding of OTA on guanine beyond the quite compelling cochromatographic implication described in ref 10.

Materials and Methods

Rats and OTA. Ten 8-week old males of the Fischer and Dark Agouti strains (B & K Universal Ltd., Grimston, Hull, UK) were ~220 and 180 g, respectively, and were maintained on standard (14.4% protein) diet (SDS Services, Witham, Essex, UK).

Design of OTA dosing strategy was based on previous experience (11) of short-term administration with only mild clinical effects and best incidence of renal DNA adducts achieved through daily oral gavage. OTA (77 mg) was dissolved in ethanol (1.2 mL) and

* To whom correspondence should be addressed. E-mail: p.mantle@imperial.ac.uk.

[†] Imperial College London.

[‡] Ecole Nationale Supérieure Agronomique.

[§] University of Guelph.

^{||} GlaxoSmithKline.

the solution made up to 20 mL with 3% w/v NaHCO₃ in water. Then 0.4 mL (1.5 mg OTA) was given from this solution to each of seven rats, at the same time of day by oral gavage on three consecutive days. The daily dose was therefore ~8.3 and 6.8 mg/kg b.w. for Dark Agouti and Fischer rats, respectively. Controls were given 0.4 mL of vehicle (5% ethanol in 3% aqueous NaHCO₃). Twenty-four hours after the last dose, four treated rats from each strain and all controls were euthanized, and blood was withdrawn from the heart. The kidneys were excised, separated into quarters, flash frozen in liquid N₂, transferred into separate Eppendorf tubes, and stored at -20 °C. Blood was centrifuged immediately and plasma separated for quantitative OTA analysis at the Central Science Laboratory, York, UK. The mean weights of the remaining three OTA-treated Fischer and Dark Agouti rats were 310.7 ± 12.1 g and 261.7 ± 10 g, respectively, 5 weeks after the first OTA dose. After 3 months (370 ± 17.3 g and 278.3 ± 4.7 g, respectively), the rats were euthanized and the blood collected as before. A narrow central transverse section was removed from each kidney for H & E-stained histology and the remaining tissues flash frozen in liquid N₂ and stored at -20 °C pending ³²P-postlabeling analysis.

All kidney samples were coded for blind analysis for adducts, to avoid suggestion of any analytical bias, and transferred personally by air in dry ice between London and Toulouse.

DNA Extraction and Postlabeling Separation of DNA Adducts. Materials. 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 (Saint Quentin Fallavier, France); spleen phosphodiesterase (centrifuged before use) was from Calbiochem (VWR, France), and nuclease P1 (NP1) and T4 polynucleotide kinase were from Roche diagnostics (Meylan, France). [γ -³²P-ATP] (444 Tbq/mmol, 6000 Ci/mmol) was from Amersham (Les Ullis, France). Rotiphenol (phenol saturated with TRIS-HCl at 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 (ref 6130932) was from VWR (France), and PEI/cellulose TLC plates used for ³²P-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.

DNA Extraction. Tissues (500 mg) were homogenized in 0.8 mL of a solution containing NaCl (0.1 M), EDTA (20 mM), and Tris-HCl, pH 8 (50 mM) (SET) in an ice bath. To the homogenate, 200 μ L of a 10% solution of sodium dodecylsulfate was added, and following incubation for 10 min at 65 °C, 800 μ L of potassium acetate (6 M, pH 5) was added. The reaction mixture was kept at 0 °C for 30 min. After centrifugation for 25 min at 0 °C (10000g), the supernatant, which contained nucleic acids, was collected, and nucleic acids were precipitated overnight at -20 °C by adding 2 volumes of cold ethanol. The DNA pellets were collected and washed once with 1 mL of 90% ethanol and dissolved in 500 μ L of SET (15 min at 37 °C). The total extract was mixed with 10 μ L of a mixture of RNase A (20 mg/mL) and RNase T1 (10 000 U/ml) and incubated for 1 h at 37 °C; this treatment was repeated twice. Samples were then treated with 25 μ L of proteinase K solution (20 mg/mL SET) for 1 h at 37 °C. After digestion, 500 μ L of rotiphenol was added, and the mixture was moderately shaken for 20 min at room temperature and centrifuged for 15 min at 15 °C (10000g). The aqueous phase was collected after two extractions. After a final extraction with one volume of chloroform/isoamyl alcohol (24:1), the aqueous phase was collected, and 50 μ L of sodium acetate (3 M, pH 6) was added. The DNA was precipitated by the addition of two volumes of cold ethanol overnight at -20 °C, and the precipitate was collected by centrifugation at 10000g for 30 min. The DNA pellet was washed four times with 90% ethanol. DNA was dissolved in deionized water and tested for purity by recording UV spectra between 220 and 320 nm.

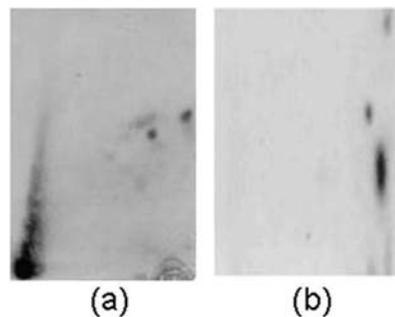


Figure 1. Comparison of the kidney DNA profile of an OTA-treated rat. (a) D1 2.3 M phosphate buffer, + transfer of 4 cm. (b) D1 3 M phosphate buffer, + transfer of 2 cm.

Postlabeling Method. The equivalent of 7 μ g of DNA was dried in vacuo, dissolved in 10 μ L of the mix containing 1 μ L of micrococcal nuclease (2 mg/mL corresponding to 500 U), spleen phosphodiesterase (15 mU/ μ g DNA), 1 μ L of sodium succinate (200 mM), and 1 μ L of calcium chloride (100 mM, pH 6) and digested at 37 °C for 4 h. The digested DNA was then treated with 5 μ L of the mix containing 1.5 μ L of nuclease P1 (4 mg/mL), 1.6 μ L of ZnCl₂ (1 mM), and 1.6 μ L of sodium acetate (0.5 M, pH 5) at 37 °C for 45 min. The reaction was stopped by the addition of 3 μ L of Tris base 500 mM. The DNA adducts were labeled as follows: to the nuclease P1 digest, 5 μ L of the reaction mixture containing 2 μ L of bicine buffer [bicine (800 μ M), dithiothreitol (400 mM), MgCl₂ (400 mM), and spermidine (400 mM) adjusted to pH 9.8 with NaOH], 10 U of polynucleotide kinase T4, and 100 μ Ci of [γ -³²P]ATP (specific activity 6000 Ci/mmol) was added and the mixture incubated at 37 °C for 45 min. Normal nucleotides, pyrophosphate, and excess ATP were removed by chromatography on PEI/cellulose TLC plates (D1) in 3 M NaH₂PO₄ buffer, pH 5.7, overnight. The origin (2 cm) areas containing labeled adducted nucleotides were cut out and transferred to another PEI/cellulose TLC plate, which was run (D2) in 4.8 M lithium formate and 7.7 M urea, pH 3.5, for 3 h. A further (D3) migration was performed after turning the plate 90° anticlockwise in 0.6 M NaH₂PO₄ and 5.95 M urea, pH 6.4, for 3 h. Finally, the chromatogram was washed in the same direction in 1.7 M NaH₂PO₄, pH 6, for 2 h (D4). Adduct profiles were analyzed qualitatively and semiquantitatively by autoradiography of the plates, carried out at -80 °C for 48 h in the presence of an intensifying screen, using a radio-analytical system of image analysis (AMBIS, Lablogis). In parallel to the adduct analysis, a series of controls were performed to verify the labeling efficiency of adducts and the efficiency of dephosphorylation of the nuclease P1. Refinement of adduct separation by use of a 3 M phosphate buffer, instead of the former 2.3 M buffer, in the D1 migration, and subsequent transfer of only 2 cm of the origin, is shown in Figure 1. Steps in the refined procedure to resolve two of our synthetic model adducts are illustrated in Figure 2.

Synthesis of OTA-dG and OTA-3'-dGMP. A sample of the OTA-dG nucleoside adduct was prepared from the photochemical reaction of OTA in the presence of excess dG, as outlined previously (12, 13). A reaction mixture (1 mL total volume) of 500 μ M OTA and 40 mol equiv of dG in a mixture of 25 vol. % DMSO and 75 vol. % 100 mM aqueous phosphate buffer (pH 7.4) was irradiated at 350 nm for 15 min using a Rayonet Chamber Reactor, Model RPR-200. The OTA-dG nucleoside adduct was isolated using an Agilent 1200 series HPLC equipped with an autosampler, auto-collector, and diode array detector, and a mobile phase consisting of 40 mM ammonium formate (pH 6.1)/acetonitrile and a semi-preparative Phenomenex Kromasil C8 column (10 mm × 250 mm) at a flow rate of 5 mL/min. The isolated sample of OTA-dG had MS and UV-vis characteristics identical to those previously reported (12, 13).

