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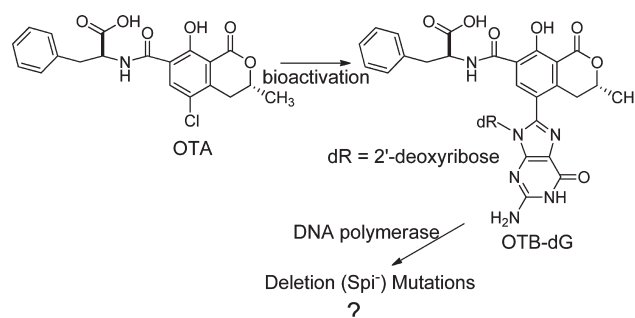
# An Update on Direct Genotoxicity as a Molecular Mechanism of Ochratoxin A Carcinogenicity

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**ABSTRACT:** Ochratoxin A (OTA) is a naturally occurring chlorophenolic fungal toxin that contaminates a wide range of food products and poses a cancer threat to humans. The mechanism of action (MOA) for OTA renal carcinogenicity is a controversial issue. In 2005, direct genotoxicity (covalent DNA adduct formation) was proposed as a MOA for OTA-mediated carcinogenicity [Manderville, R. A. (2005) *Chem. Res. Toxicol.* 18, 1091–1097]. At that time, inconsistent results had been published on OTA genotoxicity/mutagenicity, and conclusive evidence for OTA-mediated DNA adduction had been lacking. In this update, published data from the past 6–7 years are presented that provide new hypotheses for the MOA of OTA-mediated carcinogenicity. While direct genotoxicity remains a controversial issue for OTA, new findings from the Umemura and Nohmi laboratories provide definitive results for the mutagenicity of OTA in the target tissue (outer medulla) of male rat kidney that rules out oxidative DNA damage. These findings, coupled with our own efforts that provide new structural evidence for DNA adduction by OTA, has strengthened the argument for involvement of direct genotoxicity in OTA-mediated renal carcinogenesis. This MOA should be taken into consideration for OTA human risk assessment.



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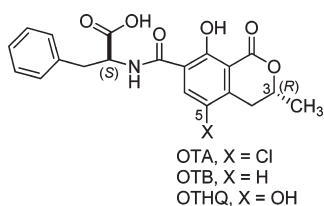
## 1. INTRODUCTION

Ochratoxin A (OTA, Figure 1) is a mycotoxin produced by several species of *Aspergillus* and *Penicillium* fungi that structurally consists of a *para*-chlorophenolic group containing a dihydroisocoumarin moiety that is amide-linked to L-phenylalanine (L-Phe).<sup>1</sup> It is a natural contaminant of corn, peanuts, storage grains, cottonseed, and decaying vegetation.<sup>2–4</sup> It has

also been detected in moldy cereals such as wheat, rye, barley, and oats and has been extracted from peanuts, coffee beans, bread, flour, rice, peas, and beans.<sup>2–4</sup> The toxin is widespread in temperate areas such as Canada,<sup>5</sup> Denmark,<sup>6</sup> Norway,<sup>7</sup> Germany, Sweden, and the United Kingdom,<sup>8</sup> and detectable amounts have been found in randomly collected human milk samples in Germany, Sweden, and Italy.<sup>8</sup>

OTA has been implicated in a diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity, neurotoxicity, and immunotoxicity.<sup>9</sup> The toxin is mainly noted for its nephrotoxicity,<sup>10</sup> and to date, it is one of the most potent renal carcinogens in rodents ever studied by the National Cancer Institute/National Toxicological Program (NCI/NTP).<sup>11</sup> In the NTP study, three doses of OTA [210, 70, and 21  $\mu\text{g}/\text{kg}$  body weight (bw)] were administered to male and female F344N rats. At 70  $\mu\text{g}/\text{kg}$  bw, 39% of the males and 4% of the females developed cancers, highlighting that females are less susceptible than males to OTA carcinogenicity.<sup>11</sup> The International Agency for Research on Cancer (IARC) has classified OTA as a group 2B carcinogen (possible human carcinogen) on the basis of sufficient evidence for carcinogenicity in animal studies.<sup>12</sup>

Given that OTA is a possible carcinogen present in human foodstuffs, government agencies propose tolerable daily intakes



**Figure 1.** Chemical structure of OTA and its analogues.

(TDIs) to manage the risk from OTA exposure. Here, the mechanism of action (MOA) by the toxin has a major influence on the methods applied for risk assessment.<sup>13,14</sup> For carcinogens causing tumor formation by genotoxicity involving covalent DNA adduction (direct mode of genotoxicity), the presence of thresholds is not considered, and the tumor incidences observed in animals given high doses of the carcinogen are used to predict potential tumor incidences in humans exposed to much lower doses. For nongenotoxic chemicals, thresholds based on the induction of cytotoxicity may be defined, and TDIs can be derived using the safety factor methodology.<sup>13,14</sup> The TDI established by the European Food Safety Authority (EFSA) has been set at  $\sim 17$  ng/kg bw/day (120 ng/kg bw/week).<sup>15</sup> This assessment considers the lowest observed adverse effect level (LOAEL) of  $8 \mu\text{g}/\text{kg}$  bw/day for early markers of renal toxicity in pigs and a threshold-based approach for risk assessment due to the absence of specific OTA-derived DNA adducts.<sup>15</sup> Because the genotoxic status of OTA is highly controversial and direct genotoxicity remains a viable MOA for OTA-induced tumor formation, Health Canada recommends that at present OTA be regulated as a nonthreshold carcinogen; hence, a more stringent TDI of 4 ng/kg bw/day (28 ng/kg bw/week) has been proposed.<sup>16</sup>

