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Review

Mycotoxins and the kidney: Modes of action for renal tumor formation by ochratoxin A in rodents

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The mycotoxin ochratoxin A (OTA) is a potent renal carcinogen in rodents. OTA is only slowly eliminated due to high affinity to plasma proteins and inefficient biotransformation. A number of mechanisms have been proposed to account for renal tumor formation. DNA adduct formation, mainly based on the ^{32}P-postlabeling assay, was postulated as a mode of action (MoA). However, studies using radiolabeled OTA or MS failed to demonstrate formation of OTA-derived DNA-adducts. While some studies have demonstrated generation of oxidative stress by OTA, the oxidative stress response appears to be not very pronounced and therefore may not explain the high potency of OTA in rodents. A number of recent investigations support the hypothesis that OTA causes disruption of mitosis resulting in blocked or asymmetric cell division. This may present an increased risk of aneuploidy acquisition and may play a critical role in OTA-induced tumor formation. The absence of DNA-adducts derived from OTA supports a thresholded MoA, and a tolerable weekly intake (TWI) of 120 ng OTA/kg bw has been derived by the European Food Safety Authority. The estimated intake of OTA in Europe is below this TWI for most of the population.

Keywords: Carcinogenicity / Kidney / Mycotoxins / Nephrotoxicity / Ochratoxin A

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1 Introduction

Mycotoxins are a structurally diverse class of secondary metabolites of fungal origin. Toxins produced by *Alternaria*, *Aspergillus*, *Fusarium*, and *Penicillium* species are frequently found in a wide variety of food items due to fungal infection of plant hosts or crops during storage, allowing for the production of these secondary metabolites in the field or postharvest. Ingestion of mycotoxin-contaminated feed may present a serious problem to human health, as mycotoxins have been shown to induce a range of adverse effects in animals, including acute toxicity, developmental and reproductive toxicity, immunosuppression, and carcinogenicity. Due to the high renal blood flow, causing delivery of high amounts of xenobiotics to the kidneys, and the presence of a variety of transporters, enabling active uptake and intercellular accumulation of toxins, the kidney is an important target of a number of mycotoxins. These include aflatoxin B1, which has been shown to alter renal function in addition to its potent hepatotoxic effects [1], and citrinin and patulin, both produced by toxic strains of the Penicillium and Aspergillus family. Importantly, fumonisin B_1 and ochratoxin A (OTA) have both been classified as having "clear evidence of carcinogenic activity" based on increased incidences of renal tumors in male, respectively male and female rats following chronic exposure [2, 3]. In fact, OTA is recognized as being one of the most potent carcinogens tested by the NTP to date, exhibiting a combined incidence of renal adenomas and carcinomas of 72% in male rats at relatively low doses (210 µg/kg bw). Interestingly, tumors induced by fumonisin B_1 and OTA have been reported to represent an exceptionally malignant phenotype, with highly invasive growth and potential to metastasize [4–6]. The mechanisms by which these two structurally unrelated mycotoxins-induce renal tumor formation, and particularly the reasons for the aggressive nature of the tumors are poorly understood. A large number of mechanistic studies on OTA-induced nephrotoxicty and renal tumor formation is available. Thus, the purpose of this review is to summarize the existing mechanistic data on the mode of action (MoA) of OTA-induced renal tumors.

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Abbreviations: BEN, Balkan endemic nephropathy; **BrdU,** 5-bromo-2′-deoxyuridine; **ERK1/2,** extracellular signal-regulated kinase; **MoA,** mode of action; **Nrf2,** nuclear factor-erythroid 2-related factor 2; **OSOM,** outer stripe of the outer medulla; **OTA,** ochratoxin A; **OTQ,** quinone derivative of OTA

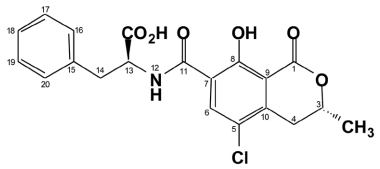
Table 1. Categorization of renal carcinogens based on mechanistic information (modified from [7])

		Examples
Category 1	Chemicals inducing renal tumors through direct interaction of the parent compound or metabolite with renal DNA	Anthraquinone, 1,2,3,–trichloropropane
Category 2	Chemicals inducing renal tumors through indirect DNA reactivity mediated by oxidative stress	Iron overload, potassium bromate
Category 3	Chemicals inducing renal tumors *via* indirect cytotoxicity and sustained tubule cell regeneration	Chloroform, d-limonene,1,4-dichlorobenzene
Category 4	Chemicals increasing the incidence of renal tumors through exacerbation of spontaneous chronic progressive nephropathy (CPN)	Hydroquinone, ethyl benzene
Category 5	Chemicals increasing the incidence of renal tumors through an unknown mechanism, but which also exacerbate CPN	Quercetin, benzofuran
Category 6	Chemicals increasing the incidence of renal tumors through an unknown mechanism, but which do not exacerbate CPN	Pyridine, monuron