The protocol for the photochemical synthesis of OTA-3'-dGMP was the same as that outlined previously (10). A reaction mixture (1 mL total volume) of 100 mM OTA and 50 mol equiv of 3'-dGMP in 100 mM phosphate buffer (pH 7.4) was irradiated for 2

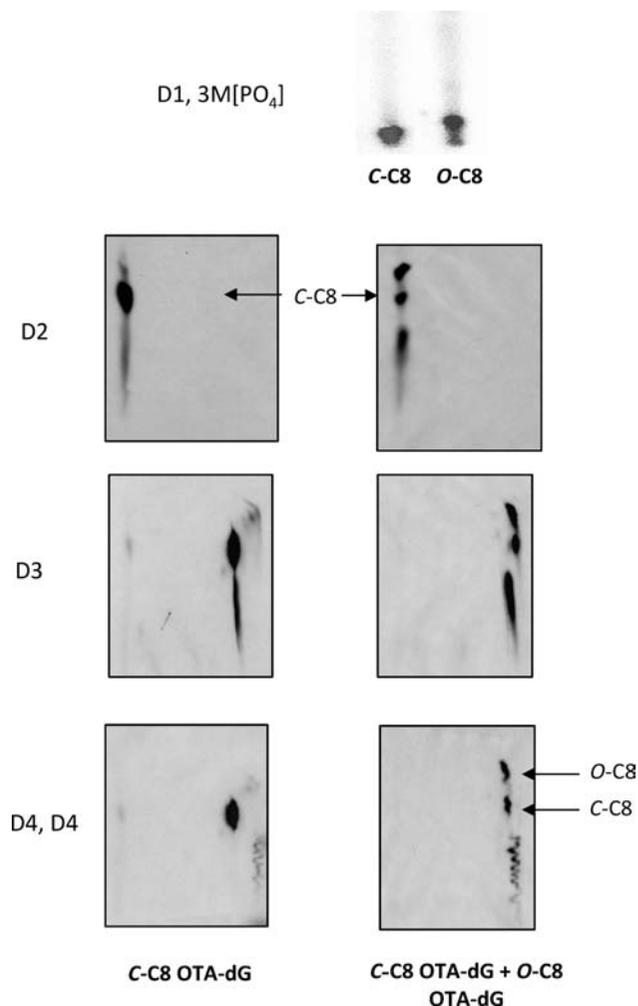


Figure 2. Autoradiographic illustration of steps in chromatographic resolution of two of our model synthetic adducts after ^{32}P -postlabeling, showing discrete separation resolved from stray radioactivity after a second D4 step. Transition from D1-D2 was by contact transfer of the radioactive region after the D1 step.

min at 350 nm. The isolated OTA-3'-dGMP adduct had MS and UV-vis characteristics identical to those reported previously (10) and was used as a postlabeling standard.

Photoreaction of OTA with DNA. A reaction mixture (1 mL total volume) of 500 μM OTA and 100 μg of calf thymus DNA (repurified) in a mixture of 10 vol. % DMSO and 90 vol. % 100 mM aqueous phosphate buffer (pH 7.0) was irradiated at 350 nm

for 13 min using a Rayonet Chamber Reactor, Model RPR-200. The sample was then divided into four 250 μL fractions, and 1 mL of 100% ethyl alcohol was added to each fraction in microcentrifuge tubes. After incubation at -20°C for 10 h, samples were centrifuged for 15 min at 12000 rpm and 6°C in an AccuSpin centrifuge. The DNA pellet was washed with 70% ethanol, then dried under vacuum and sent to the Pfohl-Leszkiwicz laboratory for ^{32}P -postlabeling analysis and preparative isolation of two prominent adducts.

Preparative Isolation of Adducts from the Photoreaction of OTA and DNA. The protocol for postlabeling was exactly the same as that for rat DNA, except that unlabeled ATP was used instead of that radiolabeled with ^{32}P . For postlabeling of 10 μg of DNA, 20 pmol of ATP was used. After postlabeling, a preparative chromatogram was prepared in which typically (Figure 3) were applied radioactive spots of our standard C-C8 adduct, products of the calf thymus DNA-OTA interaction postlabeled with ^{32}P ATP, our standard O-C8, and a mixture of C-C8 and O-C8 adducts. In the central region of the plate, a stripe of products of the calf thymus DNA-OTA interaction postlabeled only with unlabeled ATP was applied. Chromatography in the D1 direction was run with NaH_2PO_4 (2.3 M, pH 5.7) for 16 h. The plate was washed twice by soaking in ultrapure water, dried, and autoradiographed. Cellulose in the central area corresponding to spots of interest was scraped off, the cellulose suspended in methanol, shaken, centrifuged, the solution, and the extraction cycle repeated. Residual cellulose was then similarly extracted with triethanolamine. Combined extracts were evaporated to dryness in vacuo. The preparative eluates from spots 1 and 2 (Figure 3) were sent, blind as samples 1 and 2, respectively, for LC-MS analysis.

Chemicals for HPLC/MS Analysis of Adducts. OTA from *Aspergillus ochraceus* was purchased from Sigma-Aldrich Co. (Dorset, UK), formic acid was purchased from Perbio Science (Northumberland, UK), HPLC grade acetonitrile was purchased from Fisher Scientific (Loughborough, UK), and HPLC grade water was produced by an Elga Maxima Ultra Pure Water system (Buckinghamshire, UK). All solvents and chemical standards were of the highest grade commercially available. Authentic standards of C-C8 OTA 3'-dGMP (Figure 4) and O-C8 OTA 3'-dGMP (Figure 5), and OTA-DNA samples were synthesized in Guelph.

Buffers, Reagents, and Standards. A 10 $\mu\text{g}/\text{mL}$ MS standard of OTA in 50:50 acetonitrile/water was prepared by diluting a 10 μL of a 1 mg/mL solution of OTA in water with 990 μL of 50:50 acetonitrile/water. A 0.1% formic acid solution was prepared by diluting 1 μL of concentrated formic acid with 999 μL of water.

Method Development of MS Analysis of Model Synthetic Adducts in Preparation for the Analysis of Products of in Vitro OTA-DNA Interaction. Authentic synthetic standards of model OTA-guanine compounds, C-C8 OTA 3'-dGMP, and O-C8 OTA 3'-dGMP were used to develop a suitable HPLC/MSⁿ method.

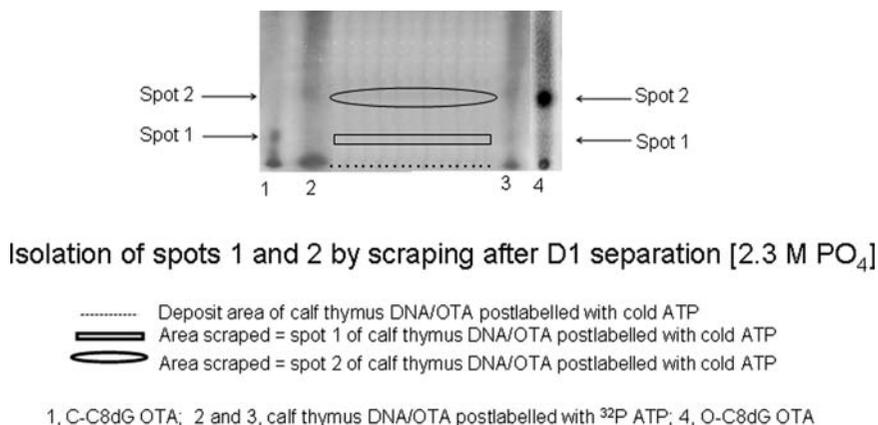


Figure 3. Annotated autoradiograph of a model chromatogram (D1 separation) illustrating the preparative isolation of samples 1 and 2 used for structural analysis. Lane 1 is synthetic C-C8dG-OTA processed via standard ^{32}P -postlabeling. Lanes 2 and 3 are products of calf thymus DNA/OTA photoreaction processed via standard ^{32}P -postlabeling to guide preparative excision of unlabeled compounds from the central region. Lane 4 is synthetic O-C8dG-OTA also processed via ^{32}P -postlabeling. It was necessary to avoid ^{32}P contamination of samples for LC-MS.

