Molecular Aspects of the Transport and Toxicity of Ochratoxin A

JIAN DAI,[†] GYUNGSE PARK,[†] JENNIFER L. PERRY,[‡,#] YURI V. IL'ICHEV,[‡,⊥] DANIEL A. J. BOW,[§] JOHN B. PRITCHARD,[§] VIRGINIE FAUCET,[∥] ANNIE PFOHL-LESZKOWICZ,[∥] RICHARD A. MANDERVILLE,*[,†,¶] AND JOHN D. SIMON*[,‡]

Department of Chemistry, Wake Forest University, Winston-Salem, North Carolina 27106, Department of Chemistry, Duke University, Durham, North Carolina 27708, Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, and Ecole Nationale Superieure Agronomique de Toulouse, Lab. Genie Chimique, UMR−CNRS 5503, Department of Toxicology and Food Safety, Agrobiopole-BP 107-F31326 Castanet-Tolosan Cedex, France

Received July 2, 2003

ABSTRACT

Ochratoxins are a class of naturally occurring compounds produced by several fungi. The most toxic is ochratoxin A (OTA), and occurrence of some human nephropathies and tumors correlate with enhanced OTA exposure. In this Account, the following areas are examined: molecular details of the binding of OTA to human serum albumin (HSA), the influences of binding to HSA on the transport of OTA across epithelial cell membranes by organic anion transport proteins, the oxidative activation of OTA, and the formation of OTA adducts with biological molecules. These studies are beginning to provide a detailed chemical model for the transport, accumulation, and genotoxic and carcinogenic effects of OTA.

Introduction

Ochratoxin A (OTA, **1**) is a mycotoxin produced by a single *Penicillium* species, *Penicillium verrucosum*, by *Aspergillus ochraceus* and several related species[1] and by *A. carbonarius*,[2] with a small percentage of isolates of the closely related *A. niger*.[3] The growth of OTA-producing molds is not readily controlled in stored animal and human foods,[4] rendering exposure to this toxin difficult to regulate. The presence of OTA in human blood samples has commonly

* To whom correspondence should be addressed. E-mail: jsimon@duke.edu (J.D.S.); rmanderv@uoguelph.ca (R.A.M.).

[†] Wake Forest University.

[‡] Duke University.

[§] National Institutes of Health.

[∥] Ecole Nationale Superieure Agronomique de Toulouse.

[⊥] Current address: Department of Chemistry, Wichita State University, Wichita, KS 67260.

[#] Current address: Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.

[¶] Current address: Department of Chemistry, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

Jian Dai received her B.Eng. degree from Shanghai Jiao Tong University and her Ph.D. degree from Wake Forest University under the guidance of Professor Ronald E. Noftle. She is currently a postdoctoral research associate in the laboratory of Professor Manderville.

Gyungse Park earned his B.A. degree from Korea University, his M.S. degree from Wayne State University, and his Ph.D. degree from the University of New Hampshire under the guidance of Professor Roy Planalp. After postdoctoral training at Wake Forest University with Professor Manderville, Gyungse has returned to Korea and is currently working with Professor Kimoon Kim as a senior researcher at Pohang University of Science and Technology.

Jennifer L. Perry received her B.S. degree from Winthrop University and her Ph.D. from Duke University. Her interests include environmental effects of chemical exposure. She is currently a research associate at the National Institute of Environmental Health Sciences.

Yuri V. Il'ichev received his Ph.D. from Moscow State University. After postdoctoral positions at Max Planck Institut für Biophysikalische Chemie, University of Basel, and Duke University, Yuri joined the faculty of the Department of Chemistry at Wichita State University in the fall of 2002. His current research interests include charge transfer dynamics in solution and in biopolymers and dynamics of conformational changes of nucleic acids and proteins coupled to proton-transfer reactions.

Daniel Bow received his Ph.D. from the University of Aberdeen, Scotland, in 2002. Since then, he has been working as a visiting research fellow at the NIEHS, focusing his interest on renal organic anion transport physiology.

John B. Pritchard is currently Chief of the Laboratory of Pharmacology and Chemistry at the National Institute of Environmental Health Sciences/NIH. He received his Ph.D. from the Department of Physiology at Harvard University in 1970. After his postdoctoral work at SUNY Upstate Medical Center, he joined the Physiology Department at the Medical University of South Carolina, serving as Assistant and Associate Professor. He joined NIEHS in 1976. His research focus has been the membrane transporters that mediate renal elimination of drugs and other xenobiotics and the roles of membrane transport in barrier epithelia. The organic anion transporter (rOAT1) was cloned in his laboratory in 1997 and was recently shown to be present in choroid plexus. His current research focuses on molecular characterization of drug transporters and control of their function.

Virginie Faucet received her master of food science in 2001 from the University of Montpellier, France. She is is a doctorate student with Professor Pfohl-Leszkowicz. She is granted by the EU Project QLK1-CT 2001-01614 on "OTA risk assessment.