2 Categorization of renal carcinogens

In experimental animals, renal tumor induction has been demonstrated with more than 100 chemicals, and nearly all types of renal neoplasms described in human pathology can also be observed in small rodents. As with many other tumors, different modes of action (MoA) for induction of tumors by chemicals have been elucidated and renal carcinogens have been categorized based on these MoAs [7]. Several renal carcinogens are thought to induce tumors through a direct interaction of the parent compound or a reactive intermediate formed in the kidney with DNA to produce premutagenic lesions as a primary MoA. Such chemicals are usually genotoxic in appropriate *in vitro* testing systems when using activation conditions considering the specific biotransformation reactions responsible for renal toxicity of these chemicals. However, a number of renal carcinogens are not mutagenic; for these chemicals, other primary MoAs have to be assumed as responsible for tumor induction. Oxidative DNA damage induced by direct oxidation of DNA constituents or through oxidative stress has been implicated in renal tumor formation in iron overload and potassium bromate (Table 1). For a large number of other chemicals, cytotoxicity and the high capacity of the proximal tubular epithelium to regenerate by increased proliferation after induction of cell death is considered as a primary MoA [7–9]. These chemicals include halogenated alkanes and alkenes, which undergo bioactivation by glutathione conjugation or bioactivation by cytochromes P450, and several chemicals, which bind to α_{2u}-globulin [7, 10–15]. Other chemicals have been shown to increase the incidence of renal tumors by exacerbation of the spontaneous chronic nephropathy in aged rats.

Since the primary MoA has a major influence on the procedures applied for assessments of health risks due to human exposure to renal carcinogens, a detailed evaluation of the MoA is mandatory to come to a science-based risk assessment. In short, for carcinogens causing tumors by genotoxic MoA, the presence of thresholds, by default, is not considered and the risk assessment process needs to extrapolate tumor incidences observed in animals given high doses of the carcinogen to predict potential tumor incidences in humans exposed to much lower doses. These predicted tumor incidences of specified exposures are then related to tumor incidences considered as acceptable by regulatory authorities. For nongenotoxic chemicals, thresholds based on the induction of cytotoxicity may be defined and tolerable daily intakes can be derived using the safety factor methodology [16, 17].



Ochratoxin A

Figure 1. Chemical structure of OTA.

3 Toxicology of ochratoxin A

OTA (*N*-[(3*R*)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl)-carbonyl]-l-phenylalanin, CAS (303-47-9)) (Fig. 1) is a mycotoxin produced by several strains of *Aspergillus* and *Penicillium* species. OTA was first isolated in 1965 from a culture of *Aspergillus ochraceus*. OTA consists of a dihydroisocoumarin subunit, which is linked to phenylalanine *via* a peptide bond.

The toxicity of OTA has been intensively studied. Dogs and pigs (LD_{50} 0.2 and 1 mg/kg bw, respectively) have been shown to be more sensitive to acute toxicity of OTA than rats (LD_{50} 20–30 mg/kg bw) and mice (LD_{50} 46–58 mg/kg bw). The kidney is the main target of OTA toxicity in all animal species tested. Exposure of pigs to doses of OTA as low as 8 μg/kg bw for 90 days was reported to cause impaired kidney function [18], and chronic feeding of pigs with OTA-contaminated diet resulted in progressive nephr-

opathy characterized by degeneration and atrophy of proximal tubules and interstitial fibrosis [19, 20].

In short-term studies in rats, OTA nephrotoxicity is characterized by polyuria and histopathological alterations consisting of disorganization of the tubule arrangement, single cell degeneration, and frequent, sometimes abnormally enlarged mitotic figures, usually restricted to the straight segment of the proximal tubule (S3) [21]. Prominent karyomegaly and polyploidy of proximal tubule cells, which occur even after short-term exposure and are indicative of nuclear division without cytokinesis, are perhaps the most striking features of OTA-induced kidney pathology [21 – 23].

In rodents, OTA is also a potent renal carcinogen, with male rats being most susceptible to renal tumor formation by OTA. In a 2-year carcinogenicity bioassay, treatment with OTA at doses of 70 and 210 μg/kg bw was shown to induce high incidences of renal adenomas and carcinomas in male F344 rats, whereas no increased tumor rates were observed following exposure to a low dose of 21 μg/kg bw, indicating a nonlinear dose-response for renal tumor formation by OTA [3] (Table 2). Tumors in rats developed from the straight segment of the proximal tubule (P3) in the outer stripe of the outer medulla (OSOM), the target site of OTA nephrotoxicity, with a relative rapid onset and unusually high rates of metastases [3, 4].

For many years, dietary human exposure to OTA has been suspected to be involved in balkan endemic nephropathy (BEN), a progressive tubulointerstitial kidney disease associated with an increased risk for the development of urothelial cancers which occurs in some rural areas of Bosnia, Bulgaria, Croatia, Romania, and Serbia. However, the epidemiological data are insufficient to establish a causal link between OTA and BEN, and more recent evidence suggests that chronic exposure to aristolochic acid *via* flour contaminated with seeds of *Aristolochia clematitis* may play a role in the etiology of BEN [24 – 26].

4 Biotransformation and toxicokinetics of ochratoxin A and relevance to renal toxicity

After oral administration OTA is absorbed predominantly from the small intestine in its lipid-soluble, nonionized form. Oral bioavailability in experimental animals is between 40 and 60% [6]. Maximal plasma concentrations were reached within 24 – 48 h after application of a single oral dose of 0.5 mg/kg bw to male and female F344 rats [27]. OTA has a very high affinity to plasma proteins and 99.9% of the circulating OTA is bound to plasma proteins. OTA is poorly metabolized and slowly excreted, with a plasma half-life of 230 h [27]. Elimination occurs in the form of the parent compound and as ochratoxin α following hydrolysis of the peptide bond, presumably by carboxypeptidases produced by the intestinal microflora. In addition,

Table 2. Renal tumor incidence in male and female F344 rats following chronic exposure to OTA [3]