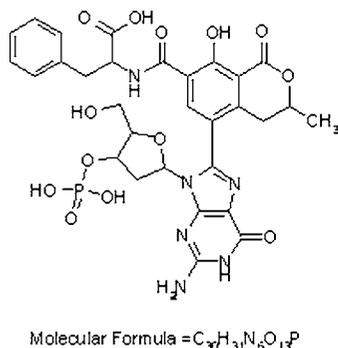


Figure 4. Structure of C-C8 OTA 3'-dGMP.

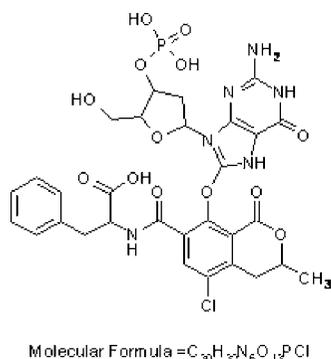


Figure 5. Structure of O-C8 OTA 3'-dGMP.

For all analytes, the instrument was tuned to give the best possible MS performance. HPLC/MSⁿ analysis was performed on an Agilent 1100 HPLC system (Santa Clara, USA) consisting of a binary pump and column thermostat, coupled to a Waters QToF Premier mass spectrometer (Manchester, UK) equipped with an electrospray ionization (ESI) source with separate Lockmass interface and operated in negative ion mode. Samples were introduced to the HPLC via a CTC-Analytics PAL autosampler (Zwingen, Switzerland) consisting of a 100 μ L loop. The autosampler, HPLC system, and mass spectrometer were controlled using Waters software MassLynx version 4.1 (Manchester, UK). Calibration over the range m/z 50–1000 was carried out using a NaI/CsI calibration standard purchased from Waters. MS tuning was performed using direct infusion of a 10 μ g/mL standard solution of OTA, at a flow rate of 10 μ L/min, using the integrated instrument syringe pump. The following mass spectrometer conditions were common to all LC/MS experiments performed: capillary voltage, 1.5 kV; sampling cone, 35 V; source temperature, 140 $^{\circ}$ C; desolvation temperature, 350 $^{\circ}$ C; cone gas flow, 50 L/h; desolvation gas flow, 400 L/h; and LM and HM resolution, 15 arbitrary units. A 1 μ g/mL solution of leucine enkephalin (m/z 554.2615) in 50:50 acetonitrile/water was pumped into the lockmass interface at a rate of 20 μ L/min using a dedicated HPLC pump (Shimadzu UK Limited, Manchester, UK). Lockmass data was collected with a scan time of 0.5 s at 10 s intervals and averaged over 10 scans. Mass spectral data was collected in MS centroid mode with a scan time of 1 s and interscan time of 0.02 s, over a mass range of m/z 100–1000.

All samples were pretreated with concentrated formic acid (50 μ L), vortex mixed, and heated on a heating block at 80 $^{\circ}$ C for 60 min. The samples were then reduced to dryness under N_2 gas, resuspended in 100 μ L of 0.1% formic acid (aqueous), and vortex mixed. Ten microliters of each sample was injected onto a C18 column (Thermo Hypersil Gold 5 μ m, 4.6 mm i.d. \times 250 mm, Thermo Fisher Scientific, Waltham, USA); 0.1% formic acid (solvent A) and acetonitrile (solvent B) were used as mobile phases. The samples were separated using a gradient elution between 5% and 95% B in 15 min. The column was kept at 95% B for 5 min and then reconditioned at 5% B for 10 min.

Results

Pharmacokinetics in Rats Treated with OTA. Plasma OTA concentration in the four Fischer rats treated with OTA was 45.7 ± 2.6 μ g/mL and in the Dark Agoutis was 36.8 ± 6.4 μ g/mL. At first sight, the lower value in the smaller rats given the same amount of OTA appears incongruous. Purely on a body weight basis, the plasma concentration in the Dark Agoutis might have been ~ 56 μ g/mL. Subsequent discovery that Dark Agoutis have much shorter plasma OTA half-life than that of Fischer rats (14) can account for the apparent disparity in accumulation in plasma after the three consecutive doses. However, substantial potential for delivery of OTA via vascular circulation to kidneys in both rat strains was demonstrated. Histology of kidneys from animals euthanized 3 months after giving OTA showed sparse karyomegaly diffused across the outer stripe of the outer medulla, consistent in our experience with a typical response in renal tubule epithelia to circulating OTA.

Results of Postlabeling Analysis. Autoradiographs relating to the kidney of each animal were copied electronically from Toulouse to London for matching to the blind code. No spots were present in samples from control (untreated) rats or from treated rats euthanized 3 months after the administration of OTA. This applied to all replicates of both strains of rats. In contrast, for the kidneys of all OTA-treated rats analyzed after the three consecutive doses, one or two radioactive spots were present in otherwise very clean autoradiographs of chromatograms, indicating the usual ^{32}P -postlabeling evidence of adducted DNA arising from consistent delivery of OTA to nephron epithelia (Figure 6). Notably, there was close replication of the strain-specific pattern of DNA adducts. In Fischer rats, there was predominantly one adduct, while in Dark Agoutis the same adduct was evident together with a minor component with slightly greater chromatographic mobility. The abundance of the principal adduct across the 10 animals was in the range of 20–70 adducts per 10^9 nucleotides.

MS Analysis. Preparative isolation of two adducts from *in vitro* interaction between OTA and DNA, occurring in approximately equal amounts, provided material for LC-MS analysis coded only as samples 1 and 2. An approximate limit of detection of 1 ng of material on column had been determined during method development.

No peaks were identified in sample 1 at mass m/z 713, the expected mass of the C-C8 OTA 3'-dGMP adduct. However, a prominent peak was identified in the m/z 517 extracted ion chromatogram at 11.7 min. The mass spectrum under the peak is shown in Figure 7. The base peak ion at m/z 517 corresponded to the loss of the phosphate conjugated deoxyribose sugar moiety from the C-C8 OTA 3'-dGMP, which is consistent with hydrolysis due to the addition of formic acid in the sample pretreatment step.

Extracted ion chromatograms of m/z 517 for samples 1 and 2, together with the analysis of the authentic standard of C-C8 OTA 3'-dGMP cochromatographed at 11.7 min, confirming the deconjugated aglycone moiety, were present in all three analyses (Figure 8).

The mass spectrum under the peak at 11.7 min in OTA-DNA sample 2 is shown in Figure 9.

The peak at m/z 517 corresponds to the loss of the phosphate conjugated deoxyribose sugar moiety from the C-C8 OTA 3'-dGMP as seen for OTA-DNA sample 1. However, additional adduct related ions are also observed in the spectrum. The ion m/z 655 corresponds to the sodium adduct of the C-C8 OTA 3'-dGMP which has lost the phosphate conjugate but still has

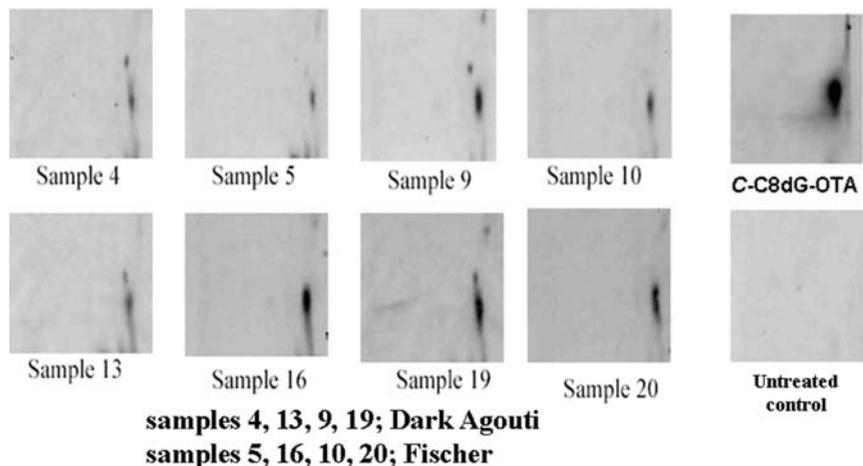


Figure 6. Representative autoradiographic displays from Dark Agouti and Fischer rats illustrating similar adduct patterns for each strain but consistent dominance of an adduct attributed to C-C8 OTA 3'-dGMP.