In 2005, a Perspective in this journal entitled “A Case for the Genotoxicity of OTA by Bioactivation and Covalent DNA Adduction” was presented as a possible MOA for OTA-mediated renal carcinogenesis.<sup>17</sup> At that time, direct genotoxicity by OTA was highly controversial, and Turesky presented a Perspective that OTA is not a genotoxic carcinogen.<sup>18</sup> Turesky noted that data on OTA toxicology are inconsistent because of the complex biological effects of OTA and the poor metabolism of OTA into reactive species, which has led to divergent interpretations of its MOA. Turesky concluded that the available data on the genotoxic effects of OTA suggest that it acts through an indirect MOA. However, he noted that direct genotoxicity by OTA could not be ruled out as a possibility and that unambiguous identification of biomarkers by LC/MS techniques could determine whether OTA is acting as a genotoxic carcinogen.<sup>18</sup>

Over the past 6–7 years, ongoing studies addressing OTA's MOA have derived new possibilities for the induction of OTA-mediated carcinogenesis. These new hypotheses stress the importance of indirect pathways and a threshold model for OTA risk assessment. The controversy surrounding OTA as a direct genotoxin has not diminished, and the MOA underlying OTA-induced carcinogenicity is still unclear. However, new studies have strengthened the argument that genotoxicity/mutagenicity is likely to contribute to OTA-induced tumor formation. This update highlights new data on the carcinogenicity of OTA, proposed indirect pathways for OTA's MOA, its current status as a genotoxic/mutagenic agent, and direct covalent binding of OTA to DNA as a MOA for OTA-mediated carcinogenesis.

## 2. CARCINOGENESIS

OTA is a potent renal carcinogen in rodents, and a striking feature of OTA-induced kidney pathology is prominent karyomegaly and polyploidy of proximal tubule cells.<sup>11,19–23</sup> Highlights from the historical NTP study<sup>11</sup> have recently been outlined by Kuiper-Goodman and co-workers at Health Canada.<sup>16</sup> Kuiper-Goodman earlier determined that characteristics of the OTA-induced tumors from the NTP data corresponded to those typically observed for genotoxic chemicals.<sup>24</sup> A more recent review of the NTP experimental rat tumor data for OTA also placed OTA in the category of “chemicals inducing renal tumors through direct interaction of the parent compound or metabolite with renal DNA” based on histopathological evidence.<sup>25</sup> The experimental evidence from the NTP study suggests that OTA acts as a complete carcinogen (initiator and promoter activity) rather than as a promoter alone.<sup>16</sup> This line of evidence stimulated Health Canada to utilize a nonthreshold approach for OTA risk assessment and assign a TDI of 4 ng/kg bw/day for OTA exposure.<sup>16</sup>

One intriguing aspect of the NTP rat tumorigenesis study is the susceptibility of male rats to OTA-mediated carcinogenesis. Tumor incidence rates in female rats were lower than in males and were only significant at the highest dose of OTA (16% as compared to 72% for males).<sup>11</sup> Earlier work by Pfohl-Leszkowicz and co-workers suggested that gender differences could stem from different degrees of expression of some bioactivation enzymes, such as cytochrome P450s.<sup>20</sup> However, Mantle and Nagy recently proposed another mechanism for rat gender difference in OTA renal carcinogenesis.<sup>26</sup> They perceive a role for  $\alpha 2 \mu$ -globulin as an OTA-carrier protein in male rats that provides an enhanced rate of transfer of some circulating OTA to the proximal tubules in the male kidney. Because female rats lack this potential OTA-carrier protein, they would sustain a lower OTA concentration in the kidney during its temporary passage through the organ. Thus, male rats are more susceptible to OTA-mediated carcinogenesis, and dose–response data for human risk assessment should only consider the female data, as there is no human analogy of the male rat urinary  $\alpha 2 \mu$ -globulin.<sup>26</sup>

Vettorazzi and co-worker examined plasma,<sup>27</sup> kidney, and liver<sup>28</sup> distribution of OTA in male and female F344 rats. Following single (0.5 mg/kg bw) and repeated (gavaged daily with OTA (0.5 mg/kg bw) for 7 and 21 days) exposure, the OTA concentration in liver and kidney after 24 and 48 h tended to be very similar between both organs.<sup>28</sup> With regard to sex and age differences, in fed conditions, adult males presented lower OTA concentration in tissues than the rest of the groups. However, in fasting conditions, the tissue OTA levels of mature males were higher than the other groups, which correlated with the plasma profile,<sup>27</sup> demonstrating parallel concentrations of OTA in plasma and tissues.<sup>28</sup> Overall, Vettorazzi and co-workers observed no significant differences between males and females regarding OTA concentrations in kidney and liver.<sup>28</sup> Given the lack of evidence showing greater exposure of male kidney to OTA, they suggested that differences in kidney or liver metabolism between both sexes could explain the higher sensitivity of male rats to OTA nephrocarcinogenicity,<sup>28</sup> as originally proposed by Pfohl-Leszkowicz and co-workers.<sup>20</sup>

While the susceptibility of male rats to OTA-mediated carcinogenesis is well documented, Stoev recently demonstrated OTA-induced carcinogenesis in Plymouth Rock chicks.<sup>29</sup> In this study, 30 chicks were divided into three

**Table 1. Summary of Neoplasms from Chicks Exposed to OTA (5 ppm) in the Absence and Presence of L-Phenylalanine (L-Phe, 25 ppm)<sup>29</sup>**