		OTA (μg/kg bw/day)			
		0	21	70	210
Adenoma	♂	1/50 (2%)	1/51 (2%)	6/51 (12%)	10/50 (20%)
	♀	0/50 (0%)	0/51 (0%)	1/50 (2%)	5/50 (10%)
Carcinoma	♂	0/50 (0%)	0/51 (0%)	16/51 (31%)	30/50 (60%)
	♀	0/50 (0%)	0/51 (0%)	1/50 (2%)	3/50 (6%)
Total	♂	1/50 (2%)	1/51 (2%)	20/51 (29%)	36/50 (72%)
	♀	0/50 (0%)	0/51 (0%)	1/50 (4%)	8/50 (16%)

pentose and hexose conjugates of OTA are the only OTA-metabolites identified by MS-based methods in rodent urine after OTA exposure (Fig. 2) [27]. The high plasma protein binding, tubular reabsorption of filtered OTA in the kidney, and the slow biotransformation to polar and excretable metabolites may explain the very long half-life of OTA in rodents and in humans.

Using subcellular fractions or isolated enzymes, OTA is transformed to additional metabolites at very low rates. In the presence of rat and mice liver microsomes OTA was oxidized to (4*R*)- and (4*S*)-hydroxy-OTA, although the rate of formation was low (Fig. 2) [28]. In rabbit liver microsomes 10-hydroxy-OTA was also identified as a biotransformation product.

Considerations of the chemical reactivity of OTA have proposed further possible metabolic pathways. Electrochemical and photochemical oxidation of OTA results in formation of a quinone derivative of OTA (OTQ)/hydroquinone derivative of OTA (OTQ/OTHQ) redox couple, which may generate reactive oxygen species and thus, oxidative DNA lesions through redox cycling [29, 30]. In addition, the electrophilic OTQ may react with tissue nucleophiles, as shown by the formation of a glutathione conjugate following autoxidation of OTHQ in the presence of glutathione [31]. However, formation of metabolites derived from OTHQ or OTQ such as glutathione conjugates or corresponding mercapturic acids have not been demonstrated in rodents or in *in vitro* systems with respective enzymatic capacities. These observations suggest that these metabolic pathways are unlikely to occur to a relevant extent in rodents. Therefore, studies on the biotransformation of OTA do not give indications that reactive electrophilic metabolites capable of covalent binding to DNA are formed in rat kidney.

There is evidence to suggest that the sex- and organ-specific toxicity of OTA may, in part, be attributable to toxicokinetics of OTA. Zepnik *et al*. [27] determined OTA concentrations in livers and kidneys of male and female F344 rats following a single oral dose of OTA (0.5 mg/kg bw) over a period of 96 days. In livers of both male and female rats, OTA concentrations were less than 12 pmol/g tissue, with a maximum at 24 h after administration. In contrast,

OTA accumulated in the kidneys, reaching a concentration of 480 pmol/g tissue in males 24 h after OTA-administration. Importantly, OTA levels in kidneys of female rats were significantly lower (<200 pmol/g tissue) than those observed in male rats, which may be related to differential expression of organic anion transporters, resulting in increased renal absorption of OTA in male rats.

In contrast to this study, no significant differences in organ concentrations were detected in male rats following repeated administration at doses of 0, 21, 70, and 210 μg/kg bw for 14, 28, and 90 days or after short-term treatment with up to 2 mg/kg bw [21, 23]. However, it should be noted that a single time-point (*i.e.*, 24 h after administration) was used to determine tissue levels, and therefore, these data may not reflect potential differences in peak concentrations in these tissues. It is also important to emphasize that OTA may preferentially accumulate in certain regions of the nephron, *e.g.*, in tubule cells of the *pars recta*, which represent the target cells of OTA toxicity. For a comprehensive understanding of the sex-, organ- and site-specificity of OTA toxicity, it is critical to appreciate regional differences in transport and accumulation of OTA.

5 Proposed modes of action for ochratoxin A kidney tumorigenicity

5.1 DNA-adduct formation

The majority of studies in bacteria suggest that OTA is not mutagenic in bacteria [3, 28, 32–35]. An evaluation of OTA by the German MAK-Commission states that the two positive bacterial mutagenicity tests on OTA [36, 37] were obtained in "unvalidated systems" [38]. More recent mutagenicity studies on OTA in bacteria using a variety of metabolic activation systems were consistently negative [28, 32]. Regarding effects in mammalian cells, a number of studies indicate weak genotoxic potential of OTA in mammalian cells, *e.g.*, DNA strand breaks, unscheduled DNA repair, sister chromatid exchange, micronuclei [39–43], whereas others were negative [3, 35, 44]. Some of the positive findings are difficult to interpret as genotoxic effects were associated with cytotoxicity [38]. In a recent study, OTA did not induce chromosomal aberrations, sister chromatid exchanges, or micronuclei in human lymphocytes and V79 cells both in the absence or presence of bioactivation systems [44]. Interestingly, however, cytogenetic analyses revealed a significant increase in endoreduplicated cells, indicating two successive rounds of DNA replication without intervening mitosis, and highly condensed and abnormally separated chromatids in both cell types [44].