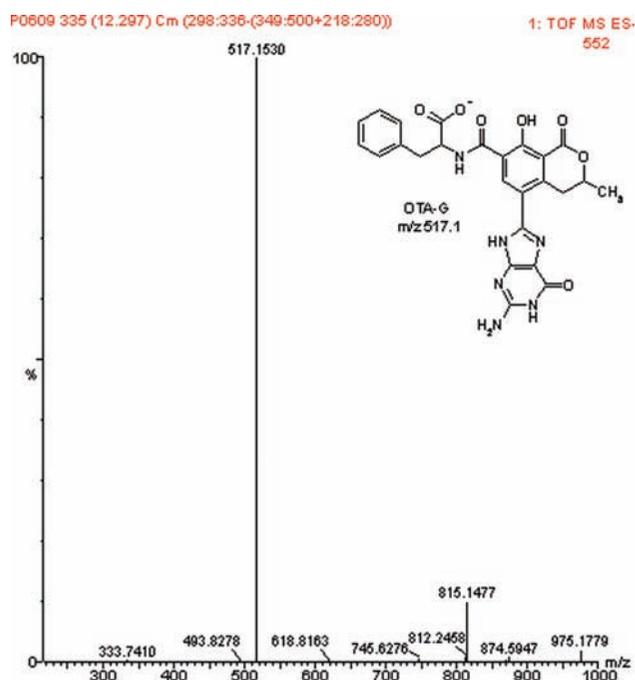


Figure 7. Background subtracted mass spectrum for OTA-DNA sample 1.

the deoxyribose sugar moiety intact (Figure 10a). The ion at m/z 815 represents the sodium adduct of the 3',5'-bisphosphate OTA-dGMP, showing additional phosphate conjugation on the 5' hydroxyl group of the deoxyribose sugar (Figure 10b).

The structure of the deconjugated aglycone observed in OTA-DNA sample 2 was confirmed by MS/MS analysis of the precursor ion m/z 517 (Figure 11). MS/MS data was generated on the Waters QT of Premier in continuum mode over the mass range m/z 100–600 at a collision energy of 25 eV, a scan time of 1 s, and an interscan time of 0.02 s.

The ions m/z 473 and m/z 429 represent the loss of the carboxylic acid group as CO_2 , with a further loss of CO_2 from the lactone moiety, thus confirming the presence of the OTA adduct in OTA-DNA sample 2. Matching of analytical findings to the blind sample code showed sample 1 to be C-C8 OTA 3'-dGMP and sample 2 to be its triphosphorylated homologue.

Discussion

The present findings have taken the search for definitive evidence on the structures of adducts, revealed consistently in

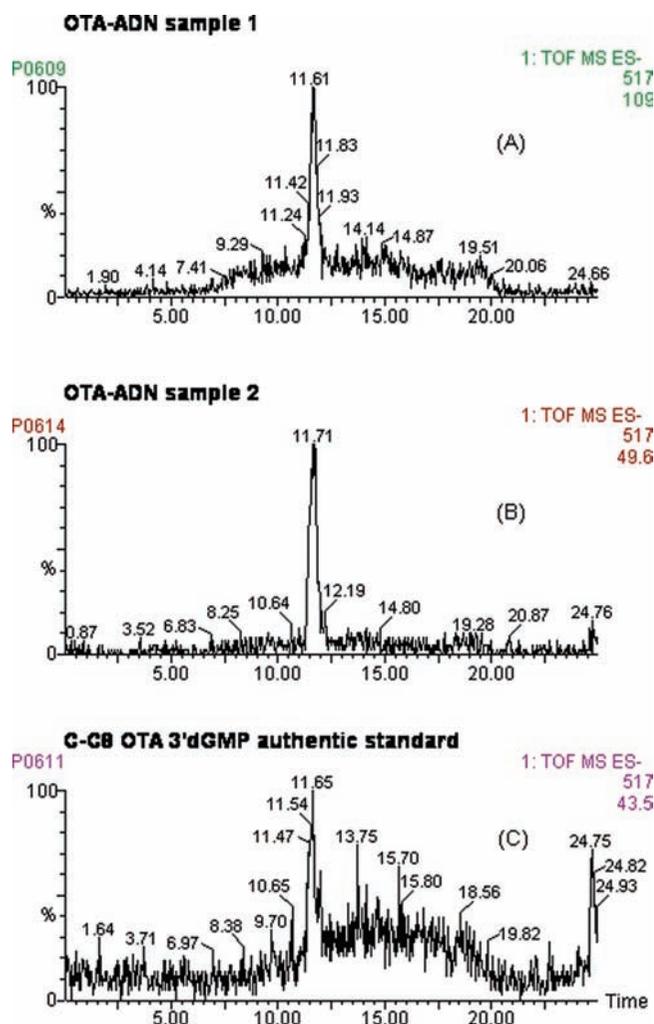


Figure 8. Extracted ion chromatograms of m/z 517 for (A) OTA-DNA sample 1, (B) OTA-DNA sample 2, and (C) the authentic standard of C-C8 OTA 3'-dGMP after pretreatment with formic acid. The data for the authentic standard is weak but clearly shows a peak at the correct retention time.

the Pfohl–Leszkowicz laboratory by ^{32}P -postlabeling in renal tissue after exposure to OTA, as far as possible with limited research resources. Limited resource of renal tissue precluded preparative isolation for LC-MS of sufficient amounts of either of the two key adducts revealed only by the 2D chromatography of ^{32}P -postlabeling after contact transfer. At least 1 ng would

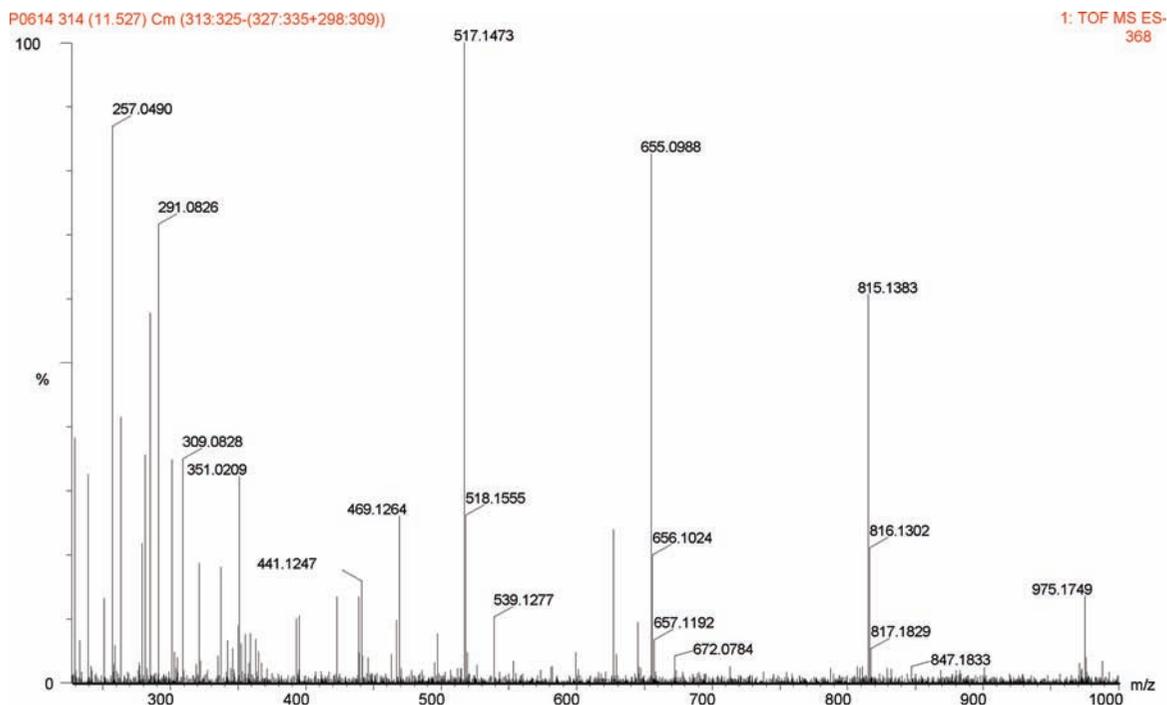


Figure 9. Background-subtracted mass spectrum for OTA-DNA of sample 2.

