Free Rad. Res., Vol. 33, pp. 129–137
Reprints available directly from the publisher
Photocopying permitted by license only

DNA Strand Scission by the Nephrotoxin [2,2′-Bipyridine]-3,3′,4,4′-tetrol-1,1′-dioxide and Related Compounds in the Presence of Iron

DANIELLE CANTIN-ESNAULT [a,*], HAMMOU OUBRAHIM [b] and JEAN-MICHEL RICHARD [a]

[a]*Labatoire de Chimie Bioorganique, Département de Pharmacochimie Moléculaire, UMR CNRS, France;* [b]*Groupe GEDEXE, Université J. Fourier de Grenoble, UFR de Pharmacie de Grenoble, Domaine de la Merci, 38706 La Tronche Cedex, France*

Accepted by Prof. H. Sies

(Received 15 November 1999; In revised form 27 December 1999)

The capacity of non-illuminated nephrotoxin orellanine ([2,2′-bipyridine]-3,3′,4,4′-tetrol-1,1′-dioxide) to induce DNA damage in the presence of ferrous iron and dioxygen has been evaluated. Maximal single-strand breaks in plasmid DNA were obtained with a metal to ligand ratio 1 : 3. Instantaneous oxidation of Fe^{2+} in presence of orellanine under air was responsible for oxy-radical production concomitant to a stable ferric complex $Fe(III)Or_3$ formation, leading to oxidative DNA breakage at physiological pH. DNA damage was lowered in the presence of SOD and catalase or DMSO, indicating a set of reactions that leads to oxy-radical generation. Iron chelators such as DTPA and EDTA had no protecting effect, Desferal slightly protected. GSH acted as an oxy-radical scavenger, whereas cysteine induced stronger damage.

Closely related bipyridine compounds were also studied in presence of Fe^{2+} and O_2 using a combination of spin-trapping and DNA-nicking experiments, none of which were able to chelate iron and induce damage at pH 7. Both catecholic moieties and aminoxide groups are required for observing breakage at physiological pH.

Keywords: DNA strand scission, ESR, iron complex, mushroom nephrotoxin, orellanine, oxy-radical

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMSO, dimethylsulfoxide; ESR, electron spin resonance; GSH, glutathione; orellanine, [2,2′-bipyridine]-3,3′,4,4′-tetrol-1,1′-dioxide; SOD, superoxide dismutase

INTRODUCTION

Several species of *Cortinarius* mushrooms have been reported to be highly toxic for animals and man. Orellanine, [2,2′-bipyridine]-3,3′,4,4′-tetrol-1,1′-dioxide, is the toxin of some *Cortinariaceae* species from different geographic origins.[1] In the last few years, severe or even fatal intoxications have occurred every autumn in Europe and North America. After a lag period (2–17 days), reversible or irreversible renal failure was induced by this lethal toxin, requiring haemodialysis or renal transplantation.

The clinical characteristics of orellanine poisoning are well documented, yet little is known

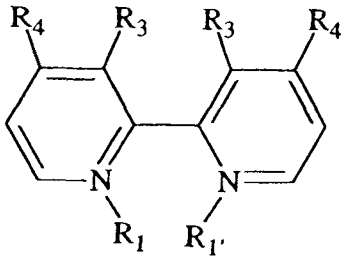
*Corresponding author. UFR de Pharmacie de Grenoble, Groupe DPM, Domaine de La Merci, F-38706 La Tronche, Cedex, France. Tel.: 04 76 63 71 00. Fax: 04 76 51 86 67. E-mail: Danielle.Esnault@ujf-grenoble.fr.

concerning the pathogenic mechanism. Two one-electron oxidation mechanisms of action of orellanine have been reported.[2,3] One mechanism involves the photochemical formation of an apparently stable *ortho*-semiquinone radical coupled to that of superoxide anion and hydroxyl radicals under aerobic conditions.[2] The reaction sequence can be maintained by GSH. In the second mechanism, production of oxy-radicals by the spontaneous aerobic oxidation of the Fe^{2+}–orellanine system concomitant with formation of a ferric ternary complex is shown by spin-trapping.[3] By these two pathways, orellanine could act as a catalyst in the formation of oxy-radicals at the renal action site of the toxin. All these properties are attributed to the *ortho*-dihydroxylated functions borne by the di-*N*-oxidized bipyridine structure of the toxin.[2,3]