L-Phe	neoplasm	malignant or benign	detection time	location	sex
no	adenocarcinoma	malignant	10th month	liver	male
no	lymphosarcoma	malignant	18th month	kidney	male
no	carcinoma	malignant	20th month	ureters	male
no	cystic adenoma	benign	24th month	kidney	male
no	cystic adenoma	benign	24th month	kidney	female
yes	adenocarcinoma	malignant	19th month	kidney	female
yes	carcinoma	malignant	21st month	liver, spleen	female
yes	rabdomyoma	benign	24th month	breast muscle	female

groups of 10 (each containing five males and five females), one group consisting of a control (no OTA exposure), and two experimental groups, one receiving feed containing 5 ppm OTA and the other receiving 5 ppm OTA in the presence of 25 ppm L-Phe to determine the possible protective effects of L-Phe in reducing OTA-mediated carcinogenesis in the exposed chicks, given that Creppy et al. had reported a protective effect of L-Phe on OTA toxicity in rats.<sup>30</sup> Given in Table 1 is a summary of neoplasms from chicks exposed to OTA (5 ppm) in the absence and presence of L-Phe (25 ppm). Malignant tumors diagnosed as lymphosarcoma in kidney, adenocarcinoma in kidney, carcinoma in the region of ureters, and carcinoma in the liver and spleen at the time of pathomorphological examination were determined. It is interesting to note that 4/5 male chicks developed tumors upon exposure to OTA in the absence of L-Phe, while only 1/5 females developed a tumor that was benign. In contrast, no males apparently developed tumors when L-Phe was included in the diet with OTA, while 3/5 females developed tumors from the OTA/L-Phe diet, two of which were malignant (Table 1). While the chick carcinogenicity study carried out by Stoev is preliminary in nature [small sample size ( $n = 5/\text{dose group}$ ), single dose of OTA (5 ppm)], the neoplasm data presented in Table 1 suggest that L-Phe does have a protective effect in male chicks but enhances tumor formation in females. This information may eventually help shed light on the gender differences noted for OTA-mediated carcinogenesis.

In addition to renal carcinoma, Schwartz hypothesized that OTA exposure may be related to increased incidence of testicular cancer.<sup>31</sup> Although no epidemiological studies have been done to confirm this hypothesis, implication of such a cancer is substantiated by a recent report that acute exposure of pregnant mice to OTA induces adducts in testicular DNA of male offspring.<sup>32</sup> While Mantle is strongly opposed to the notion that OTA may cause testicular cancer,<sup>33</sup> observations of DNA adducts in the testes of mice exposed prenatally to OTA, and the absence of any such adducts in the testes of control mice, are evidence of the carcinogenic potential of OTA in the testes, as DNA adducts are markers of exposure and possibly of biological effect.<sup>34</sup> Prenatal exposure to OTA in mice also significantly depresses expression of the *DMRT1* gene in male offspring.<sup>35</sup> *DMRT1* is a tumor suppressor gene in the testis and its loss produces testicular tumors in mice.<sup>36</sup> A study from the United Kingdom recently confirmed a role for *DMRT1* in testicular tumors in humans.<sup>37</sup> Thus, molecular evidence supports the hypothesis that OTA may be a cause of testicular cancer,<sup>34</sup> as proposed by Schwartz.<sup>31</sup>

### 3. INDIRECT MECHANISMS

**3.1. Oxidative Stress-Mediated MOA.** In a recent review, oxidative stress is proposed as a plausible role in OTA carcinogenicity.<sup>38</sup> This proposal is based on evidence that OTA treatment facilitates the generation of reactive oxygen species (ROS)<sup>39,40</sup> with subsequent oxidative damage to DNA.<sup>41–47</sup> Oxidative DNA damage mediated by OTA has been indirectly detected using the comet assay (single cell gel electrophoresis) with the aid of the repair enzyme formamido-pyrimidine-DNA-glycosylase (Fpg), which recognizes oxidized DNA bases, such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (dOG) that is a biomarker for oxidative damage.<sup>48</sup> The enzyme Fpg cleaves DNA at a variety of oxidized guanine adducts, producing DNA fragmentation that can be detected with the comet assay, and suggests that OTA is capable of producing oxidative DNA damage.<sup>41–47</sup> Recent studies show that the onset of OTA-induced cytotoxicity in cell cultures is highly correlated with the induction of oxidative DNA damage<sup>45,46</sup> and that antioxidants counteract the cytotoxicity mediated by OTA.<sup>49,50</sup>

The proposal is strengthened by studies showing that OTA treatment causes reduction of cellular antioxidant defenses.<sup>51,52</sup> In these studies, OTA was found to reduce the expression of genes regulated by nuclear factor-erythroid 2 p45-related factor (Nrf2) that is involved in the induction of genes encoding detoxification, cytoprotective, and antioxidant enzymes.<sup>38</sup> A correlation between OTA-induced reduction of the Nrf2 pathway and an increased production of oxidative damage were observed.<sup>52</sup> Further studies showed that OTA increases the expression of inducible nitric oxide synthase (iNOs) and stimulates protein nitration,<sup>53</sup> indicating that OTA exposure may be considered as a source of both ROS and RNS.<sup>38</sup>

On the basis of the evidence outlined above, a model for oxidative stress-mediated MOA for OTA has been proposed.<sup>38</sup> In this model, OTA exposure leads to ROS/RNS production with increased levels of oxidative DNA, lipid, and protein damage. Direct redox cycling reactions involving OTA may yield ROS, as OTA contains a phenolic ring system, and a potential mechanism for oxidative stress mediated by phenols has been described as futile thiol pumping.<sup>54,55</sup> This causes thiol oxidation and antioxidant depletion, and OTA is known to reduce GSH levels in mammalian cell lines.<sup>45</sup> Such direct redox cycling pathways are coupled to indirect mechanisms resulting from reduction of cellular antioxidant defenses,<sup>51,52</sup> which is expected to amplify the oxidative-mediated effects of OTA.<sup>38</sup> For the selective induction of tumors in the kidney, increased oxidative stress in connection with cytotoxicity and increased cell proliferation might represent initiating factors.