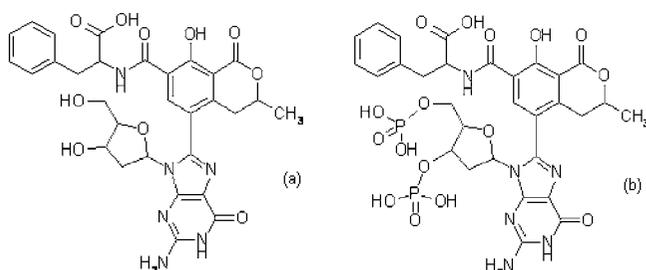


Figure 10. (a) Structure of desphosphate C-C8 OTA 3'dGMP (m/z 655) and (b) structure of 3',5'-bisphosphate OTA-dGMP (m/z 815).

be needed on the column and would require ~ 100 rats. The present finding confirms previous recognition of C-C8-dG-OTA as an *in vivo* DNA-OTA adduct by cochromatography in a 2D system after contact transfer with an authentic standard (10). We also show (Figure 12) similarly consistent chromatographic behavior through five sequential steps in two dimensions for C-C8-dG-OTA as a product of DNA-OTA interaction both *in vitro* and *in vivo*. However, research effort may better be focused on understanding the genetic changes in rat renal tumors caused by OTA and the mechanism for marked gender difference in sensitivity and pharmacokinetics (14, 15). Approximately 10^{18} molecules of OTA necessarily pass via a male rat's kidneys during several months to cause any renal cancer (16).

The experimental design described in ref 17 is presumably based on previous OTA toxicokinetics (18) in which a single dose (500 $\mu\text{g}/\text{kg}$ body weight) was given by oral gavage. For male rats in that study, assuming that the quoted rat weight (~ 250 g) is more reliable than the stated 8 week age (cf. Charles River Web site for Fischer rats), approximately 130 μg of OTA had been given per os in 500 μL of corn oil. Maximum concentration of OTA in blood, measured in plasma 48 h later, had reached ~ 1.8 $\mu\text{g}/\text{mL}$, corresponding to ~ 0.1 $\mu\text{g}/\text{g}$ measured in kidney from which blood had been flushed by syringing with physiological saline after excision. Thus, in a rat given 130 μg of OTA, less than 0.2% was actually found resident in its kidneys. If blood containing 1.8 μg OTA/mL of the plasma component circulates through the kidney in which blood

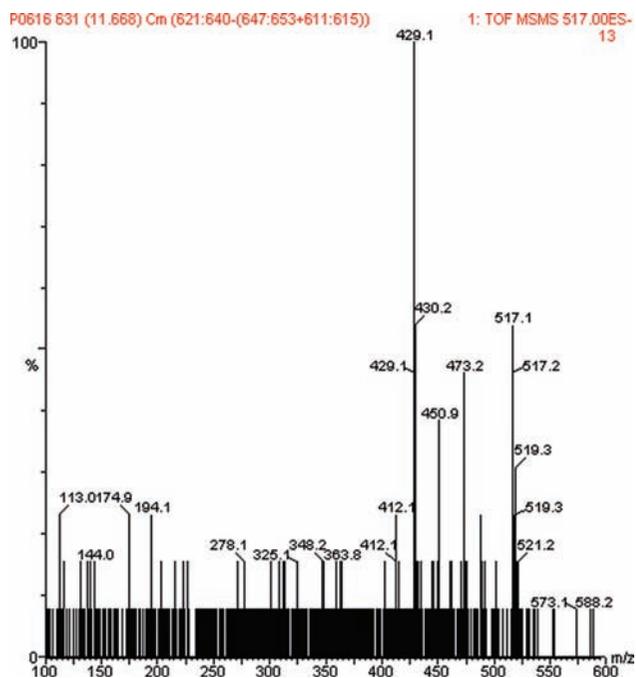


Figure 11. MS/MS spectrum (collision energy 25 eV) of m/z 517, showing CID fragmentation of the deconjugated aglycone of C-C8 OTA 3'dGMP. The spectrum shows the two principal fragment ions m/z 473 and m/z 429.

constitutes 20–25% of tissue (19), OTA concentration in the organ would be ~ 0.4 $\mu\text{g}/\text{g}$. Since removal of blood from the kidney can only be achieved efficiently, particularly from the peritubular capillaries, by perfusion *in situ* under normal blood pressure (19), the concentration of OTA in nonvascular tissue within the kidney would have been considerably less than the 0.2% of the given dose recorded in ref 18. The similar dosing of three adult male rats in ref 17, with assumed weight of ~ 250 g and each rat given ~ 130 μg ^{14}C -OTA (0.25 mCi/mmol), thus delivered ~ 0.08 μCi ^{14}C to each animal. This translates to $\sim 170,000$ dpm of ^{14}C , of which only about 50 dpm might have been intracellular in the 300–400 mg of kidney used for the

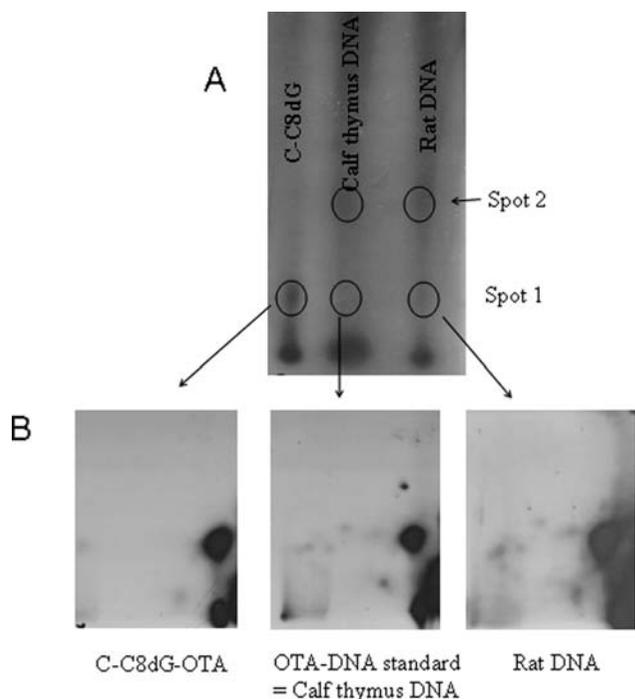


Figure 12. Autoradiographic comparison of chromatographic mobility after ^{32}P -postlabeling of synthetic C-8dG-OTA, the product of photoradiation of calf thymus DNA with OTA, and kidney DNA of a Dark Agouti rat given the oral OTA regimen. (A) D1 separation, showing sources of contact transfer of spot 1 to new plates for subsequent D2, D3, and D4 steps and (B) outcome of the completed protocol showing the mobility of the standard adduct similar to that of a principal radioactive component after both in vitro and in vivo interaction with OTA.

extraction of DNA for ACMS analysis. Unfortunately, there was no measurement of ^{14}C content of kidney of the dosed animals. Even with very efficient extraction and isolation (which seems not to have been the case), the total content of radioactivity of the DNA above background, about one ^{14}C atom to disintegrate per second, could hardly have significantly changed its natural abundance even if all potentially disintegrating carbon atoms had been involved in DNA/OTA adduct(s). This simple calculation should have been possible from the authors' own data. It is also unfortunate that no preliminary studies to optimize the delivery of ^{14}C -OTA to kidney had been performed (17), considering the costly resource investment made elsewhere to produce the ^{14}C -OTA. Optimum concentration of OTA circulating in blood may only be achieved after several weeks of continuous daily intake of the amount of ^{14}C -OTA given in ref 17, as indicated by in vivo rat data (14, 16, 20) and corroborated by predictive pharmacokinetic modeling (15). All these factors compound the unsatisfactory design of the radiolabeled study, which, together with failure to have the planned positive control evidence of successful ^{32}P -postlabeling of parallel material in the Pfohl-Leszkowicz laboratory, fails to justify the claim of nongenotoxicity. The claim was also premature because substitution, of commercial ^{14}C phenylalanine for the corresponding unlabeled moiety in the ^{14}C -OTA prepared biosynthetically (21), by well-established methodology (22–24) was not done.

Experiments described in ref 25 showed integrated findings on concentration of OTA in blood and ochratoxin α (OTalpha) in urine over a 2-week period of 5-day-a-week gavage dosing in vegetable oil. This information provides insight into pharmacokinetics applicable to the NTP study and the renal tumors produced. Clearly, with increased dose, blood OTA concentration increased progressively irrespective of the two nondosing days in the middle and end of the 2-week experimental period.

This is consistent with strong binding of OTA to serum albumin and a plasma half-life of about 10 days in the Fischer rat (14). However, the pattern of OTalpha excretion is striking and informative, although no discussion of this finding was made in ref 25. OTalpha in urine indicates metabolism of OTA in nephron epithelia and, at higher OTA doses, this activity is prominent. However, OTalpha excretion falls quickly nearly to zero during the two-day nondosing period, but exactly the same pattern is repeated during the second week. Thus, repeated pulses of OTA dosed by oral gavage are rather quickly subject to metabolism in nephron epithelia. Therefore, waiting for 3 days after a single OTA dose in ref 17 can be seen as counterproductive in an experiment designed to find evidence of DNA/OTA adducts in whole kidney.