The role of covalent DNA binding of OTA or a metabolite has been a matter of much debate [45–47]. Based on positive ^{32}P-postlabeling results, some authors postulate that OTA forms covalent DNA adducts [48, 49], while several *in vitro* and *in vivo* studies using radiolabeled OTA consistently failed to detect radioactivity associated with DNA [50–53], suggesting that the spots observed by postlabeling may not contain OTA or parts of the OTA molecule. Although one of the spots observed by ^{32}P-postlabeling was reported to cochromatograph with a carbon-bonded OTA-deoxyguanosine adduct (dGuoOTA, Fig. 3) generated by photoirradiation of OTA in the presence of 2′-deoxyguanosine [54], formation of this adduct in rat kidney *in vivo* could not be confirmed by ^{32}P-postlabeling and stable isotope dilution LC-MS/MS [55, 56], with LODs as low as 3.5 dGuoOTA/10^9 nucleotides.

The absence of covalent DNA adducts is consistent with the lack of bioactivation to reactive metabolites [28, 51, 52] and the finding that OTA-mediated genotoxicity in mammalian cells is independent of metabolic activation [39, 57]. In taking all the available data into consideration, the EFSA scientific panel on contaminants in the food chain concluded that there was no evidence for the existence of specific OTA–DNA adducts and that the genotoxic effects of OTA were most likely attributable to oxidative stress [58].

5.2 Oxidative stress

Sustained cytotoxicity and DNA damage mediated by oxidative stress is known to play an important role in tumor formation in rat kidney, and increased incidences of renal tumors have been reported in response to several chemicals thought to induce tumors *via* generation of oxidative stress such as potassium bromate and Fe(III) nitrilotriacetate [7]. Thus, considering the lack of DNA reactivity, induction of oxidative damage has been proposed as an alternative mechanism of OTA-mediated carcinogenicity. Rahimtula *et al*. [59] investigated the effects of OTA on lipid peroxidation as a marker of oxidative stress by measuring ethane exhalation following treatment of male Wistar rats with a single oral dose of OTA (6 mg/kg bw). Under these conditions, an up to seven-fold increase in the level of ethane in exhaled air was evident in OTA-treated rats as compared to vehicle treated controls. In contrast, no effects on a range of markers indicative of lipid peroxidation, including 1,N^6-etheno-deoxyadenosine, 1,N^2-propano-deoxyguanosine, hydroxyalkenals, and 8-isoprostaglandin F_{2a} were evident following repeated administration of doses up to 2 mg/kg bw for 2 wk, suggesting that lipid peroxidation may only occur in response to relatively high (toxic) doses [21]. Similarly, 4-week treatment of rats with doses in the range that caused kidney tumors in rats after longterm administration, did not result in increased protein carbonyls [60]. Consistent with these findings, OTA did not enhance formation of 8-hydroxy-2′-deoxyguanosine in male rat kidney following single (1 mg/kg bw) [61] or repeated administration (up to 2 mg/kg bw for 2 wk) [21] as determined by LC-MS/MS analysis. In apparent contrast, DNA strand breaks indicative of oxidative DNA lesions were observed in livers and kidneys of OTA-treated male rats using the comet assay in the

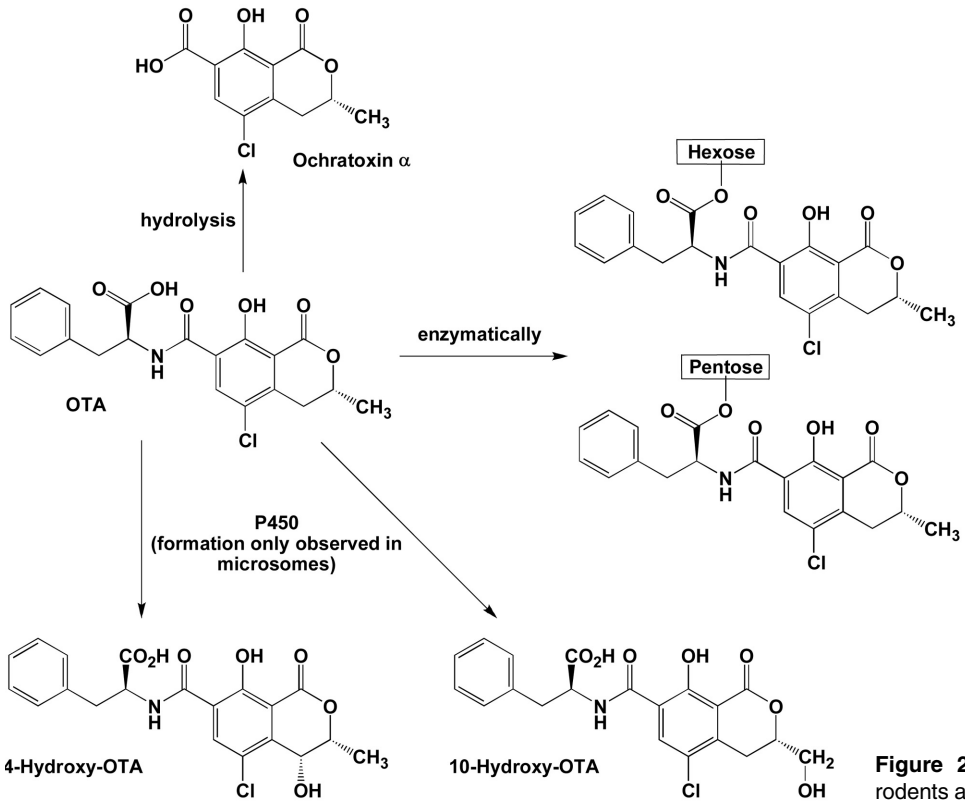


Figure 2. Biotransformation of OTA in rodents and in subcellular fractions.

presence of formamidopyrimidine DNA glycosylase at doses as low as 0.03 mg/kg bw [56, 60, 62]. These *in vivo* findings are further supported by a number of investigations demonstrating DNA breakage in various *in vitro* systems, although there is disagreement with respect to requirement of bioactivation and the role of cytotoxicity in mediating these effects [40, 42, 63 – 66]. In a recent study, OTA was found to induce weak mutagenic effects in mammalian cells independent of bioactivation, with a mutation spectrum similar to that of spontaneous mutants, consistent with the hypothesis that OTA genotoxicity seen in some studies is attributable to oxidative stress [57].

