## Ochratoxin A acts as a photoactivatable DNA cleaving agent

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The ability of ochratoxin A to photoinduce DNA cleavage is described; in the presence of DNA the photoreaction yields the non-chlorinated derivative, ochratoxin B, while a hydroquinone derivative is produced under anaerobic conditions.

Ochratoxin A (OTA, 1: X = Cl) is a fungal toxin produced by a species of Aspergillus and Penicillium.<sup>1,2</sup> It contaminates a wide range of foodstuffs and is implicated in the disease Balkan endemic nephropathy in which patients suffer from urinary tract tumors.<sup>3</sup> OTA induces single-strand DNA cleavage<sup>4</sup> and DNA adduction *in vivo*;<sup>5</sup> properties that establish a basis for its genotoxicity. However, the mechanism of OTA-induced DNA damage is currently not known.



Despite this, it is known that the chlorine atom is essential<sup>6</sup> and antioxidants inhibit its genotoxic activities.<sup>7</sup> While activation of OTA appears to be oxidative,<sup>3–7</sup> we have found, using fluorescence spectroscopy ( $\lambda_{ext} = 380 \text{ nm}$ ,  $\lambda_{em} = 441 \text{ nm}$ ), that the toxin is particularly susceptible to light and decomposes over time unless a suitable filter is used to suppress light intensity. Since certain halogenated compounds have been shown to efficiently photocleave DNA,<sup>8–10</sup> we hypothesized that **1** may photoinduce DNA damage. Such activity was also expected to provide new insight into the toxin's DNA targeting activities as photoactivatable agents have proven useful as probes of DNA structure,<sup>11</sup> sequence,<sup>12</sup> and mechanism, both in terms of DNA strand-scission<sup>13</sup> and DNA alkylation.<sup>14</sup>

Here we describe our preliminary findings on the photonuclease activity of **1**. While *in vivo* activation of **1** may be mediated by cytochrome P450<sup>15</sup> with subsequent formation of hydroxyl radicals,<sup>16,17</sup> our results show that light can also activate **1** and the products from the photoactivation provide new insight into species that may participate in its *in vivo* DNA targeting activities.

The ability of **1** to facilitate DNA photocleavage was examined using supercoiled plasmid DNA and agarose gel electrophoresis. As shown in Fig. 1, no cleavage resulted in the absence of light (lane 2), highlighting that **1** alone does not induce DNA cleavage. However, in the presence of light, DNA cleavage by **1** occurred in a concentration dependent fashion (lanes 4–7). Table 1 shows the effect of various additives on the extent of photocleavage by **1**. In an ambiently oxygenated atmosphere, inhibition was provided by the oxygen radical scavengers, DMSO, *tert*-butyl alcohol and sodium azide. However, a marked increase in the extent of photocleavage occurred in an N<sub>2</sub>-flushed atmosphere, precluding the requirement for activated oxygen species. Inhibition of photocleavage was also provided by copper(II) ions; a metal that binds **1** effectively and quenches its fluorescence spectrum.<sup>18</sup> That both Cu<sup>II</sup> and O<sub>2</sub> inhibit DNA cleavage suggests that they quench the excited state of **1**.<sup>19</sup>

Additional insight into the mode of photocleavage by **1** was obtained from the finding that the non-chlorinated derivative, OTB (**2**: X = H), and the derivative **4**,<sup>†</sup> which lacks the dihydroisocoumarin (lactone) ring system of **1**, failed to photoinduce DNA strand-scission (Fig. 2). These findings are remarkably similar to the *in vivo* toxicity of **1** and indicate a requirement for both the chlorine atom<sup>6</sup> and the lactone.<sup>20</sup>

Product analysis from the photoreaction of 1 in aqueous buffered media also provided information regarding the mode of photocleavage by the toxin. In an ambiently oxygenated atmosphere, no products were identified in the photoreaction of 1 alone, even though the toxin was consumed as evidenced by