**3.2. Disruption of Mitosis MOA.** The research team of Dekant and Mally also favors indirect mechanisms for OTA-mediated renal carcinogenesis and a threshold model for risk assessment.<sup>56</sup> However, they do not favor oxidative stress as an indirect MOA for OTA-induced carcinogenesis; a view that is shared by others.<sup>16</sup> They have also found that OTA treatment of rats leads to enhanced DNA breakage by the comet assay following Fpg treatment, but the extent of DNA damage is not target (kidney) specific with similar levels being detected in liver and kidney.<sup>44</sup> Furthermore, the nonchlorinated analogue *N*-{[(3*R,S*)-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-isochromen-7-yl]carbonyl}-*L*-phenylalanine (OTB), which displays cytotoxicity but fails to display nephrotoxicity with the pathology consistent with OTA-mediated carcinogenesis,<sup>57</sup> generates a similar degree of DNA breakage at the same dose of OTA.<sup>44</sup> These results suggested that additional events are required for renal tumor formation by OTA. This team of researchers then utilized LC-MS/MS techniques to determine the levels of modified DNA bases directly and failed to detect etheno-DNA adducts (associated with lipid peroxidation) and dOG in the kidney and liver of rats exposed to OTA.<sup>22</sup> These findings suggest strongly that oxidative stress mediated by OTA is unlikely to explain the high carcinogenic potency of OTA in rodents.<sup>16,56</sup>

The team of Dekant and Mally have also reported negative results for a direct genotoxic MOA involving covalent DNA adduction by OTA.<sup>22,44,58,59</sup> Thus, they categorize the toxin as a nonmutagenic, non-DNA-reactive carcinogen and propose that OTA-induced carcinogenicity operates through a unique mechanism involving disruption of mitosis and chromosomal instability.<sup>56,60–62</sup> This proposal is based on observations that early pathological changes observed in kidneys of rats treated with OTA exhibit prominent karyomegaly,<sup>63–65</sup> as evidenced by the presence of large polyploid cells, which might be related to OTA carcinogenesis,<sup>66</sup> and is associated with impairment of cell division.<sup>67</sup> Karyomegalic nephropathy was first identified in 1974 by Burry<sup>68</sup> in a 22 year old female who died from liver cell carcinoma. As pointed out by Bhandari and co-workers,<sup>67</sup> the occurrence of polyploidy in karyomegalic nephropathy may be due to a G2 block, as also suggested for the cytostatic effects of OTA.<sup>69</sup> Thus, the team of Dekant and Mally carried out a series of experiments to determine how OTA disrupts mitosis. Treatment of immortalized human kidney epithelial (IHKE) cells with OTA blocked metaphase/anaphase transition and led to the formation of aberrant mitotic figures and giant cells.<sup>60,61</sup> OTA was also found to inhibit microtubule assembly in a concentration-dependent manner in an *in vitro* assay. More recent findings show that OTA blocks histone acetyltransferase (HAT) activity.<sup>82</sup> Thus, an indirect MOA for OTA is proposed that involves disruption of mitosis with HAT as a primary cellular target that acts as a driving force in tumorigenesis and acquisition of a malignant phenotype.

For this indirect MOA, it is uncertain that disruption of mitosis plays a critical role in OTA-induced tumor formation, especially if karyomegaly is indicative of disruption of mitosis that triggers carcinogenesis. For example, in male DA and Lewis rats, OTA-mediated karyomegaly has been shown to be significantly decreased by the addition of 2-mercaptoethane sulfonate (MESNA), but MESNA showed no beneficial effect on renal tumor incidence.<sup>70</sup> Bhandari and co-workers<sup>67</sup> note that in human karyomegalic nephropathy, only one case appears to have been associated with carcinoma, and this occurred in nontarget (liver) tissue. The

NTP study<sup>11</sup> showed that karyomegaly was observed at all three dose levels in female rats; yet, female rats are far less susceptible than males to tumor formation.

## 4. DIRECT GENOTOXICITY

**4.1. Bioactivation.** This MOA for OTA-induced renal carcinogenesis evokes a relatively simple concept. OTA undergoes bioactivation to generate electrophilic species that attach covalently with DNA to generate DNA adducts that stimulate mutagenicity and subsequent renal carcinogenesis. Insight into the bioactivation of OTA can be gleaned from examination of established genotoxic pathways for other chlorophenol toxins, such as pentachlorophenol (PCP). In the presence of CYP450, PCP undergoes oxidative dechlorination to form the electrophilic tetrachlorobenzoquinone (TCBQ) that reacts covalently with sulfhydryl groups,<sup>71</sup> 2'-deoxyguanosine (dG),<sup>72</sup> and other DNA bases<sup>73</sup> to form benzetheno type adducts. TCBQ is known to form covalent DNA adducts in rat liver and is expected to play a key role in PCP-mediated carcinogenesis.<sup>74</sup>

The activation of PCP by enzymes with peroxidase activities furnishes the electrophilic phenoxyl radical.<sup>75</sup> In the presence of glutathione (GSH), the futile thiol pump mechanism will yield GSSG<sup>•-</sup> that can reductively activate O<sub>2</sub> to generate O<sub>2</sub><sup>•-</sup> that can generate free Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub>. The Fenton reaction will then furnish HO<sup>•</sup> to cause oxidative DNA damage that contributes to the toxicity of phenolic xenobiotics.<sup>54,55</sup> The phenolic radical derived from PCP can also react covalently with the C8 site of dG to generate an oxygen (O)-linked C8-PCP adduct<sup>76</sup> that is also formed from reaction of the PCP phenolic radical with DNA.<sup>77</sup>

Pathways that generate TCBQ and the PCP phenolic radical are oxidative. However, reductive processes can lead to dehalogenation (+e/-Cl<sup>-</sup>) to produce reactive aryl radicals.<sup>78</sup> Aryl radicals are well-known to react at the C8 site of purine bases to yield carbon (C)-linked DNA adducts.<sup>79–81</sup> In biological systems, Fe<sup>2+</sup> may act as the reductant to facilitate reductive dehalogenation.<sup>82</sup>