Collectively, these studies provide indirect evidence for OTA to cause oxidative damage, yet how OTA mediates these effects, and particularly the contribution of oxidative stress to the mechanism of OTA toxicity and carcinogenicity remain poorly understood. While some authors hypothesize that the potential of OTA to cause oxidative stress may be linked to its iron chelating properties which may facilitate reduction of Fe^{3+} [67], recent toxicogenomics data suggest that altered expression of genes regulated by the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) may result in impaired antioxidant defense rather than generation of reactive oxygen species [68, 69]. Interestingly, modulation of the Nrf2 pathway, which regulates detoxication and protection from oxidative stress, appears to be target-organ specific [69]. In contrast, two independent studies demonstrate that (oxidative) DNA damage is not restricted to the kidneys, but occurs to a similar extent in livers of OTA-treated animals, even at relatively low doses of OTA [56, 60]. Thus, oxidative stress by itself does not appear to be sufficiently supported by data to be considered as a primary MoA for OTA tumorigenesis. A simple explanation might be that OTA-induced tumor formation occurs as a result of oxidative DNA damage combined with enhanced renal cell turnover, allowing for the conversion of lesions into permanent mutations and clonal expansion of initiated cells. However appealing this hypothesis might be,

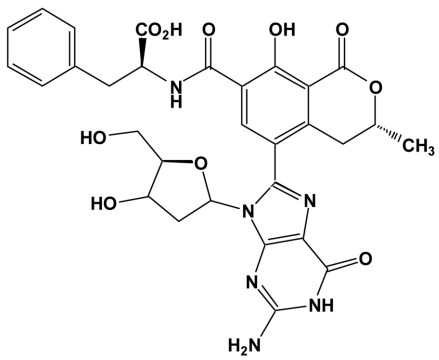


Figure 3. Structure of a carbon-bonded C8-deoxyguanosine adduct of OTA formed by photoirradiation of OTA in the presence of 2′-deoxyguanosine.

it needs to be emphasized that characteristic features of OTA-induced kidney pathology, *i.e.*, the frequency and early onset of karyomegaly and polyploidy, which are rarely seen with other renal carcinogens, as well as the exceptionally aggressive nature of tumors, are difficult to reconcile with a mechanism solely based on sustained cytotoxicity and DNA damage mediated by oxidative stress.

5.3 Cell proliferation (including α_{2u})

Considering the higher susceptibility of male rats to OTA-induced tumor formation, Rasonyi *et al*. [70] speculated that sustained cytotoxicity and compensatory cell proliferation mediated through binding of OTA to the male rat specific urinary protein α_{2u}-globulin (α_{2u}) might play a role in the mechanism of OTA carcinogenicity. However, in contrast to *d*-limonene, which was used as a positive control, treatment of male rats, which are susceptible to α_{2u}-globulin-induced nephropathy, with OTA (1 mg/kg bw for 7 days) did not cause hyaline droplet accumulation or enhanced cell proliferation in the S2 segment of the proximal tubule, the site of reabsorption of α_{2u}-globulin, suggesting that OTA does not bind to α_{2u}-globulin. The lack of involvement of α_{2u}-globulin in OTA toxicity is also consistent with the known target site of OTA nephrotoxicity being the S3 segment of the proximal tubule, in which single cell degeneration was also observed.

In a 90-day oral toxicity study in male rats, early effects of OTA at doses known to cause tumor formation following long-term administration (70 and 210 μg/kg bw) consisted of cell loss accompanied by markedly increased 5-bromo-2′-deoxyuridine (BrdU)-labeling indicative of stimulation of cell proliferation, and prominent nuclear enlargement within the straight segment of the proximal tubule epithelium (S3) in the OSOM [23], from which OTA-induced tumors arise [6]. In contrast, exposure to a low dose of 21 μg/kg bw, which did not result in increased tumor rates in the 2-year bioassay, had no effects on BrdU labeling. Moreover, stimulation of cell proliferation was not evident in livers of treated animals. Using Eker rats, which are uniquely susceptible to both spontaneous and chemically induced renal tumor formation, Stemmer and Dietrich [71] recently reported a higher increase in cell proliferation in male as compared to female rats following administration of 210 μg/kg bw OTA for 14 days, consistent with the known sex differences in tumor response to OTA. Taken together, the sex-, organ-, and site-specificity, the time- and dose-dependency, and close correlation between cell proliferation and tumor incidence in the 2-year bioassay provide substantial evidence to suggest that stimulation of renal cell proliferation is a key event in the mechanism of OTA carcinogenicity.