**Fig. 1** Cleavage of supercoiled plasmid DNA by **1**. Reaction mixtures (20  $\mu$ l total volume) contained 400 ng of plasmid DNA in 10 mM MOPS buffer, pH 7.4, and were irradiated on ice through a Pyrex filter with an ILC Technology 300 W Xenon arc lamp. After 5 min, the reaction mixtures were analyzed on a 1.2% agarose gel. Lane 1: DNA alone. Lane 2: DNA + 1000  $\mu$ M **1**. Lane 3: irradiated DNA. Lane 4–7: irradiated DNA + 40, 200, 400 and 1000  $\mu$ M **1**, respectively.

Table 1 Effect of additives on the extent of photocleavage of supercoiled DNA (Form 1) by 1

Conditions <sup>a</sup>	Form $I^{b}$ (%)	Form $II^c$ (%)	Form $III^d$ (%)
Control	89	11	0
+1	27	73	0
+100 mм NaN <sub>3</sub>	78	22	0
+1 µl DMSO	66	34	0
+1 µl Bu <sup>t</sup> OH	62	38	0
+ SOD <sup>e</sup>	27	73	0
+ catalase <sup>f</sup>	27	73	0
+10 mM Cu(OAc) <sub>2</sub>	74	26	0
Anaerobic <sup>g</sup>	0	61	39

<sup>*a*</sup> Reactions were run at pH 7.4 (10 mM MOPS buffer) using 200 μM **1** and 400 ng of supercoiled plasmid DNA in 20 μl total volume, and were irradiated in ice through a Pyrex filter with an ILC Technology 300 W Xenon arc lamp for 5 min. Densitometric quantitation of the gels was performed using a Microtek Scanmaker E<sub>6</sub> equipped with PhotoImpact and UTHSCSA Image Tool software. <sup>*b*</sup> Supercoiled DNA. <sup>*c*</sup> Nicked circular DNA. <sup>*d*</sup> Linear DNA. <sup>*e*</sup> 1000 units ml<sup>-1</sup> super oxide dismutase. <sup>*f*</sup> 1000 units ml<sup>-1</sup> catalase. <sup>*s*</sup> Reaction with **1** was purged with N<sub>2</sub> prior to photocleavage.

**Fig. 2** Structure–activity relationships in DNA cleavage of supercoiled plasmid DNA by **1**. Reactions were carried out for 5 min as described in the caption below Fig. 1. Lane 1: DNA alone. Lane 2: DNA + 200 mM **1**. Lane 3: irradiated DNA. Lane 4: irradiated DNA + 200  $\mu$ M **1**. Lane 5: irradiated DNA + 200  $\mu$ M **2**. Lane 6: irradiated DNA + 200  $\mu$ M 4.



**Scheme 1** Summary of the photoreaction of **1**. Excitation by light (>290 nm) to produce **1**\* is accompanied by production of **2** in the presence of a sugar. Under anaerobic conditions (N<sub>2</sub>, H<sub>2</sub>O) substitution of chlorine by H<sub>2</sub>O yields the hydroquinone **3**, which is also produced under reducing conditions in the presence of O<sub>2</sub>.

HPLC. However, in the presence of calf thymus DNA or dextrose, photoirradiation of **1** yielded the non-chlorinated derivative **2**.<sup>‡</sup> Under anaerobic conditions in the absence of a sugar, photoreaction of **1** produced the hydroquinone derivative, OTOH (**3**: X = OH),§ a finding that we attribute to S<sub>RN1</sub> displacement<sup>21</sup> of the chlorine atom by H<sub>2</sub>O. Interestingly, **3** was also detected in the presence of O<sub>2</sub> when a reducing agent (sodium ascorbate) was added to the photoreaction. This observation suggests that the hydroquinone **3** may have originated from a reactive quinone precursor, in analogy to photooxidation of halogenated phenols that yield benzoquinone derivatives in the presence of O<sub>2</sub>.<sup>22,23</sup> The results of these studies are summarized in Scheme 1.