Annie Pfohl-Leszkowicz received a diploma in Pharmacy in 1976, a Master of Toxicology in 1979, a Ph.D. in toxicology in 1981, and a Doctorate of Sciences in 1985. Since 1994, she has been a full professor of Toxicology and Food Safety at the National High School of Agriculture of Toulouse in France. She is Advisor for INC on Evaluation of OTA level in Food and for GOEMAR Society on "Improvement of wheat ozonation for destroying fungi". She is the author of 150 scientific publications. Her research focused on the relation between food contaminants (natural toxins such mycotoxins, phycotoxins, and aristolochic acid, but also environmental pollutants, such as polycyclic aromatic hydrocarbons) and human health (e.g., nephrotoxicity and cancers). She is a specialist of adduct detection by the postlabelling method.

Richard A. Manderville received his B.Sc. degree in chemistry and biology from Queen's University in Kingston, Canada, and his Ph.D. degree from Queen's University under the supervision of Professor Erwin Buncel. After postdoctoral training with Professor Sidney M. Hecht at the University of Virginia, he joined the faculty at Wake Forest University in 1995, where he holds a Junior Faculty Fellow and is Associate Professor of Chemistry. His current research interests include DNA alkylation by chlorinated phenols and redox active pyrromethenes. On July 1, 2004, he returned to Canada and joined the Department of Chemistry and Biochemistry, University of Guelph in Guelph, Ontario.

John D. Simon received his B.A. in chemistry from Williams College and his M.A. and Ph.D. from Harvard University. After a postdoctoral position with Professor Mostafa A. El-Sayed at UCLA, he joined the faculty at UCSD in 1985. In 1998, he accepted the position as the George B. Geller Professor at Duke University and chaired the Department of Chemistry from 1999 to 2004. He has accepted the appointment of Vice-Provost of Academic Affair at Duke University starting in January 2005. His current research interests include protein−mycotoxin interactions and time- and spatially resolved studies of human pigments.

10.1021/ar0302134 CCC: $27.50
Published on Web 09/24/2004

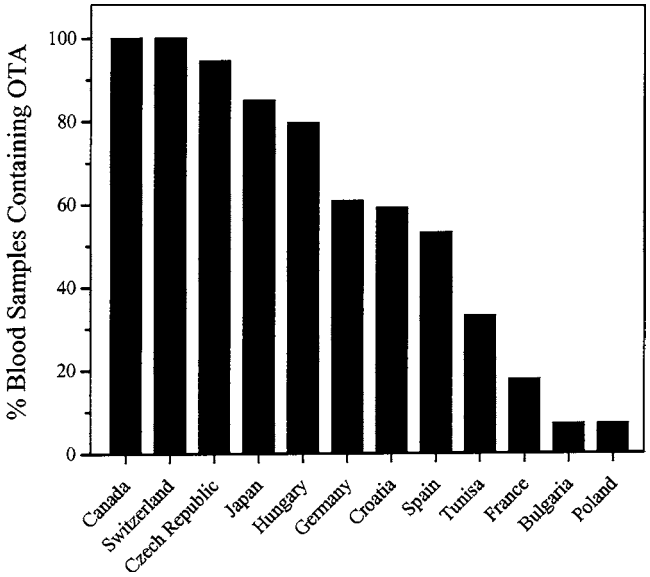


FIGURE 1. OTA exposure in humans through prevalence in blood of healthy persons worldwide. See ref 6 for dates of the sample collection and sample size.

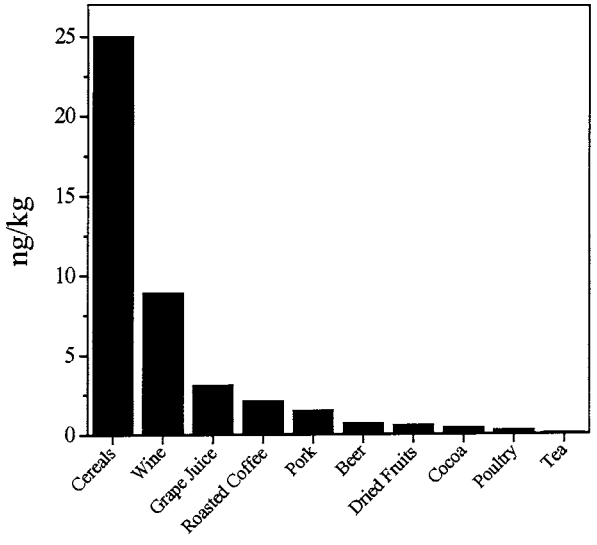
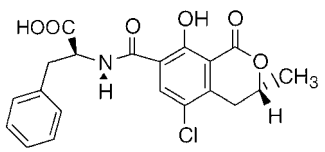


FIGURE 2. Average OTA contamination of human agricultural products. Values were obtained from ref 6.

been utilized to assess exposure to the toxin.[5] While the United States has not undertaken a study to monitor OTA levels in human blood, many other countries have compiled such data over the past 20 years (Figure 1).[6]



OTA 1

Mean contamination values for agricultural food products affected by OTA-producing molds are given in Figure 2.[6] The International Agency for Research on Cancer (IARC) and the Joint FAO/WHO Committee on Food Additives and Contaminants (JCFAC) are responsible for reviewing OTA toxicity data and recommending maximum safe exposure levels. Currently, the JCFAC has set a provisional tolerable weekly intake (PTWI) at 100 ng/kg body weight.[6] Both organizations have plans to revise the limits as more research data become available.