However, the quality of the proliferative response mediated by OTA appears to be significantly different from what is typically observed with renal carcinogens that induce cell proliferation, such as chloroform or potassium bromate, in that regenerative hyperplasia does not appear to be a feature of OTA-induced kidney pathology. Moreover, treatment with OTA leads to a significant decrease in relative kidney weight [23], whereas increased cell proliferation is generally associated with increased organ weight. This suggests that, despite stimulating cell proliferation, kidneys of OTA-treated animals fail to replace degenerate cells, resulting in an overall loss of kidney mass. In this respect, it is important to recognize that the markers commonly used to determine cell proliferation, *i.e.*, proliferating cell nuclear antigen (PCNA) or incorporation of BrdU into DNA, specifically label cells in S-phase. Thus, even though it is evident from both studies that OTA stimulates DNA replication, this does not imply that cells successfully complete cell division to give rise to viable daughter cells. In contrast, the nuclear enlargement accompanied by a net loss of cells suggests that OTA may interfere with mitosis and/or cytokinesis, resulting in cell death at mitosis or aberrant exit from mitosis leading to production of polyploid cells, which is supported by a recent *in vitro* study demonstrating endoreduplication and polyploidy in human lymphocytes and V79 cells in response to OTA [44]. While proximal tubule cell degeneration seems to predominate over mitotic exit initially, which might be seen as a protective means to prevent generation of genetically unstable polyploid cells, there appears to be a shift toward mitotic exit, cell survival, and re-entry of polyploid cells into a second round of DNA synthesis with continuing OTA exposure, accompanied by an increased risk of aneuploidy acquisition and subsequent tumor formation (Fig. 4). In summary, we hypothesize that it might be the failure of cells to divide in the presence of OTA which causes single cell death, mitotic abnormalities, and stimulation of cell proliferation in an attempt to compensate for the cell loss.

5.4 Modulation of apoptosis

Independent of their primary MoA, a unifying feature of nongenotoxic carcinogens appear to be their ability to interfere with molecules involved in the regulation of cell growth and cell death. Within the multi-step process of carcinogenesis, cell death by apoptosis is generally viewed to protect from oncogenic transformation by eliminating "initiated" or predisposed cells and counterbalancing cell proliferation, whereas inhibition of apoptosis is thought to contribute to tumor formation by promoting survival of initiated cells which would normally be eliminated from the tissue [72]. Evidence for evasion of cell death by apoptosis as a critical event in nongenotoxic carcinogenesis has come from the finding that a number of rat liver tumor promoters such as peroxisome proliferators, suppress apoptosis *in vivo* or *in vitro* [73 – 75].

However, numerous *in vitro* studies indicate that OTA induces rather than inhibits apoptosis [65, 76 – 81], and this