As outlined in Figure 2 for the bioactivation of OTA, pathways analogous to those for PCP are envisioned. Thus, in the presence of CYP450, OTA undergoes oxidative dechlorination to generate the electrophilic quinone OTQ that reacts covalently with GSH to generate the GSH conjugate.<sup>83</sup> In the presence of ascorbate, OTQ is reduced to the hydroquinone *N*-{[(3*R,S*)-5,8-dihydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-isochromen-7-yl]carbonyl}-*L*-phenylalanine (OTHQ).<sup>84</sup> The hydroquinone metabolite OTHQ has been detected in the urine of rats,<sup>59</sup> in the kidneys of male rats,<sup>85</sup> and in blood and urine of Serbian people exposed to OTA.<sup>86</sup> It is also proposed that peroxidase enzymes will cause the one-electron oxidation of OTA to generate the electrophilic phenolic radical<sup>87</sup> that is expected to undergo the futile thiol pump mechanism<sup>54,55</sup> to stimulate oxidative stress through ROS production.<sup>38</sup> This provides a rationale for OTA to deplete GSH in cells.<sup>45</sup> This pathway also suggests that the nonchlorinated analogue OTB should be equally efficient at promoting oxidative damage, as observed.<sup>44</sup> Replacement of the C5–Cl atom of OTA with H will not dramatically impact the reactivity of the phenoxyl radical, given that the phenolates of phenol and 4-chlorophenol have almost identical one-electron oxidative potentials ( $E^{\circ} \sim 0.85$  V vs NHE).<sup>88</sup>

The liberated Fe<sup>2+</sup> from OTA-induced O<sub>2</sub><sup>•-</sup> toxicity, or other reducing equivalents, is proposed to cause reductive dehalogenation

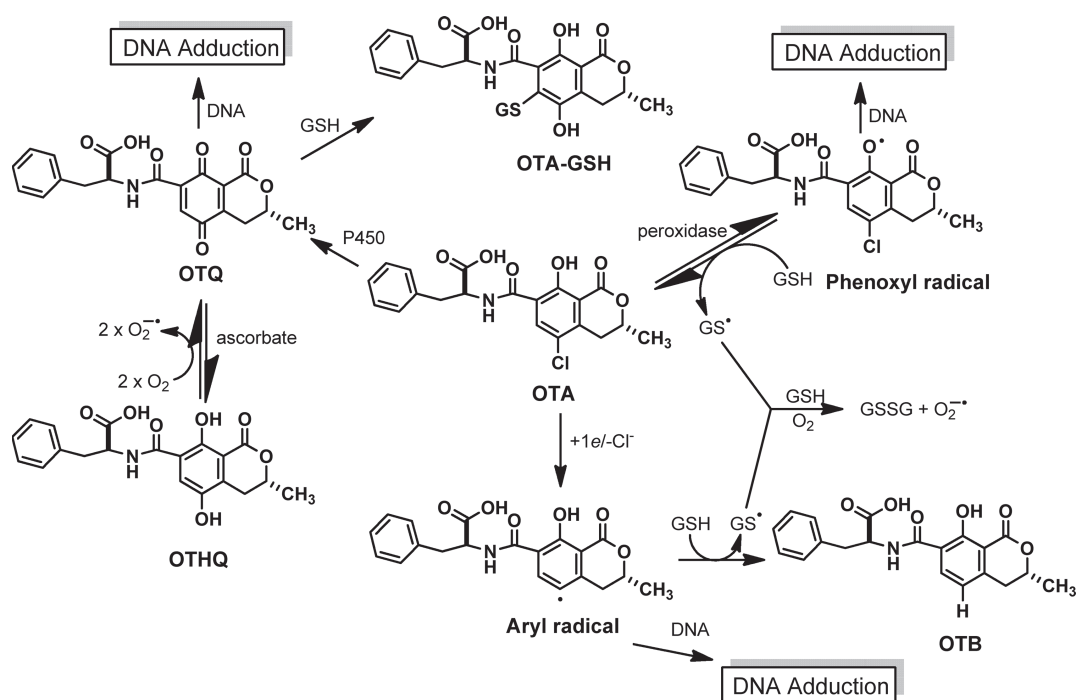


Figure 2. Proposed pathways for the bioactivation of OTA.

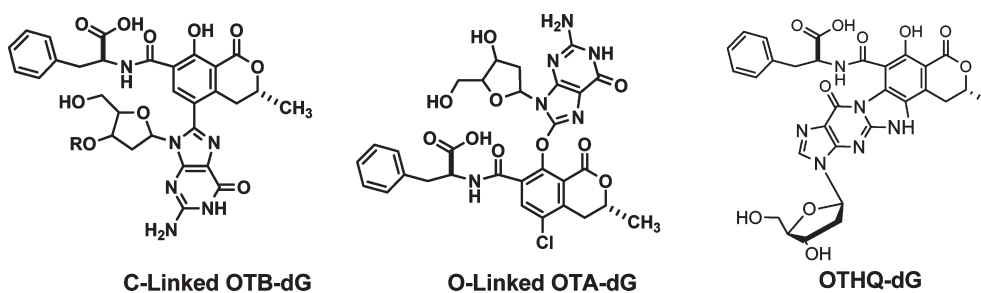


Figure 3. Structures of OTA nucleoside adducts generated photochemically from the reaction of OTA or OTHQ with dG.

of OTA to generate the reactive aryl radical. This hypothesis stems from the structure generated from the photoreaction of OTA with dG.<sup>89</sup> Reaction of the aryl radical with an H-donor also provided a rationale for the production of the nonchlorinated OTB metabolite. Thus, the chemistry of OTA suggests that it will generate reactive radical species (phenolic and aryl radicals) and an electrophilic quinone OTQ.