The prevalence of OTA exposure among humans worldwide has prompted concern because adverse effects are apparent in animals and in cell cultures.[7,8] The kidney is the main organ affected by OTA toxicity in mammals.[9] Human renal disease remains only causatively linked to OTA,[10] but the occurrence of human bladder and urothelial urinary tract tumors appears to be associated with above normal exposures to OTA.[11] Other impacts of OTA include genotoxicity,[12] carcinogenity,[13] and hepatotoxicity.[14] The development of protocols to reduce OTA toxicity requires knowledge of the transport, cellular entry, and mechanistic details of biochemical activation. It is these aspects of OTA chemistry that are discussed in this Account.

Molecular Details and Implications of the Binding of OTA to Human Serum Albumin (HSA)

Interaction of OTA with the predominant plasma protein, serum albumin, has been described for several mammalian species. A 585 amino acid protein, HSA transports many endogenous and exogenous ligands throughout the body by binding them mostly within two of its three similar α-helical domains.[15] These two binding sites (referred to as Sudlow sites I and II) have been extensively characterized by X-ray crystallography and are located in domains II and III, respectively.[15] Optical spectroscopic measurements reveal two binding sites for OTA in HSA, with binding constants that differ by a factor of 50. Studies of competitive binding with other HSA ligands and binding studies of OTA to recombinant domains of HSA established that the high-affinity site lies within domain IIA ($K_b = 5.2 \times 10^6\ M^{-1}$) and the weaker binding site is in domain III (site II, $K_b = 1.0 \times 10^5\ M^{-1}$).[16,17] The magnitude of the binding constant is comparable to binding constants for many drugs, which generally fall in the range from 10^4 to $10^7\ M^{-1}$. For example, the binding constant for OTA in its high-affinity site is greater than that of the anticoagulant warfarin and the nonsteroidal anti-inflammatory drug piroxican ($2.3 \times 10^5\ M^{-1}$) and ibuprofen ($9.6 \times 10^5\ M^{-1}$) but less than that of iophenoxic acid ($3.3 \times 10^7\ M^{-1}$).[17]

One of the most interesting details associated with the complexation of OTA by HSA is the effect of binding on the pK_a of the hydroxyl group. OTA contains two ionizable protons in aqueous solutions, and reported pK_a values for the carboxyl and hydroxyl groups range from 4.2 to 4.4 and from 7.0 to 7.3, respectively.[18−20] At physiological pH, both the mono- and dianion are present in solution. Binding to HSA lowers the pK_a of the hydroxyl group to 3.9, and so only the dianion is bound to the protein at physiological pH.[16]

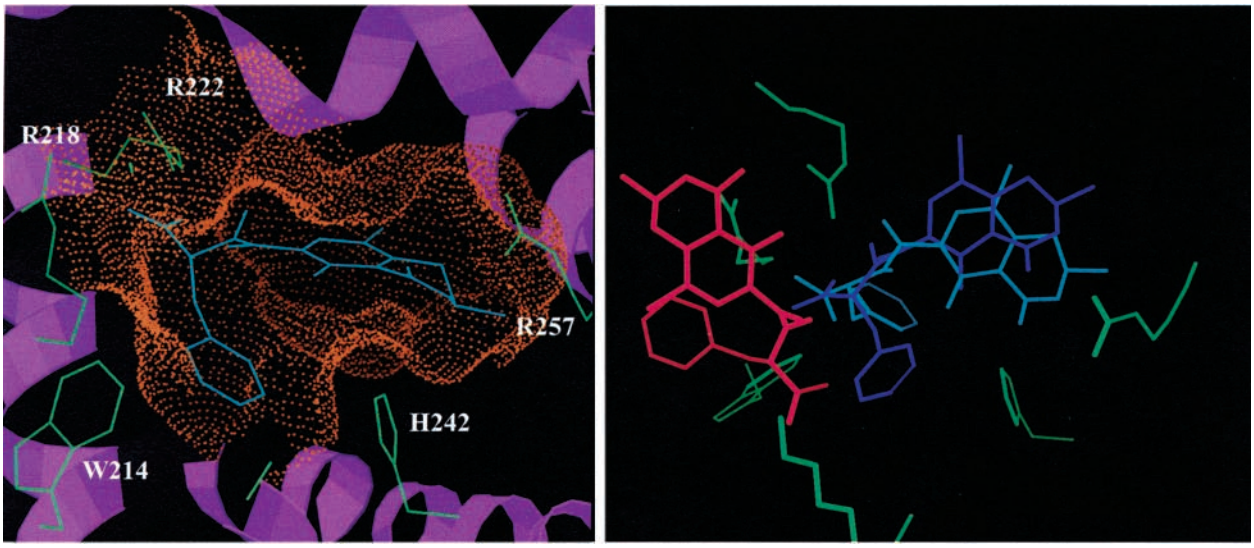


FIGURE 3. (Left) Lowest energy conformer of OTA^{2-} in the site-I-binding pocket. Amino acids surrounding the cavity are labeled. Both the carbonyl of the lactone and phenolic oxygen point toward R257, whereas the carboxy terminus interacts with R218 and R222. The image was rendered in Mage.6.30.030528 on a Linux Workstation. (Right) Overlay of the lowest docking energy OTA^{2-} conformer for WT HSA and HSA mutants. WT-HSA residues are shown for clarity (green) with OTA^{2-} in the case of (a) R218A (dark blue), (b) R257A (pink), or (c) WT HSA (cyan). Reprinted from ref 22.