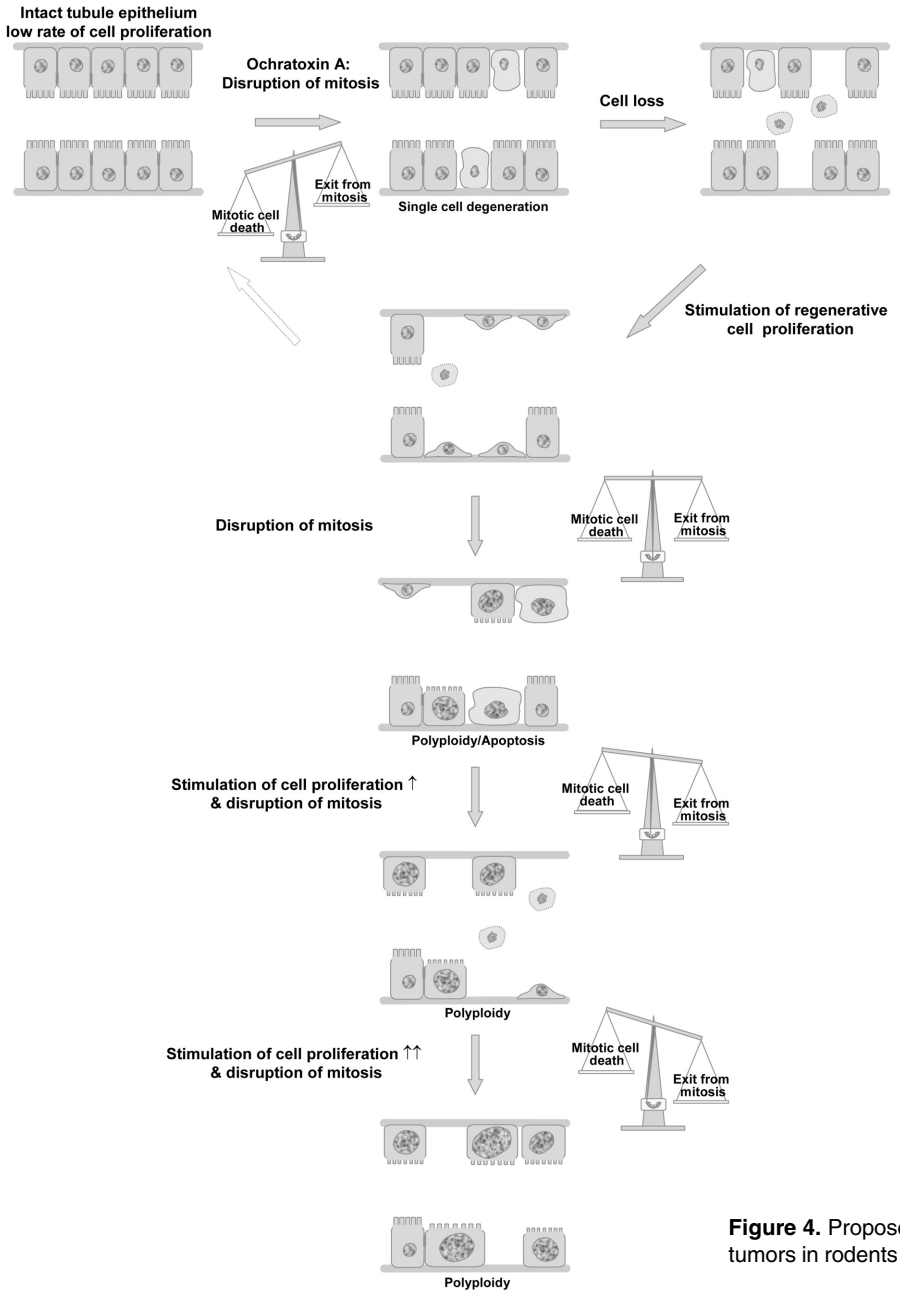


Figure 4. Proposed primary MoA of OTA to induce renal tumors in rodents

is consistent with the detection of apoptotic cells in kidneys of OTA-treated rats, either degenerating while attached to the basement membrane or detached into the tubule lumen [21, 23, 70, 82]. Despite the apparent contrast to the concept of evasion of apoptosis, it should be pointed out that the kidney may respond to apoptosis much in the same way as to necrotic cell death by turning on cell proliferation to compensate for the cell loss. In order to do so, signaling pathways conveying growth and survival signals must be turned on, whereas pro-apoptotic or growth-inhibitory signals must be switched off.

Several studies indicate that OTA may simultaneously stimulate signals implicated in apoptosis and stress response, *e.g.*, activation of stress-activated protein kinase c-Jun N-terminal kinase (JNK) and the MAP kinase p38, and signals thought to promote cell survival, *i.e.*, the extracellular signal-regulated kinase (ERK) 1/2 and the transcription factor NF-κB [76, 81, 83, 84], although the latter appear to predominate in rat kidney under conditions of carcinogenicity [85]. Further support for a critical role of extracellular signal-regulated kinase (ERK1/2) has come from the finding that pharmacological inhibition of ERK1/2 by U0126 1,4-diamino-2,3-dicyano-1,4 bis-(2-aminophenylthio)-butadiene-augmented OTA toxicity in proximal tubule cells [77]. Thus, stimulation of ERK1/2 survival signaling in response to OTA toxicity may protect cells from

cell death and confer resistance to apoptosis. Similarly, NF-κB activation has been shown to suppress apoptosis and promote cell survival. Collectively, these data indicate that OTA toxicity may involve modulation of opposing pro- and anti-apoptotic signaling pathways, and that the balance between both may determine cell fate. With sustained exposure, a shift toward cell growth through activation of ERK1/2 and NF-κB signaling may favor survival and clonal expansion of damaged or preneoplastic cells, thereby promoting tumor formation. However, the initial events that trigger activation of these pathways remain to be identified.

Based on the observation that degenerate proximal tubule cells in OTA-treated rat kidney were frequently found to be detached from the basement membrane, it has been suggested that loss of cell adhesion may contribute to cell death induced by OTA. However, while OTA has been demonstrated to alter both cell–cell and cell–matrix interactions, a causal role for modulation of cell-adhesion in OTA-mediated cell death could not be established [79, 80, 84]. In contrast, there is evidence to suggest that OTA may induce cell death by interfering with mitosis [84], which might also explain OTA-mediated effects on cell adhesion, as cells loose contact to their neighboring cells during cell division. Several *in vitro* studies indicate that OTA may be cytostatic rather than cytotoxic, and that this is mediated by transiently arresting cells in G2/M [57, 65, 86], supporting the hypothesis that OTA acts during mitosis. This is also consistent with the recent finding that OTA-induces endoreduplication and aberrant condensation and separation of chromatids [44]. In human kidney epithelial cells, OTA-blocked metaphase/anaphase transition, and this was associated with mitotic spindle defects, increased apoptosis and nuclear abnormalities in surviving cells [84]. Cell death during mitosis constitutes a special case of apoptosis, with the primary goal of eliminating cells which would otherwise exit mitosis without cell division or divide asymmetrically to give rise to aneuploid daughter cells. However, suppression of mitotic cell death, which – in the case of OTA – might be brought about by activation of ERK1/2 and NF-κB survival signaling in response to the initial cell loss and failure to replace cells, represents a violation of the ‘`better dead than wrong’ principle and may promote genetic instability and tumor formation. Thus, understanding the molecular events that precede OTA-induced cell death may be critical to understand how evasion of apoptosis may contribute to the development of such exceptionally malignant tumors in high incidences and with a relatively rapid onset in the absence of direct genotoxicity.