In conclusion, the fungal carcinogen 1 does not facilitate DNA cleavage alone. However, we have demonstrated that light is one way to activate 1 to induce DNA damage. The finding that 2 is a product of 1 photoirradiation in the presence of DNA, suggests that strand-scission is mediated by H-atom abstraction from deoxyribose sugars through initial C-Cl bond homolysis.<sup>8–10</sup> In the absence of a sugar and  $O_2$ , 1 is converted in high yield to the hydroquinone derivative, 3. This species is also formed in the presence of  $O_2$ , provided that a suitable reducing agent is added to the photoreaction. This result suggests strongly that photooxidation of 1 produces a reactive quinone derivative, a species that may be responsible for the toxin's DNA adduction properties.<sup>5</sup> Presently, experiments are in progress to identify the cleavage sites and the mechanistic pathways for photocleavage. We are also studying the oxidation of 1 to determine the exact nature of oxidized 1, its chemical reactivity and lifetime in aqueous buffered media and the potential of such a species to induce DNA adduction.

We thank Dr Fred W. Perrino (Department of Biochemistry, Wake Forest University) for the sample of plasmid DNA. This work was supported by Grant #IRG from the American Cancer Society and Wake Forest University.

## **Notes and References**

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† The derivative **4** was prepared in 70% yield from 5-chloro-2-hydroxybenzoic acid and L-β-phenylalanine using a DCC coupling procedure; mp 172–173 °C,  $\delta_{H}$ [(CD<sub>3</sub>)<sub>2</sub>CO, 200 MHz] 12.3 (br, 1 H), 8.62 (d, 1 H, *J* 7.9), 7.92 (m, 1 H), 7.54–7.10 (m, 6 H), 6.94 (d, 1 H, *J* 8.9), 5.01 (m, 1 H), 3.4–3.1 (m, 2 H);  $\delta_{C}$ [(CD<sub>3</sub>)<sub>2</sub>CO] 173.0, 169.2, 160.6, 138.3, 134.6, 130.0, 129.2, 127.7, 127.5, 120.3, 116.7, 55.0, 37.7. (Calc. for C<sub>16</sub>H<sub>14</sub>ClNO<sub>4</sub>; C, 60.10; H, 4.41; N, 4.38. Found C, 60.02; H, 4.47; N, 4.39%.)

<sup>‡</sup> After 5 min irradiation time, the yield of **2** from the reaction of 100  $\mu$ M **1** with 100 mM dextrose was *ca.* 20% based on HPLC analysis. The isolated sample was identical to authentic **2** purchased from Sigma.

§ The hydroquinone derivative  $\hat{\mathbf{3}}$  was obtained in 80% from the photoreaction of (100 μM) in N<sub>2</sub>-flushed phosphate buffer (0.1 м, pH 7.4).  $\delta_{\rm H}([^{2}{\rm H}_{6}]{\rm DMSO})$  13.08 (s, 1 H), 12.04 (s, 1 H), 9.88 (s, 1 H), 8.95 (d, 1 H, *J* 6.0), 7.72 (s, 1 H), 7.29 (m, 5 H), 4.29 (m, 2 H), 3.12 (m, 3 H), 2.66 (dd, 1 H, *J* 11.9, 11.6), 1.43 (d, 3 H, *J* 6.2).  $\delta_{\rm C}([^{2}{\rm H}_{6}]{\rm DMSO})$  172.5, 169.9, 163.1, 152.2, 145.8, 136.9, 130.2, 129.3, 128.4, 126.7, 123.4, 118.3, 109.4, 76.2, 53.9, 36.7, 28.1, 20.3. These peaks are identical to a separately prepared sample starting from 4-methoxyphenol. Full details will be published elsewhere.

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Received in Corvallis, OR, USA, 17th November 1997, 7/08275D