This change in acidity must arise from interactions with specific amino acid(s) within domain IIA.[16] Isothermal calorimetry data reveal that the proton liberated from OTA^- upon binding to HSA remains in the protein.[21] Furthermore, the direct binding of OTA^{2-} is accompanied by a quantitative uptake of a proton from the surrounding solvent. A similar shift in pK_a of the hydroxyl group was observed for the binding of the toxin to bovine, rat, and porcine serum albumins. Assuming that binding by the nonhuman albumins also occurs in domain IIA, the only conserved basic amino acids that lie close enough to the binding cavity to serve as a proton acceptor and thereby stabilize the binding of OTA through formation of an ion pair with the phenoxide group are R218 and R257. These two amino acids sit across the binding cavity from each other in site I, and it has not been possible to experimentally distinguish the orientation of OTA in the binding site to determine which amino acid interacts with the phenoxide group with the data available.

Details of this binding site were revealed through genetic algorithm simulations performed in Autodock 3.0.[22] Figure 3A shows the lowest energy conformer of OTA^{2-} in the D2A pocket, as well as the average coordinate position of each of the 250 runs performed (indicated in green dots). The calculated binding constant for the OTA dianion, 3.7×10^6 M^{-1}, is in excellent agreement with the experimental value. R218 and R222 associate with the carboxyl terminus of OTA. The lowest energy structure reveals that the phenolic group of OTA is pointed toward R257.

The importance of R218 and R257 to the binding of OTA has recently been addressed experimentally using R218A and R257A mutants of HSA. The experimental binding constant for the R218A mutant is 2.2×10^5 M^{-1}, reduced by more than an order of magnitude from wild-type (WT) HSA. Calculations reveal an even larger reduction on the binding constant (1.8×10^4 M^{-1}). However, the lowest energy geometry of the complex is essentially the same as WT HSA (Figure 3B). Thus, R218 stabilizes the binding of OTA in this site but is not necessary for establishing the overall binding geometry.

The binding constant of OTA to the R257A mutant is 1.6×10^5 M^{-1} and is also an order of magnitude smaller than the WT protein. The corresponding calculated binding constant, 8.3×10^4 M^{-1}, is also in good agreement with the experimental results. Unlike the R218A mutant, for the R257A mutant, the phenoxide ring associates with K199. Thus, OTA adopts a completely different conformation compared to that found in WT HSA. This suggests that R257 is critical for the orientation of the lactone portion of OTA. While R218 and to some extent R222 are involved in electrostatic interactions with the carboxyl moiety, R257 stabilizes the lactone end of the molecule and is in position to accept a proton from the monoanion of OTA or the surrounding solvent.

The mechanism(s) of OTA entry and accumulation in the renal proximal tubule (PT), which is believed to cause resultant nephrotoxicity, is poorly understood.[23,24] All segments of rabbit renal PT show peritubular uptake of OTA, suggesting that several basolateral organic anion transporters (OATs) handle the transport of OTA.[25,26] The cloned human organic anion transporters 1 (hOAT1) and 3 (hOAT3) in the secondary and tertiary segments of the mouse PT have also been shown to mediate the transport of OTA.[27,28] To date, the binding of OTA to OATs has not been quantified.

Data on OTA transport across the basolateral PT cell membrane is generally collected in the absence of plasma proteins. In light of the fact that OTA will be complexed to HSA in vivo, it is important to establish whether binding to HSA inhibits transport. We recently examined OTA accumulation by hOAT1 expressing *Xenopis laevis* oocytes.[29]

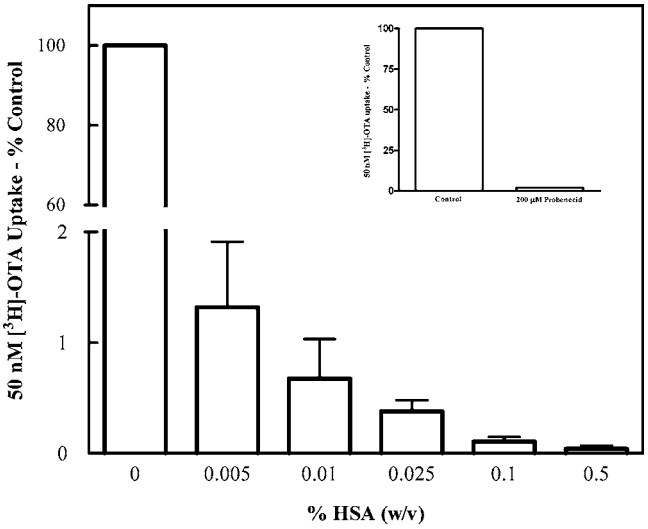
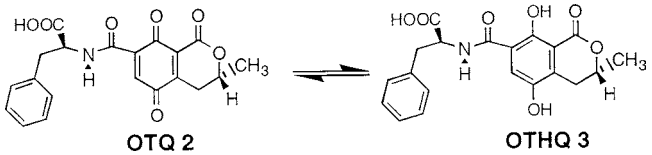