**4.2. DNA Adduction.** Experiments carried out by Obrecht-Pflumio and Dirheimer suggested that OTA forms guanine-specific DNA adducts.<sup>90,91</sup> This prompted the Manderville laboratory to examine the reactivity of OTA toward dG. Figure 3 shows structures of OTA nucleoside (dG) adducts. The C-linked OTB-dG adduct was initially derived from the photoreaction of OTA in the presence of excess dG and was definitively identified by mass spectrometry and NMR.<sup>89</sup> The C-linked adduct was also produced from reaction of OTA/dG in the presence of  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ , and horseradish peroxidase (25 units/mL)/1 mM  $\text{H}_2\text{O}_2$ .<sup>89</sup> While each system converted OTA into the C-linked adduct, free  $\text{Fe}^{2+}$  was the most efficient, yielding the adduct in  $\sim 5$  orders of magnitude greater than  $\text{Cu}^{2+}$  and HRP/ $\text{H}_2\text{O}_2$ .<sup>89</sup> At the time of these experiments, it was speculated that the OTB-dG adduct

was generated from attachment of the OTA phenoxyl radical.<sup>89</sup> However, given that photolysis of OTA yields the OTA phenolic radical and solvated electrons ( $e_{\text{aq}}^-$ ),<sup>92</sup> it was speculated that  $e_{\text{aq}}^-$  may initiate decomposition of the toxin to afford the carbon-centered aryl radical (Figure 2) and  $\text{Cl}^-$  and that the C-linked OTB-dG adduct was in fact derived from direct aryl radical attachment to the C8 site of dG.<sup>17</sup> That  $\text{Fe}^{2+}$  was most effective at producing OTB-dG<sup>89</sup> suggested that it may have acted as a reducing agent to cause reductive dehalogenation of OTA to afford the aryl radical species.

Upon further examination of the photolysis of OTA in the presence of dG, a second adduct (produced in lower yield than OTB-dG) was identified by mass spectrometry and UV spectroscopy.<sup>93</sup> The UV spectrum of the adduct displayed a single phenolic absorbance at 290 nm that was insensitive to pH changes, suggesting loss of the phenolic H-atom. Its MS spectrum showed the characteristic chlorine isotope peak and had a molecular ion consistent with attachment of OTA to dG with loss of two H atoms. Given that photolysis of OTA is known to generate the phenolic radical<sup>92</sup> that reacts at the C8 site of dG, as noted for  $\text{HO}^\bullet$ ,<sup>94</sup> the adduct was ascribed to the O-linked

OTA-dG adduct shown in Figure 3.<sup>93</sup> The final adduct shown in Figure 3 OTHQ-dG was derived from the photoreaction of OTHQ in the presence of excess dG.<sup>85</sup> The photolysis of OTHQ generates the quinone electrophile OTQ.<sup>83</sup> The adduct OTHQ-dG exhibits a UV spectrum with  $\lambda_{\text{max}} = 376$  nm and a mass of 632. This suggested attachment of dG (267) to OTHQ (385) (267 + 385 = 652) with loss of 20 mass units [OH (17) + 3H]. The proposed structure for OTHQ-dG shown in Figure 3 is consistent with its mass observed by LC-MS and the known tendency of quinone electrophiles to react with dG to form benzetheno type adducts.<sup>72,73,77</sup> Thus, the adduct structures shown in Figure 3 are derived from the electrophilic radicals and quinone OTQ species outlined in Figure 2 for the bioactivation of OTA.

The Manderville Perspective in 2005<sup>17</sup> outlined the <sup>32</sup>P-post-labeling evidence for DNA adduction by OTA in animal tissue that was carried out prior to 2004 without adduct standards for comparison.<sup>95–101</sup> These experiments implicated an oxidative pathway using kidney microsomes and certain biotransformation enzymes for OTA bioactivation<sup>98–101</sup> and highlighted a potential role for the quinone (OTQ) pathway in OTA DNA adduction.<sup>70</sup> This prediction was substantiated by examination of DNA adduction by OTHQ that undergoes autooxidation to generate OTQ that can react with DNA in mammalian cells, as evidenced by <sup>32</sup>P-postlabeling experiments.<sup>102</sup> Finally, evidence showing that the C-linked OTB-dG adduct (Figure 3) standard comigrates with <sup>32</sup>P-postlabeling adducts detected in the kidney of rats and pigs following OTA exposure<sup>93,103</sup> was also discussed.<sup>17</sup>

Despite these published <sup>32</sup>P-postlabeling data on DNA adduction by OTA, others, notably the Dekant and Mally research team<sup>22,44,58,59</sup> and the Nestlé Research Centre,<sup>104</sup> have been unable to repeat these findings. They observe no adduct spots using <sup>32</sup>P-postlabeling upon treatment of rats with OTA<sup>44,59</sup> and have been unable to detect covalent DNA adducts by OTA in rats using LC-MS/MS.<sup>22,104</sup> That <sup>32</sup>P-postlabeling does not provide structural information and high resolution makes it possible that the observed spots do not actually contain attached OTA. While we have presented arguments for their lack of success,<sup>9,85,93,105,106</sup> it was imperative to obtain structural evidence for the adduct spots consistently observed using <sup>32</sup>P-postlabeling.