The general lack of DNA adduct detection in several publications (2, 17, 25, 26) could be explained by several drawbacks in the methodologies used. Indeed, the experimental conditions and the methodologies used in these papers are not optimal and did not reproduce those described by the others. Notably, there is no quality verification of the DNA used for ACMS analysis or ^{32}P -postlabeling in the key publication (17) used to underpin much of the current claims that OTA is not a genotoxin. For some in vivo experiments, a low dose (0.2–1 mg/kg b.w.) was used for a short exposure time, 24 h; and for ACMS detection, the conditions were 0.5 mg/kg b.w. for 72 h. It has long been stated clearly that most of the adducts at a low (0.6 mg/kg b.w.) dose or a medium (1.2 mg/kg b.w.) dose become undetectable by 72 h (1, 27). Thus, the conditions were not optimal for adduct recognition. Moreover, the ACMS measurement had been performed without piloting authentic samples for comparison, and they used a Turbo Ionspray source and heated the samples to 400 °C. Most of the DNA adducts and OTA-GSH will probably decompose at such high temperatures. For generation of OTA-DNA adduct standards, the photoreaction was carried out with OTA at 500 mM and dG at 20 mM, conditions that are at an exceedingly high concentration and do not match the conditions reported in ref 12 for successful isolation and characterization of the adduct standards. Also, it appears that no purification of the photoreaction was carried out and, at best, the solution would have been a mixture of C-C8 and O-C8 adducts.

Conditions for optimizing DNA extraction are not correct in ref 17. Mainly for the purification, they used a Qiagen column, a technique shown in three interlaboratory studies on postlabeling to induce a loss of DNA adduct (EU project 5 V0448 Comparison and validation of ^{32}P -postlabeling method; EU project BEQALM; EU project OTA Risk assessment QKL1-2001-0614). The purity of the DNA was also not good enough, probably being contaminated by RNA and protein, which would have decreased postlabeling efficiency (28). Figure 13 shows comparison of the UV spectrum of DNA prepared in the Wurzburg laboratory contemporaneously with the studies of ref 17 and markedly contaminated with RNA with that from which the adduct data in Figure 6 was obtained. Other spectra showed contamination with protein. The chromatographic conditions of separation were also not adequate for OTA-DNA adduction. The chromatographic pH conditions for ^{32}P -postlabeling analysis, with D1 at pH 6.8 and D3 at pH 3.5, do not conform to standard published procedures, where D1 is run at a pH value below 6.0, and D3 is run at pH \sim 6.4 (10). Indeed, in ref 17, the separation conditions described were those for polycyclic aromatic hydrocarbons (PAH). PAH conditions were too strong for OTA and, even for the aristolochic acid positive control, the pattern was not optimal. The authors speculate that the DNA

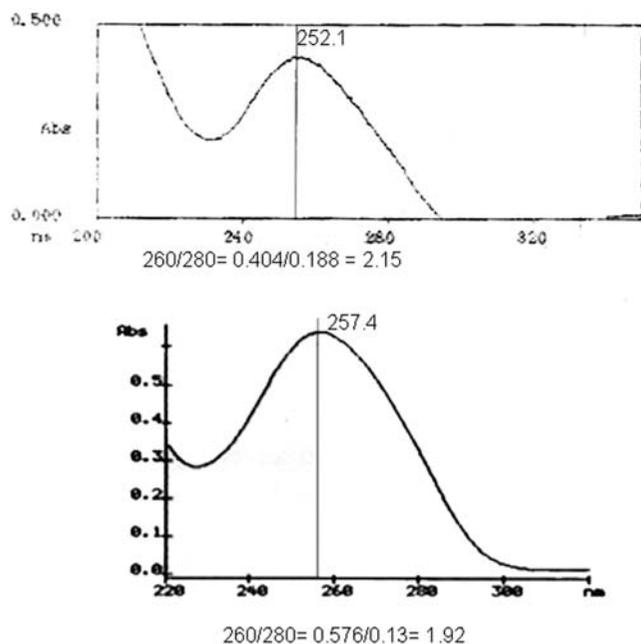


Figure 13. Comparison of UV-spectra of rat kidney DNA isolated by EU partners in Wurzburg (above) and Toulouse (below) as part of a 2003 interlaboratory study, as illustrated in attachments 11 and 7, respectively, submitted to the EU project coordinator for the official report but not included. Spectra are matched here for x-axis scale. Considering the DNA quality criterion that UVmax should not be <257 nm and that the UV absorbance ratio (260:280 nm) should be 1.8–2.0, all Wurzburg samples were contaminated by RNA; the spectrum illustrated here represents nearly pure RNA (UVmax 252; 260:280 nm absorbance ratio 2.2).

adduct observed is due to the cytotoxicity of oxygen reactive species leading for example to 8-oxoguanine. This modification can, however, not be detected with the postlabeling method using nuclease P1 enrichment, as it detects only bulky lipophilic adducts and not hydroxylated hydrophilic nucleotides. In the same way, etheno bases cannot be detected by this technique (29). Recently, we explained how an oxidative pathway leads to the covalent OTA-DNA adduct via biotransformation of OTA into the quinone derivative OTHQ (10, 30, 31).

If histopathological change is a general indicator of renal cells that are significant active or passive sites of OTA uptake and may be sites of persistent genetic change necessary for tumorigenesis, the outer stripe of the outer medulla is ideally the preferred tissue for finding adducts because prominent aneuploid karyomegaly in response to some mycotoxins is generally located in and around this region (4, 32–34). Even this region is not absolutely defined; packing of nephron P3 segments does not rigidly conform to a sharp line of demarcation in renal tissue, particularly concerning those from peripheral glomeruli. Recognition of precise anatomical transition in segment structure and function from P2 to P3 in a single nephron cannot easily be made in histology sections. There is no doubt that OTA can cause genetic damage both in vitro and in vivo. Demonstration of this (25) in the kidney of rats dosed for a short period has been complemented by data from cells in tissue culture (35); similar demonstration was also made (Mosesso, unpublished data) in kidneys at the 12-month stage of the lifetime experiment described in ref 36.

More recent analysis by isotopic dilution to determine whether OTA forms DNA adducts in vivo has also concluded that it does not (37). However, as with the findings in ref 17, data from sophisticated spectrometric methodology can only be as reliable as is the quality of the material analyzed. The use of

kidney from other protocols (25, 37), not specifically designed to optimize the recognition of very small amounts of adducted nucleotide, is disappointing. Kidneys from rats given the higher OTA dose (25) were obtained 3 days after the last OTA dose and from animals with marked polyuria, which indicated some renal dysfunction. It is unreasonable to assume efficient delivery of OTA to proximal tubule epithelia if there is overt evidence of renal dysfunction. We calculate that circulating OTA concentration in blood plasma can account for all measured OTA in kidneys so that no renal parenchyma accumulation of OTA could be perceived.

Material sourced from a 90-day study (38) for the study of ref 37 had plasma OTA concentration for the highest dose group, designed to mimic that of the NTP study (4), only at a mean value of 3 $\mu\text{g}/\text{mL}$ (with rather large standard deviation). In any case, such animals would probably not have had a sufficient period of OTA exposure for renal tumorigenesis to have been put in place (16), and there was no evidence that plasma OTA concentration had reached a steady state. This is an important consideration since covalent binding of OTA to DNA could relate only rather specifically to renal tumorigenicity. Adducts can occur without implication that they constitute an actual carcinogenic risk if the toxin insult is below threshold (39). DNA adducts were recognized by ^{32}P -postlabeling in our studies on kidneys of rats with plasma OTA concentration stabilized around 8 $\mu\text{g}/\text{mL}$ (19), which was typical of that associated with long-term renal tumorigenesis in male Fischer rats (36). The rats were at the 12 month stage from a large cohort continuing for life on dietary OTA and causing renal cancer (36), but at that time, it was not realized that further exposure to OTA might be unnecessary for tumorigenesis because no one had yet explored latency for this toxin. This emphasizes that sensitive ^{32}P -postlabeling analysis should have been applied to the kidney tissues selected for the study of ref 37 to give assurance that spectrometric methodologies could be used with confidence to assess whether adducts were present. Otherwise, acquisition of negative evidence from protocols that have not been challenged concerning their fitness for purpose is an extremely weak basis for constructing a putative mechanism in carcinogenesis.