FIGURE 4. Uptake of 5 nM [^{3}H]OTA by hOAT1-expression oocytes as a function of HSA concentration. Inhibition observed in the presence of 200 μM probenecid (inset). Standard deviations indicated with error bars are based upon data from 5 to 6 oocytes per treatment with OTA and HSA or OTA and probenecid.

In the absence of HSA, hOAT1 mediates OTA transport (Figure 4). Probenecid, an inhibitor of hOAT1-mediated transport, reduced OTA accumulation by >90%. The data presented in Figure 4 show that OTA transport by hOAT1 is significantly inhibited by HSA (99% inhibition at 0.025% w/v HSA). Considering the HSA concentration in blood (~4.5% w/v),[15] these results reveal that albumin binding impacts transport in vitro and thus must be considered when thinking about the clearance of OTA in vivo.

Oxidative Activation of OTA

One hypothesis for OTA-mediated carcinogenicity is based on the prooxidant properties of the toxin that lead to an oxidative stress response,[30,31] oxidative DNA damage,[32] and bulky DNA adducts from covalent attachment of activated OTA.[33−35] Under this scenario, OTA requires oxidative activation to exert its genotoxic, mutagenic, and carcinogenic mechanisms.



To gain insight into the oxidative chemistry of OTA, we have employed electrochemical,[36] photochemical,[37,38] and biochemical[39] methods. Together, these point to the intermediacy of the OTA phenoxyl radical and the quinone/hydroquinone redox couple, OTQ (**2**)/OTHQ (**3**), formed from oxidative dechlorination of OTA. For example, the electrochemical behavior of OTA in acetonitrile (MeCN) shown in Figure 5 is nearly identical to that of 4-chlorophenol (4-ClPhOH).[36] Both OTA and 4-ClPhOH exhibit high half-peak primary oxidation potentials in MeCN [$E_{p/2}$ = 1.81 V versus saturated calomel electrode (SCE) for OTA; peak 1 in Figure 5] that produce secondary redox peaks