5.5 Alteration of gene expression

Toxicogenomics is a promising tool to gain insight into mechanisms of toxicity and has been employed by a number of groups to identify OTA-related changes in genes expression both *in vitro* and *in vivo*. Luhe *et al*. [87] investigated alterations in gene expression in kidneys of male Wistar rats treated with a single or three consecutive doses of OTA (1 and 10 mg/kg bw) for 24 or 72 h, respectively. While no significant histopathological changes were evident after 24 h, both dose levels produced degenerative lesions within the inner part of the cortex and OSOM after 72 h [87]. Under these conditions, OTA-induced changes in the expression of genes involved in acute phase response, DNA repair/apoptosis, cellular metabolism/detoxification and oxidative stress. Interestingly, there was a relatively good concordance between *in vivo* expression profiles and *in vitro* experiments using rat primary kidney cells. Similarly, Arbillaga *et al*. [88] reported deregulation of genes associated with oxidative stress and mitochondrial transport, DNA repair, apoptosis, and cell-cycle control in HK-2 human kidney cells in the presence of OTA concentrations which reduced cell survival to 50% of controls. However, it should be noted that alterations in gene expression observed in response to the relatively high doses/concentrations producing overt toxicity may not adequately reflect changes which occur under conditions of carcinogenesis. To address this, Marin-Kuan *et al*. [69] applied Affymetrix RG-U34A chips to liver and tissue samples obtained from a study in which dietary administration of OTA to male F344 rats for up to 2 years resulted in a significant increase in renal tumors in the absence of overt nephrotoxicity. Genes modulated by OTA treatment in rat kidney were grouped according to their biological functions, and included downregulation of genes involved in xenobiotic metabolism, transport and oxidative stress response, and upregulation of a number of genes with known function in cell proliferation, survival, and oncogenesis. In contrast to the acute study by Luhe *et al*. [87], only few genes associated with DNA synthesis, damage, and repair were differentially expressed in kidneys of OTA-treated animals. Genes previously identified as being specifically modulated by direct-acting genotoxins [89] were not affected by dietary OTA exposure, further supporting the hypothesis that OTA-induces tumors through mechanisms other than direct interference with DNA. The finding that OTA disrupts pathways regulated by the transcription factor Nrf2, as evidenced by downregulation of a battery of genes controlled by Nrf2 and related to detoxication and protection from oxidative stress, provides evidence to suggest that OTA may cause chronic oxidative stress through impaired antioxidant defense [69, 90]. Other genes modulated by OTA in rat kidney related to inhibition of protein synthesis, calcium homeostasis, cell adhesion and cell signaling. However, a unifying hypothesis as to how these changes might contribute to tumor formation by OTA could not be derived.

Stemmer *et al*. [91] used gene expression profiling to compare early effects of OTA in the Eker rat model of increased susceptibility to renal tumor formation *versus* wild type rats. Overall, a higher number of genes were significantly deregulated in the susceptible Eker rat as com-

pared to wild type animals, consistent with a significant increase in atypical tubules in OTA-treated Eker but not wild type rats on day 14, although no strain differences in PCNA labeling were apparent at this time-point. In line with previous findings, effects of short-term OTA treatment in this study involved alterations in the expression of genes associated with metabolism, and oxidative and cellular stress, including mitogen activated protein kinases (MAPK) signaling. More importantly, however, both genes with pro- and anti-apoptotic function as well as genes implicated in reduced and enhanced cell survival/proliferation were found to be deregulated, reflecting OTA-induced pathology, *i.e*., cell degeneration and proliferation, and involvement of opposing signaling pathways. In addition, gene expression analysis provided evidence for OTA-mediated effects on mitotic spindle formation, cell adhesion, motility, cytoskeleton organization, and angiogenesis. Thus, an important conclusion from this study is that early features of OTA carcinogenicity – as evidenced by transcription profiling – correspond with the hallmarks of cancer [92], including evasion of apoptosis and insensitivity to growth inhibitory signals, self-sufficiency in growth and limitless potential to replicate, angiogenesis, and tissue invasion.

Using a focused quantitative real time PCR (qRT-PCR) array, we were recently able to show that doses of OTA known to cause tumor formation after chronic application resulted in overexpression of key regulators of mitosis and chromosome segregation, including $Cdk1^{cdc2}$, Aurora B kinase, polo-like kinase 1, and spindle checkpoint kinase Bub1b [93], which are frequently overexpressed in various tumors and have been linked to chromosomal instability, aneuploidy, poor prognosis, and high metastatic potential [94, 95]. These data support the hypothesis that disruption of mitosis, resulting in blocked or asymmetric cell division, accompanied by an increased risk of aneuploidy acquisition, may play a critical role in OTA carcinogenicity.

6 Ochratoxin A risk assessment

The main producers of OTA, *i.e*., *Penicillium verrucosum*, *A. ochraceus*, *A. carbonarius*, preferentially grow under moderate climatic conditions as prevalent in many parts of Europe. Cereals, cereal products, beer, coffee, wine, dried fruits, and spices are among the food commodities most frequently contaminated with OTA. In addition, meat and meat products may contain significant levels of OTA due to carryover from contaminated feed for farm animals [6]. Based on food consumption data and the level of OTA in various food commodities, a conservative assessment of the mean daily intake of OTA by the population of the EU member states was recently performed. Daily intakes of OTA were estimated to range between 2 and 3 ng/kg bw *per* day for the average adult consumer and 6 – 8 ng/kg bw *per* day for high consumers giving weekly doses between 15 to 20 ng/kg bw and 40 and 60 ng/kg bw *per* week [58] (SCOOP, Directorate-General Health and Consumer Protection 2002, http://ec.europa.eu/food/fs/scoop/3.2.7_en.pdf.). However, infants, children, and distinct segments of the population, consuming high amounts of certain locally produced foods, may experience higher exposures.

EFSA recently established a tolerable weekly intake (TWI) of 120 ng/kg body weight for OTA based on a lowest observed adverse effect level (LOAEL) of 8 µg/kg bw *per* day for early markers of renal toxicity in pigs, the most sensitive animal species, and a threshold-based approach for risk assessment due to absence of specific OTA-derived DNA-adducts. While the exposure of the general population in Europe is below this TWI, additional data regarding OTA-exposure of infants and children were considered necessary to account for their specific dietary preferences.

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