It has recently been pointed out that the work presented in ref 37 was flawed because the limit of detection (LOD) (10 fmol dG-OTA, corresponding to 3.5 adducts per 10^9 nucleotides) was obtained from DNA hydrolysates (500 μg DNA equivalent) and not from the actual rat kidney sample for which the negative results were presented (40). This argument stemmed from the fact that a high amount of sample spiking (1250 fmol) with an isotopically labeled dG-OTA adduct of 96–98% isotopic purity was used, and the nonisotopically labeled dG-OTA adduct impurity was observed in the DNA hydrolysates but not in the rat kidney sample. Clearly, the LOD for the DNA hydrolysates versus the rat kidney sample were not the same. DNA purification from the rat tissue sample is more complicated, requiring extra steps, and interfering species could dramatically change the LOD. It was also pointed out that in the rat kidney sample a peak for the dG-OTA adduct following 90 days of incubation was actually observed in the ref 37 study using the m/z 633 \rightarrow 429 fragmentation transition in MS/MS detection. This apparent peak was not attributed to the dG-OTA adduct because an adduct peak was not observed using the transition m/z 633 \rightarrow 517. In response to the criticism, the authors argued (41) that their method of quantification was sound, but they avoided discussing whether they were justified in their assumption that the LOD from the DNA hydrolysates could serve as an accurate LOD for the rat kidney sample. They also point

out that the absence of a signal at transition m/z 633 \rightarrow 517 is a strong argument against the presence of dG-OTA in the sample, even though an adduct peak was observed using the m/z 633 \rightarrow 429 transition. However, the m/z 633 \rightarrow 429 transition is more intense than the m/z 633 \rightarrow 517 (20% greater), and here a clear adduct peak above background was observed that comigrates with the adduct standard. Given that the m/z 633 \rightarrow 429 transition provides a more intense adduct signal and showed positive evidence for dG-OTA in rat kidneys (for OTA-treated rat at a dose of 210 $\mu\text{g}/\text{kg}$ b.w. for 90 days), we can argue that the findings from the ref 37 study are in agreement with our positive evidence for dG-OTA presented here.

However, a genotoxicity mechanism operating in animals in which renal tumors will form and in which genetic damage has been demonstrated will evoke a relatively simple concept. The concept turns on a reasonable probability of occasional persistent genetic change related to tumorigenesis residing in perhaps just one nucleus in the \sim 30,000 highly differentiated nephrons (42) in just one of a rat's kidneys. Such could certainly be expected after a lifetime of continual bombardment of rat tubular epithelial cells with OTA molecules, as in the principal lifetime carcinogenicity studies to date (4, 6, 36). It is not unreasonable that other recent studies have shown that lifetime exposure is not mandatory (16) but that less than half a lifetime or even as little as 6 months of continuous toxic insult during the first half of an \sim 2-year rat lifespan would also be sufficient to determine that renal tumorigenesis could arise in some individuals. Experimental OTA tumorigenesis seems to be highly focal in rat kidneys, according to observations on about 50 tumorous kidneys throughout recent studies in London. Bilateral OTA tumorigenesis seems, in our experience in the rat, to be dose-related, and this would be consistent with the statistical probability of causing one focus of the necessary genetic damage in each kidney of an animal.

All experimental dose-response data for OTA's renal carcinogenesis, combined and plotted in the format of ref 43, makes a compelling case for OTA being a thresholded carcinogen in the male rat (16). There should be no philosophical conflict that the mycotoxin can also make covalent adducts with DNA and thereby, after many months of exposure to toxin molecules passing through renal epithelium, occasionally cause the particular combination of persistent DNA change that programs an occasional renal cell toward tumorigenesis, discovered late in life. Recently, it has been demonstrated (44) that genotoxic thresholds can exist for some genotoxins. Thus, determining the lowest dose that does not increase background mutation rate is essential and represents an important new aspect of genotoxicology. We recently showed that continuous treatment of Dark Agouti rats with feed contaminated with 50 ng OTA/kg for 4 weeks led to the formation of DNA adduct (20, 28); this dosage is 8-fold less than that having no adverse clinical effect during lifetime exposure (16).

The current findings relate solely to defining the fundamental genotoxicity of OTA as revealed by ^{32}P -postlabeling and the structure determination of the principal adduct to demonstrate direct covalent binding of OTA to DNA. Further understanding of the precise mutations involved in rat renal tumorigenesis in response to OTA will assist evidence-based application of animal model data to human risk assessment.

Acknowledgment. Part of this work was funded by the 5th RTD Framework Programme of the European Union, Project QLK1-2001-01614. R.A.M. thanks the Natural Sciences and Engineering Research Council of Canada for funding. A.P.-L.

thanks Region Midi-Pyrénées 'food safety program', Ligue Nationale Française Contre le Cancer (2007) and Association pour la Recherche Contre le Cancer (2005; 2008) for funding. We thank Mariana Tozlovanu and Maud Juzio for skilled technical assistance in Toulouse and Guelph, respectively, and the Drug Metabolism and Pharmacokinetics Department, Glaxo-SmithKline for the use of their analytical facilities.

References

- (1) Pfohl-Leschkowicz, A., Chakor, K., Creppy, E. E., Dirheimer, G. (1991) DNA-Adduct Formation, in Mice Treated with Ochratoxin A, in *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Castegnaro, M., Plestina, R., Dirheimer, G., Chernozemsky, I. N., and Bartsch, H., Eds.) IARC Scientific Publications No 115, pp 245–253, IARC Scientific Publications, Lyon, France.
- (2) Gautier, J.-C., Richoz, J., Welti, D. H., Markovic, J., Gremaud, E., Guengerich, F. P., and Turesky, R.J. (2001) Metabolism of ochratoxin A: Absence of formation of genotoxic derivatives by human and rat enzymes. *Chem. Res. Toxicol.* 14, 34–45.
- (3) EFSA. (2006) Opinion of the scientific panel on contaminants in the food chain on a request from the Commission related to ochratoxin A in food. *EFSA J.* 365, 1–56. www.efsa.eu.int.
- (4) Boorman, G. A. (1989) Toxicology and Carcinogenesis Studies of Ochratoxin A (CAS No. 303-47-9) in F344/N Rats (Gavage Studies), 1989 National Toxicology Program, Technical Report 358.
- (5) Bendele, A. M., Carlton, W. W., Krogh, P., and Lillehoj, E. B. (1985) Ochratoxin A carcinogenesis in the (C57BL/6J x C3H)F1 mouse. *J. Natl. Cancer Inst.* 75, 733–742.
- (6) Castegnaro, M., Mohr, U., Pfohl-Leschkowicz, A., Esteve, J., Steinmann, J., Tillmann, T., Michelon, J., and Bartsch, H. (1998) Sex- and strain-specific induction of renal tumours by ochratoxin A in rats correlates with DNA adduction. *Int. J. Cancer* 77, 70–75.
- (7) Turesky, R. J. (2005) Perspective: Ochratoxin A is not a genotoxic carcinogen. *Chem. Res. Toxicol.* 18, 1082–1090.
- (8) Manderville, R. A. (2005) A case for the genotoxicity of ochratoxin A by bioactivation and covalent DNA adduction. *Chem. Res. Toxicol.* 18, 1091–1097.
- (9) Pfohl-Leschkowicz, A., and Manderville, R. A. (2007) Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Mol. Nutr. Food Res.* 51, 61–99.
- (10) Faucet, V., Pfohl-Leschkowicz, A., Dai, J., Castegnaro, M., and Manderville, R. A. (2004) Evidence for covalent DNA adduction by ochratoxin A following chronic exposure to rat and subacute exposure to pig. *Chem. Res. Toxicol.* 17, 1289–1296.
- (11) Miljkovic, A., Pfohl-Leschkowicz, A., Dobrota, M., and Mantle, P. G. (2003) Comparative responses to mode of oral administration and dose of ochratoxin A or nephrotoxic extract of *Penicillium polonicum* in rats. *Exp. Toxicol. Pathol.* 54, 305–312.
- (12) Dai, J., Wright, M. W., and Manderville, R. A. (2003) Ochratoxin A forms a carbon-bonded C8-deoxyguanosine nucleoside adduct: Implications for C8 reactivity by a phenolic radical. *J. Am. Chem. Soc.* 125, 3716–3717.
- (13) Frenette, C., Paugh, R. J., Tozlovanu, M., Juzio, M., Pfohl-Leschkowicz, A., and Manderville, R. A. (2008) Structure-activity relationships for the fluorescence of ochratoxin A: Insight for detection of ochratoxin A metabolites. *Anal. Chim. Acta* 617, 153–161.
- (14) Mantle, P. G. (2008) Interpretation of the pharmacokinetics of ochratoxin A in blood plasma of rats, during and after acute or chronic ingestion. *Food Chem. Toxicol.* 46, 1808–1816.
- (15) Vettorazzi, A., Gonzales-Penas, E., Troconiz, I. F., Arbillaga, L., Corcuera, L. A., Gil, A. G., and Lopez de Cerain, A. (2009) A different kinetic profile of ochratoxin A in mature male rats. *Food Chem. Toxicol.* 47, 1921–1927.
- (16) Mantle, P. G. (2009) Minimum tolerable exposure period and maximum threshold dietary intake of ochratoxin A for causing renal cancer in Dark Agouti rats. *Food Chem. Toxicol.* 47, 2419–2424.
- (17) Mally, A., Zepnik, H., Wanek, P., Eder, E., Dingley, K., Ihmels, H., Volkel, W., and Dekant, W. (2004) Ochratoxin A: Lack of formation of covalent DNA adducts. *Chem. Res. Toxicol.* 17, 234–242.
- (18) Zepnik, H., Volkel, W., and Dekant, W. (2003) Toxicokinetics of the mycotoxin ochratoxin A in F344 rats after oral administration. *Toxicol. Appl. Pharmacol.* 192, 36–44.
- (19) Garcia-Sanz, A., Rodriguez-Barnero, A., Bentley, M. D., Ritman, E. L., and Romero, J. C. (1998) Three-dimensional microcomputed tomography of renal vasculature in rats. *Hypertension* 31, 440–444.
- (20) Castegnaro, M., Canadas, D., Vrabcheva, T., Petkova-Bocharova, T., Chernozemsky, I. N., and Pfohl-Leschkowicz, A. (2006) Balkan endemic nephropathy: role of ochratoxin A through biomarkers. *Mol. Nutr. Food Res.* 50, 519–529.