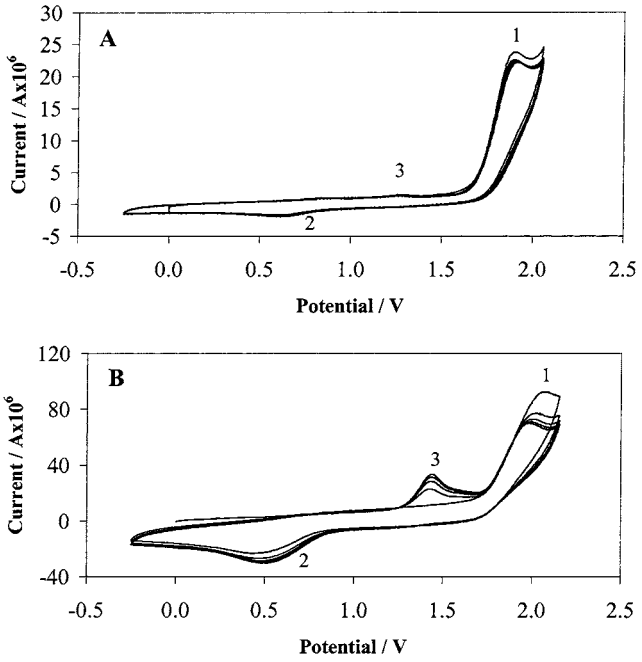


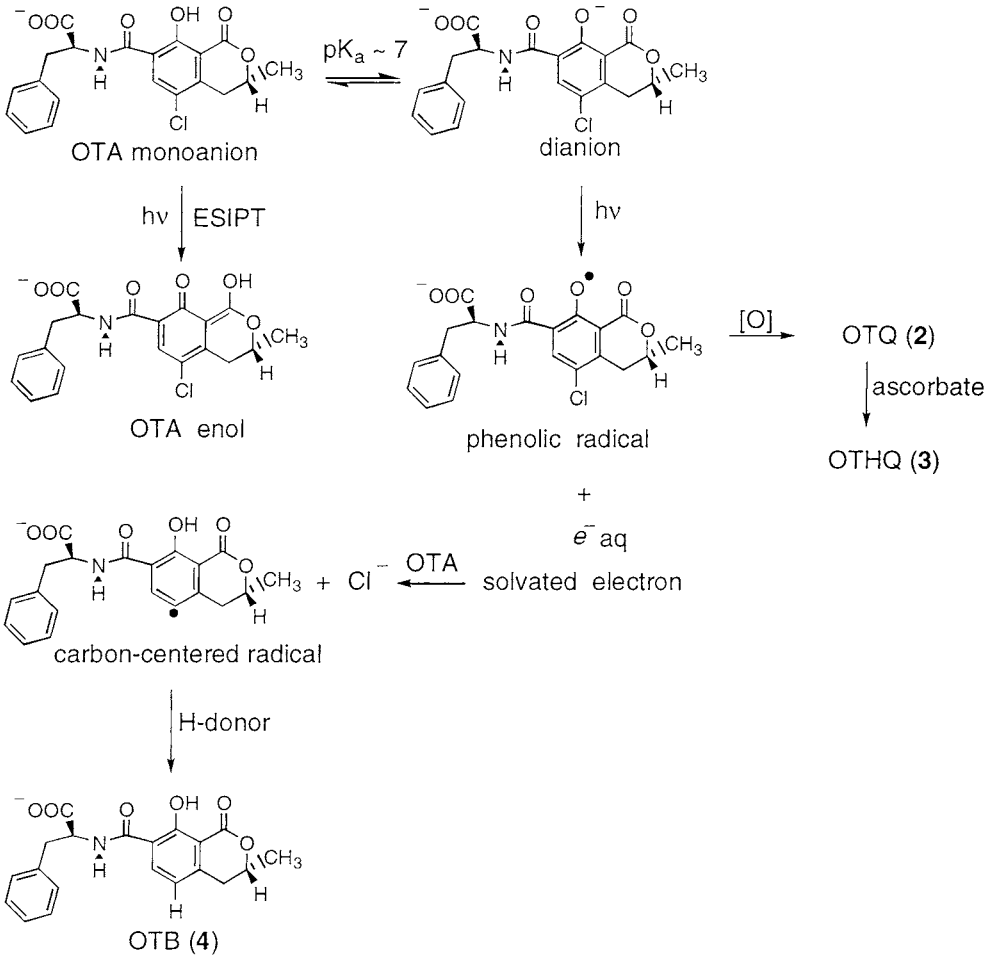
FIGURE 5. Cyclic voltammetry of OTA (5 mM) and tetrabutylammonium perchlorate (0.5 M) in MeCN. Voltammograms recorded using a glassy carbon (diameter of 1.5 mm) working electrode and a Ag wire pseudoreference electrode. (A) Scan rate (ν) = 0.025 V s^{-1}; 1 = primary oxidation wave, and 2 and 3 are secondary waves ascribed to the **2/3** redox couple. (B) ν = 0.5 V s^{-1}, highlighting growth in the secondary redox waves at the higher scan rate.

ascribable to their quinone/hydroquinone redox couple (peaks 2 and 3 in Figure 5). It is generally accepted that such couples stem from 2*e* oxidation of the phenol to a phenoxonium cation that reacts with traces of water in the solvent to liberate HCl and form the quinone electrophile. Deprotonation to yield the phenolates lowered $E_{p/2}$ by 0.8 V and caused a change in the oxidative mechanism from a 2*e* to a 1*e* process. Phenolates exhibit lower oxidation potentials than their parent phenols and undergo 1*e* oxidations to phenolic radical species. The electrochemical oxidation of OTA in aqueous buffer at pH 7.4 resembled the oxidative behavior of the phenolate in MeCN, in accordance with the fact that the phenolic group of OTA (pK_a ~ 7) is predominantly deprotonated.

Photoirradiation of OTA also generates the **2/3** redox couple and promotes DNA-strand scission.[37] Photophysical measurements suggest that the phenolic form of OTA undergoes excited-state intramolecular proton transfer (ESIPT), which inhibits its photodecomposition.[38] Photoexcitation of the phenolate initiates DNA cleavage and produces the OTA phenolic radical and solvated electrons (e^-_{aq}) that are either scavenged by O_2 to generate $O_2^{\bullet -}$ or by OTA in the ground state.[38] As outlined in Scheme 1, the interaction of OTA with e^-_{aq} may initiate the decomposition of the toxin via an $S_{RN}1$-type process that affords an OTA carbon-centered radical and Cl^-.

Interaction of the carbon-centered radical with an H donor provides a route to the nonchlorinated derivative OTB (**4**) and a mechanism for an H-atom abstraction process leading to DNA-strand scission. Further oxidation ([O]) of the OTA phenolic radical is proposed to yield the

Scheme 1. Proposed Pathway for the Photooxidation of OTA



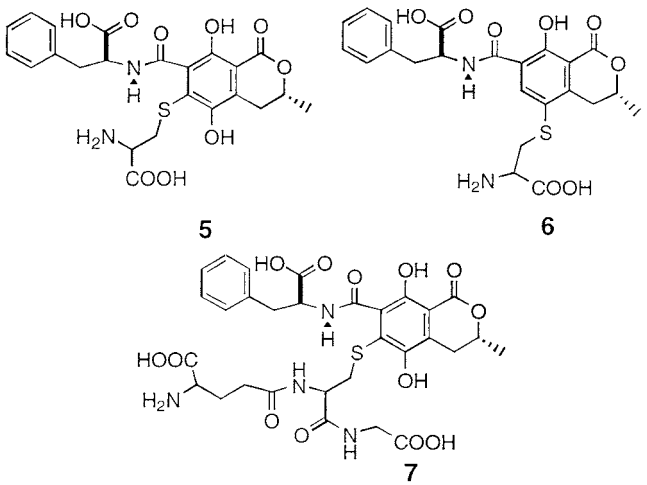
quinone **2**, which in the presence of ascorbate generates the hydroquinone species **3**.