To address this challenge, rat kidney tissue from OTA-treated rats were initially analyzed for DNA adducts by <sup>32</sup>P-post-labeling.<sup>107</sup> Rat kidney samples were coded for blind analysis for adducts, to avoid suggestion of analytical bias. Autoradiographs relating to the kidney of each animal were copied electronically for matching to the blind code. No adduct spots were present in samples from control (untreated), while the kidneys of all OTA-treated rats analyzed after three consecutive doses of OTA showed one or two radioactive spots, indicating the usual <sup>32</sup>P-postlabeling evidence of adducted DNA following OTA treatment.<sup>93</sup> The abundance of the major adduct across the 10 rats was in the range of 20–70 adducts per 10<sup>9</sup> nucleotides.<sup>107</sup>

Further evidence for the nature of the major adduct spot was obtained using LC-MS/MS with the authentic C-linked OTB-dG adduct standard (Figure 3) for comparison. For these experiments, preparative isolation of adducts generated from in vitro interaction between OTA and calf thymus (CT) DNA provided material for LC-MS/MS analysis. The protocol for <sup>32</sup>P-postlabeling of the preparative OTA/CT-DNA reaction was exactly the same as that used for the in vivo rat kidney DNA, except that unlabeled cold ATP was used instead of that radiolabeled with hot <sup>32</sup>P. Chromatography of the cold preparative samples adjacent to the hot

kidney samples on PEI/cellulose TLC plates was then carried out with the <sup>32</sup>P-postlabeled kidney DNA adduct spots, providing a guide for preparative excision of unlabeled DNA material for LC-MS/MS analysis. The LC-MS/MS data confirmed the presence of the C-linked OTB-dG adduct (Figure 3) as the major adduct from the in vitro OTA/CT-DNA sample that comigrated with the principle adduct spot observed in the rat kidney DNA samples.<sup>107</sup>

**4.3. Mutagenicity.** Prior to the Manderville and Turesky Perspectives in 2005,<sup>17,18</sup> positive evidence for OTA mutagenicity had been reported. De Groene and co-workers showed that OTA induced mutagenicity in NIH/3T3 cell lines expressing human CYP450s 1A1, 1A2, 2C10, and 3A4 and a shuttle vector containing the *lacZ'* as the reporter gene.<sup>108</sup> OTA was then shown to induce revertants in the Ames reversion assay using *Salmonella typhimurium* with metabolic activation by mouse kidney in tester strains TA98, TA1535, and TA1538, but not in strains TA100 or TA102.<sup>109</sup> However, the Dekant laboratory then published negative evidence for OTA-mediated mutagenicity,<sup>110</sup> which seemed to negate the positive evidence. This led to controversies and doubt surrounding the positive findings, as pointed out by Turesky to support the claim that OTA is not a genotoxic carcinogen.<sup>18</sup> Evidence presented by Palma and co-workers in 2007 provided further confirmation that OTA does not act by a direct genotoxic mechanism.<sup>111</sup> They reported that OTA-mediated mutagenicity seems consistent with oxidative DNA damage and that bioactivation is not a requirement.<sup>111</sup>

While the status of OTA as a mutagenic agent seemed in doubt, Kuiper-Goodman and co-workers from Health Canada recently re-examined the mutagenicity data and noted that the experiments carried out in 1999 by Obrecht-Pflumio et al.<sup>109</sup> and the study by the Dekant laboratory<sup>110</sup> in 2001 were actually in agreement.<sup>16</sup> As outlined by Health Canada,<sup>16</sup> mutagenicity experiments conducted by the Dekant laboratory<sup>110</sup> were carried out using strains TA100 and TA2638 (genetically related to TA102), which gave negative results in the Obrecht-Pflumio study. They did not use the *S. typhimurium* strains (TA98, TA1535, and TA1538) that gave positive results but claimed that their findings do not support the positive responses and that OTA is not a mutagenic agent. Thus, in the view of Health Canada,<sup>16</sup> the significance of positive mutagenic responses by OTA in the modified Ames test<sup>109</sup> has been overlooked. This claim now appears to be justified, as new positive evidence for OTA mutagenicity has been reported by Hibi and co-workers at the National Institute of Health Sciences in Tokyo, Japan.<sup>112</sup>

In the Hibi study, the *gpt*  $\delta$  transgenic rat model was employed, which is particularly useful for investigating the genotoxicity of reagents in vivo and can identify both point mutations by the *gpt* assay and certain types of deletions and frameshift mutations using the Spi<sup>-</sup> assay.<sup>113,114</sup> Groups of 4–5 male and female *gpt*  $\delta$  rats were administered a carcinogenic dose of OTA at a concentration of 5 ppm in the basal diet for 4 or 13 weeks. In both sexes, apoptosis and karyomegaly of tubular epithelial cells were prominent in the outer stripe of the outer medulla, while other regions of the kidney were not affected. Hibi et al. note that the distribution of OTA in the kidney is not uniform, and the affected tubules were the S3 segment of the proximal tubules in both male and female *gpt*  $\delta$  rats showing essentially equal extent of histopathological changes (apoptosis and karyomegaly).<sup>112</sup>

DNA extracted from whole kidneys from both sexes failed to show mutagenicity in the reporter gene mutation assay. The kidney was then cut into three parts, the cortex, outer medulla,



and inner medulla, and the reporter gene mutation assay was performed using DNA extracted from the cortex (nontarget site) and outer medulla (target site). Results from the kidney of male *gpt*  $\delta$  rats are presented, and the Spi<sup>-</sup> mutation frequencies (MFs) of the outer medulla were significantly increased as compared to controls, while *gpt* MFs were not changed. In the cortex from male kidney, no increase in MFs was observed.