- (21) Nestler, S. (2008) Experimental Renal Ochratoxicosis: A Molecular and Pathological Study, Ph.D. Thesis, University of London.
- (22) Hult, K. (1986) Ochratoxin A labelled with ^{14}C or ^3H in the phenylalanine moiety. *J. Labelled Comp. Rad.* 23, 801–805.
- (23) Breitholz-Emanuelsson, A., Fuchs, R., Hult, K., and Appelgren, L.-E. (1991) Distribution of ^{14}C -ochratoxin A and ^{14}C -ochratoxin B in Rats: A Comparison Based on Whole Body Autoradiography, in *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Castegnaro, M., Plestina, R., Dirheimer, G., Chernozemsky, I. N., and Bartsch, H., Eds.) pp 201–203, International Agency for Research on Cancer, Lyon, France.
- (24) Breitholz-Emanuelsson, A., Fuchs, R., Hult, K., and Appelgren, L. E. (1992) Synthesis of ^{14}C -ochratoxin A and ^{14}C -ochratoxin B and a comparative study of their distribution in rats using whole body radiography. *Pharmacol. Toxicol.* 70, 255–261.
- (25) Mally, A., Pepe, G., Ravoori, S., Fiore, M., Gupta, R., Dekant, W., and Mosesso, P. (2005) Ochratoxin A causes DNA-damage and cytogenetic effects, but no DNA-adducts in rats. *Chem. Res. Toxicol.* 18, 1253–1261.
- (26) Schlatter, C., Studer-Rohr, J., and Rasonyi, T. (1996) Carcinogenicity and kinetic aspects of ochratoxin A. *Food Addit. Contam.* 13, 43–44.
- (27) Pfohl-Leszkowicz, A., Grosse, Y., Kane, A., Creppy, E. E., and Dirheimer, G. (1993) Differential DNA adduct formation and disappearance in three mice tissues after treatment by the mycotoxin, ochratoxin A. *Mutat. Res.* 289, 265–273.
- (28) Pfohl-Leszkowicz, A., and Castegnaro, M. (2005) Further arguments in favour of direct covalent binding of Ochratoxin A (OTA) after metabolic biotransformation. *Food Addit. Contam., Suppl. 1*, 75–87.
- (29) Bartsch, H., and Nair, J. (2000) Ultrasensitive and specific detection methods for exocyclic DNA adducts: markers for lipid peroxidation and oxidative stress. *Toxicology* 153, 105–114.
- (30) Manderville, R. A., and Pfohl-Leszkowicz, A. (2008) Bioactivation and DNA adduction as a rationale for ochratoxin A carcinogenesis. *World Mycotoxin J.* 1, 357–367.
- (31) Tozlovanu, M., Faucet-Marquis, V., Pfohl-Leszkowicz, A., and Manderville, R. A. (2006) Genotoxicity of the hydroquinone metabolite of ochratoxin A: Structure-activity relationships for covalent DNA adduction. *Chem. Res. Toxicol.* 19, 1241–1247.
- (32) Mantle, P. G., McHugh, K. M., Adatia, R., Gray, T., and Turner, D. R. (1991) Persistent karyomegaly caused by *Penicillium* nephrotoxins in the rat. *Proc. R. Soc. London, Ser. B* 246, 251–259.
- (33) Turner, D. R., Mantle, P. G., McHugh, K. M., Adatia, R., and Gray, T. (1993) The Effect of *Penicillium* Moulds on the Kidney and Their Relationship with Balkan Nephropathy, Abstract: Proceedings, 14th European Pathology Congress, Innsbruck.
- (34) Brown, A. L., Odell, E. W., and Mantle, P. G. (2007) DNA ploidy distribution in renal tumours induced in male rats by dietary ochratoxin A. *Exp. Toxicol. Pathol.* 59, 85–95.
- (35) Mosesso, P., Cinelli, S., Pinero, J., Bellacima, R., and Pepe, G. (2008) In vitro cytogenetic results supporting a DNA nonreactive mechanism for ochratoxin A, potentially relevant for its carcinogenicity. *Chem. Res. Toxicol.* 21, 1235–1243.
- (36) Mantle, P., Kulinskaya, E., and Nestler, S. (2005) Renal tumourigenesis in male rats in response to chronic dietary ochratoxin A. *Food Addit. Contam., Suppl. 1*, 58–64.
- (37) Delatour, T., Mally, A., Richoz, J., Ozden, S., Dekant, W., Ihmels, H., Otto, D., Gasparutto, D., Marin-Kuan, M., Schilter, B., and Cavin, C. (2008) Absence of 2'-deoxyguanosine-carbon 8-bound ochratoxin A adduct in rat kidney DNA monitored by isotope dilution LC-MS/MS. *Mol. Nutr. Food Res.* 52, 472–482.
- (38) Rached, E., Hard, G. C., Blumbach, K., Weber, K., Draheim, R., Lutz, W. K., Ozden, S., Steger, U., Dekant, W., and Mally, A. (2007) Ochratoxin A: 13-week oral toxicity and cell proliferation in male F344/N rats. *Toxicol. Sci.* 97, 288–298.
- (39) Waddell, W. J., Fukushima, S., and Williams, G. M. (2006) Concordance of thresholds for carcinogenicity of *N*-nitrosodiethylamine. *Arch. Toxicol.* 80, 305–309.
- (40) Pfohl-Leszkowicz, A., Gabryelski, W., and Manderville, R. A. (2009) Formation of 2'-deoxyguanosine-carbon 8-bound ochratoxin A adduct in kidney DNA. *Mol. Nutr. Food Res.* 53, 154–155.
- (41) Delatour, T., Mally, A., Dekant, W., Marin-Kuan, M., Schilter, B., and Cavin, C. (2009) Answer to Prof. Pfohl-Leszkowicz's letter to the editor. *Mol. Nutr. Food Res.* 53, 156–157.
- (42) Solomon, S. (1977) Developmental changes in nephron number, proximal tubular length and superficial nephron glomerular filtration rate of rats. *J. Physiol.* 272, 573–589.
- (43) Waddell, W.J. (2006) Critique of dose response in carcinogenesis. *Hum. Exp. Toxicol.* 25, 413–436.
- (44) Johnson, G. E., Doak, S. H., Griffiths, S. M., Quick, E. M., Skibinski, D. O. F., Zair, Z. M., and Jenkins, G. J. (2009) Non-linear dose-response of DNA reactive genotoxins: recommendations for data analysis. *Mutat. Res.* 678, 95–100.

TX900295A