The relevance of our chemical studies has been confirmed by the finding that **3** is detected in the urine of rats following OTA feeding by gavage.[40] This establishes **3** as an authentic metabolite of OTA, suggesting that in vivo OTA undergoes oxidative dechlorination to form the reactive quinone electrophile **2** that is subsequently reduced to form **3**.[36−39]

Formation of Covalent OTA Adducts

The fact that OTA is poorly metabolized by P450s, peroxidases, and glutathione (GSH) transferases in vitro has made it difficult to characterize covalent adduct formation by OTA. Here, some groups have reported ^{32}P-postlabeling evidence for covalent DNA adduction by OTA in vivo[33] and in vitro,[34,35] while others have been unable to confirm these findings.[40,41] Thus, the ability of OTA to react covalently with DNA remains a controversial issue.

From our chemical studies it was anticipated that the photoactivation process could provide valuable insight into the reactivity of the toxin. Our first efforts employed LC/MS to elucidate the primary products from the photoreaction of OTA in the presence of cysteine (CySH).[42] Photoirradiation of OTA in the presence of CySH affords the S-cysteinyl conjugates **5** and **6**. The adduct **5**, which is also formed by copper activation,[43] was ascribed to the Michael-addition conjugate resulting from covalent attachment of CySH to the quinone **2** generated by photooxidation of OTA.



5 **6**

7

After the details for CySH adduction by OTA were established, attention was focused on the interaction of OTA with reduced GSH.[44] An authentic sample of the conjugate **7** was generated from the photoreaction of **3** in the presence of excess GSH. With **7** in hand, it was then determined that the same conjugate is produced from treatment of 100 μM OTA in the presence of excess GSH

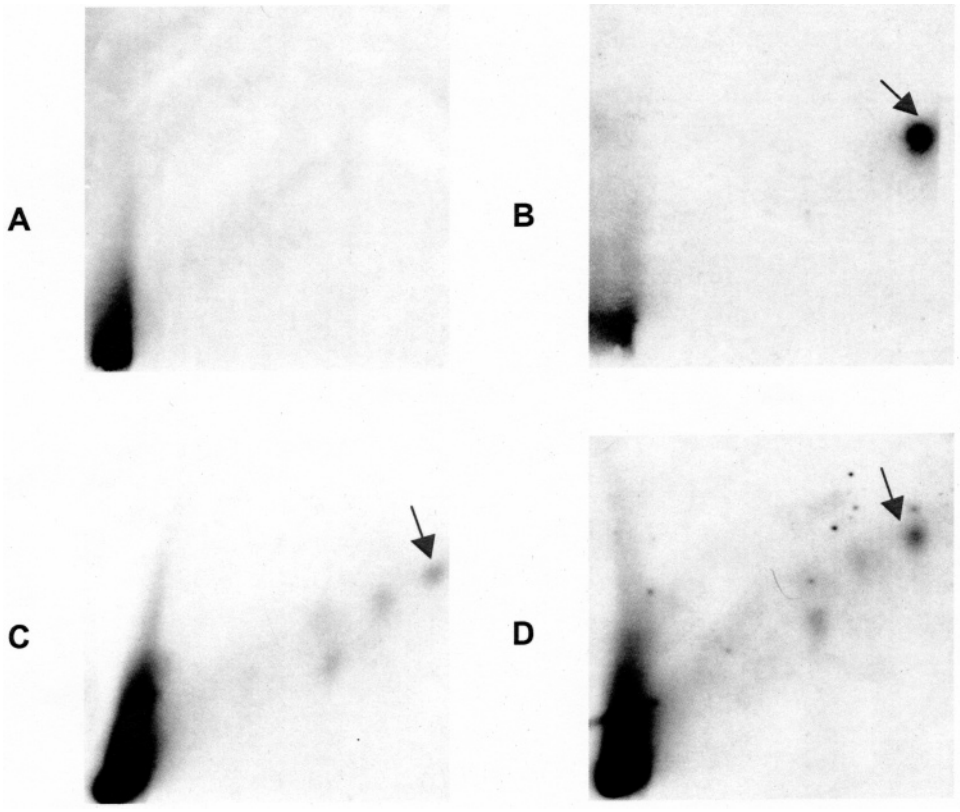
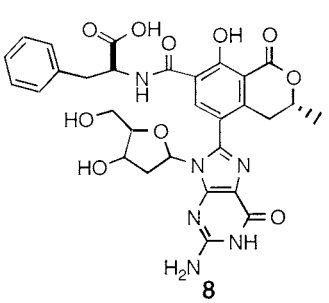


FIGURE 6. Autoradiographs from ^{32}P postlabeling of OTA-mediated DNA adducts from the kidney medulla of pigs. (A) Control DNA isolated from pigs without OTA treatment. (B) 3′-dGMP-OTA C-adduct standard. (C) DNA from OTA-treated pigs showing four adduct spots, with the 3′-dGMP-OTA C adduct indicated by the arrow. (D) Coelution of the 3′-dGMP-OTA C-adduct standard with the DNA from OTA-treated pig DNA (the C adduct is indicated by the arrow). Reprinted from ref 48.