Because oxidative damage can promote a positive response in the Spi<sup>-</sup> assay, levels of dOG in the outer medulla DNA were determined. However, no changes in dOG levels were detected, and OTA exposure did not significantly increase the frequencies of GC:TA transversion mutations, which are characteristic of dOG mutagenicity.<sup>112</sup> Hibi and co-workers state that their findings strongly support the notion that oxidative DNA damage does not contribute to renal carcinogenesis following OTA exposure of rats and that a direct genotoxic MOA appears consistent with their findings.<sup>112</sup>

## 5. CONCLUSIONS AND FUTURE STUDIES

The effects of ochratoxins have massive socioeconomic implications, which make the elucidation of the MOA of this toxin imperative.<sup>115</sup> The MOA should explain all of the effects observed both in vivo and in vitro and provide a role for the C5–Cl atom of OTA, as its removal to generate the nonchlorinated OTB analogue is known to decrease toxicity considerably. For the indirect MOA for OTA discussed in this Perspective, new evidence does not support the role of oxidative stress as the initiating factor leading to OTA-mediated carcinogenesis. The Dekant and Mally research team has shown that Fpg-mediated DNA strand scission, as evidenced by the comet assay and indicative of oxidative DNA damage, is not target (kidney) specific with similar levels being detected in liver and kidney.<sup>44</sup> This indirect MOA also does not provide a rationale for the lack of toxicity mediated by OTB, as a similar degree of DNA breakage at the same dose of OTA occurs with OTB in rat.<sup>44</sup> Direct analysis of DNA for biomarkers of oxidative DNA damage<sup>22,112</sup> and adducts consistent with lipid peroxidation<sup>22</sup> failed to generate positive evidence in rat, and in vivo mutations generated by OTA in male rat kidney are not consistent with oxidative DNA damage.<sup>112</sup> This evidence suggests strongly that oxidative stress, as an indirect MOA, does not appear to be the major contributing factor in OTA renal carcinogenicity.<sup>16,22,112</sup>

The second indirect MOA outlined in this Perspective is that OTA causes disruption of mitosis that leads to renal carcinogenesis.<sup>56,60–62</sup> This indirect MOA is consistent with the histopathology of OTA-mediated nephrotoxicity that exhibits prominent karyomegaly,<sup>63–65</sup> as evidenced by the presence of large polyploid cells, which is indicative of blocked nuclear division during mitosis.<sup>67</sup> In this MOA, OTA is proposed to interfere with histone acetyltransferases (HATs) that are the primary cellular target of OTA. The interference of HAT promotes disruption of mitosis that triggers renal carcinogenesis.<sup>62</sup> However, for this indirect MOA, it is uncertain that disruption of mitosis plays a critical role in OTA-induced tumor formation. Female rats are known to be far less susceptible to OTA-induced tumor formation, yet show prominent karyomegaly in the outer stripe of the outer medulla<sup>112</sup> that is indicative of disruption of mitosis. Furthermore, in male DA and Lewis rats, OTA-mediated karyomegaly has been shown to be significantly decreased by the addition of MESNA, but MESNA showed no beneficial effect on renal tumor incidence.<sup>70</sup> OTA-mediated karyomegaly is also inhibited

by addition of aspartame.<sup>66</sup> It would be informative to determine whether aspartame inhibits HAT disruption, and subsequent deletion mutations that now have been shown to be a characteristic of OTA-mediated in vivo mutagenicity in the *gpt*  $\delta$  transgenic rat model.<sup>112</sup>

The final MOA outlined in this Perspective involves direct genotoxicity (covalent DNA adduct formation and mutagenicity) by OTA. The C-linked OTB-dG adduct (Figure 3) has been fully characterized by NMR spectroscopy,<sup>89</sup> and new evidence suggests its formation in male rat kidney exposed to OTA.<sup>107</sup> OTA is also able to induce reporter gene mutations at the target site of male rats, strongly suggesting involvement of direct genotoxicity.<sup>112</sup> This MOA can explain the lack of tumorigenicity mediated by the nonchlorinated analogue OTB, as OTB does not generate the OTB-dG adduct; the C5–Cl atom of OTA plays a critical role in OTB-dG formation.<sup>17</sup> Future studies should address the biological impact of the C-linked OTB-dG adduct and determine its mutational spectrum. Simple alkylating agents that preferentially induce base substitutions generate *gpt* mutations but not Spi<sup>-</sup> mutations in the *gpt*  $\delta$  transgenic rat model.<sup>113</sup> However, arylamine carcinogens, such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), which induce frameshift mutations, exhibit an Spi<sup>-</sup> phenotype in the *gpt*  $\delta$  transgenic rat model.<sup>116</sup> The induction of Spi<sup>-</sup> mutations by PhIP is ascribed to formation of the N-linked C8-PhIP-dG adduct<sup>116</sup> that is structurally related to the C-linked OTB-dG adduct, in that a bulky group is attached to the C8-site of dG.

In summary, that OTA is mutagenic in the target tissue of male rat kidney is a significant new finding. The LC-MS/MS evidence for DNA adduction by OTA in male rat kidney also provides new evidence that OTA can react directly with DNA. At present, the DNA adducts are indicators of exposure only, and not mutagenesis and carcinogenesis. The link between DNA adduction and deletion mutations generated by OTA has not been developed and nor has the link between deletion mutations and OTA-mediated carcinogenicity. That OTA can react covalently with DNA and generate mutations does not necessarily mean that it operates by a nonthreshold MOA. Regardless, the IARC classification of OTA in 1993 as a group 2B carcinogen based on the absence of MOA and absence of human data should be updated given the new mutagenicity and genotoxicity findings for OTA outlined in this Perspective. Furthermore, the EFSA TWI re-established in 2006 based only on OTA-mediated nephrotoxicity should be revised given that the toxin is clearly carcinogenic and mediates in vivo mutagenicity at the target organ.

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## ABBREVIATIONS

OTA, *N*-{[(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-isochromen-7-yl]carbonyl}-*L*-phenylalanine; OTB, *N*-{[(3*R,S*)-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-isochromen-7-yl]carbonyl}-*L*-phenylalanine; OTHQ, *N*-{[(3*R,S*)-5,8-dihydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-isochromen-7-yl]carbonyl}-*L*-phenylalanine; MOA, mechanism of action; dG, 2'-deoxyguanosine; MESNA, 2-mercaptoethane sulfonate.

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