(1 mM), following incubation for 1 h with either horseradish peroxidase (HRP)/H_2O_2, rat-liver microsomes (RLM)/NADPH, or free Fe^{II}. From the RLM/NADPH system, the conjugate **7** was formed at a rate of ~1−3 pmol min^{-1} (mg of protein)$^{-1}$. Using our MS data, other laboratories have been able to reproduce our results and have confirmed that OTA bioactivation in the presence of GSH generates the conjugate **7**.[40]



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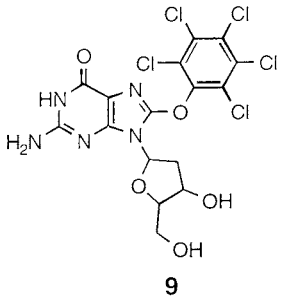

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The photoreactivity of OTA toward deoxyguanosine (dG) was also examined.[45] These experiments led to the isolation and identification of the carbon-bonded C8-dG OTA adduct, **8**. Treatment of OTA (100 μM) with 50 equiv of dG in 100 mM phosphate buffer (pH 7.4) at 37 °C in the presence of either 100 μM $Fe(NH_4)_2(SO_4)_2$, 100 μM $Cu(OAc)_2$, or HRP/H_2O_2 also yielded the adduct **8**, as determined by LC/MS. This observation confirmed the ability of activated OTA to react with a DNA-derived nucleophile and suggested the intermediacy of the OTA phenoxyl radical in reaction with dG. This hypothesis stemmed from the well-known susceptibility of the C8 position of dG to radical attack, as amply proven through formation of the hydroxyl radical-derived lesion, 8-oxo-dG.[46] In this regard, it is interesting to note that the pentachlorophenoxyl radical generated during HRP/H_2O_2 oxidation of pentachlorophenol (PCP) reacts with dG to form the corresponding oxygen-bonded C8-PCP-dG adduct, **9**.[47] These results highlight the ambident electrophilicity of phenoxyl radicals toward the C8 position of dG.

We have now isolated the C-bonded C8 adduct generated from the photoreaction of OTA with 3′-monophosphate-dG (3′-dGMP). Our authentic 3′-dGMP-OTA C adduct was used as a standard for ^{32}P-postlabeling analyses of OTA-mediated DNA adduction in the kidney medulla of pigs following chronic exposure to OTA.[48] Figure 6 shows that the DNA from the kidney medulla of OTA-treated pigs contains four distinct adduct spots (C), one of which comigrates (D) with our 3′-dGMP-OTA C-adduct standard (B). Because OTA is a proven carcinogen in the kidney and DNA adducts are generally regarded as precursors of tumors,[49−51] the ^{32}P-postlabeling data in Figure 6 suggest that the C-adduct **8** may play a role in carcinogenesis mediated by OTA.

Conclusions

The studies discussed above provide new molecular insight into the transport and activation of OTA. Studies in vivo reveal that the lifetime of OTA in living systems is dependent on the presence of serum albumin, while in vitro experiments establish that OTA binds to HSA with a binding constant similar to that characteristic of many exogenous molecules. Computation studies and binding studies to WT and mutant HSA provide a molecular picture for the binding geometry. The toxin binds within D2A of HSA. Binding lowers the pK_a of the hydroxyl group, thereby facilitating the formation of an ion pair between the phenoxide group and protonated R257. The binding with HSA mitigates in vitro transport of the toxin by human organic anion transport proteins. The mechanism by which OTA gains access to epithelial cells remains unresolved. Oxidative activation of OTA generates the phenoxyl radical and the hydroquinone/quinone redox couple. These species can form adducts with sulfhydryl groups and deoxyguanine. Recent biological studies confirm the presence of the hydroquinone (OTHQ), the GSH conjugate, and support the presence of the guanine adduct as evidenced by ^{32}P-postlabeling measurements.

This work was supported by the National Cancer Institute (R01-CA080787), Duke University, and the North Carolina Biotechnology Center. We thank our many co-workers, Florian Ruker, Michael Dockal, Eric H. Toone, Trine Christensen, Michael R. Goldsmith, David N. Beratan, Colin Chignell, Jamal McClendon, Ivan Gene Gillman, Jason A. Ardus, Mark E. Brow, M. Wade Calcutt, Ronald E. Noftle, Marcus, W. Wright, Mark S. Miller, Gary G. Schwartz, and Steven A. Akman.

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AR0302134