The aim of this paper was to establish the effects of the Fe^{2+}–orellanine system on supercoiled DNA under normal aerobic conditions at physiological pH. The formation of oxygen species is known to be responsible for DNA-strand breaks. The dianionic form of orellanine at pH 7 was not favourable to an electrostatic binding of the toxin to the negatively charged phosphate diester backbone of DNA. Therefore the ability of the orellanine, Fe^{2+} and O_2 system to inflict DNA damage had to be checked.

Iron was chosen as a transition metal. Its orellanine-complex ($Fe(III)Or_3$) is well soluble at physiological pH and is the best understood metal–orellanine complex at present.[3]

The relationship between chemical structure of orellanine-related compounds and DNA-nicking capacity was investigated. Orelline, [2,2′-bipyridine]-3,3′,4,4′-tetrol, the major metabolite of orellanine, and two other bipyridine compounds, [2,2′-bipyridine]-4,4′-diol-1,1′-dioxide ($DHBPO_2$) and [2,2′-bipyridine]-1,1′-dioxide (BPO_2), were also studied. The structure of these molecules are presented in Figure 1. Little or nothing was known concerning the physicochemical or chelating properties of these compounds. Effect of iron had never been studied.



Compounds	R_1	$R_{1'}$	R_3	R_4
Orellanine: [2,2′-bipyridine]-3,3′,4,4′-tetrol-1,1′-dioxide	O	O	OH	OH
Orellanine: [2,2′-bipyridine]-3,3′,4,4′-tetrol			OH	OH
$DHBPO_2$: [2,2′-bipyridine]-4,4′-diol-1,1′-dioxide	O	O	H	OH
BPO_2: [2,2′-bipyridine]-1,1′-dioxide	O	O	H	H

FIGURE 1 Chemical structure of orellanine and related compounds.

MATERIALS AND METHODS

Chemicals

All media employed were as free as possible of transition metals other than the deliberately added iron. Enzymes were from Boehringer and other chemicals from Sigma or Suprapur reagent grade from Merck. Ferrous sulphate was standardized manganimetrically and stored under nitrogen. Ferric iron was dissolved at pH 1.

Preparation of Studied Compounds

Orellanine was extracted from *C. orellanus* mushrooms.[4] Stock solutions (50 mM) of the photosensitive and easily oxidizable toxin were prepared in 100 mM disodium hydrogen phosphate pH 10, adjusted to pH 7, stored in the dark at 4°C and used within 2 days. Orelline, $DHBPO_2$ and BPO_2 were synthesized according to Refs. [5,6].

DNA Cleavage Experiments

Damage was evaluated with an *in vitro* technique based upon the electrophoresis of different forms

of plasmid DNA. Reaction mixtures contained 0.27 μg of pBR322 DNA in 5 mM phosphate buffer pH 7 incubated in the dark for 10 min at 37°C with different substances in a total volume of 40 μl under ambient dioxygen pressure. Samples were diluted with 10 μl of loading buffer (90 μg ml^{-1} bromophenol blue, 18% v/v glycerol); 10 μl of mixtures were loaded into the slots of a 5 mm thick 0.8% m/v agarose aqueous gel stained with ethidium bromide (0.1 μg ml^{-1}) and electrophoresed in running TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.8). Horizontal electrophoresis was carried out for 60 min at 6 V cm^{-1}. Immediately after the run, the gel was laid onto a UV transilluminator (312 nm) for viewing and irradiated for 30 s. Amounts of nicked circular Form II were quantified from a same gel in regard to DNA controls.

ESR Experiments

For ESR experiments, iron (3.6 mM) was added last to 10.8 mM orelline solution in 100 mM phosphate buffer pH 10.6 under air. The red-brown mixtures were immediately frozen with liquid nitrogen in cylindrical quartz tubes. Spectra were recorded at −174°C on a Bruker ESP 330 E spectrometer at 9.41 GHz.

For spin-trapping experiments, X-Band ESR were obtained using a quartz flat cell, a Bruker cavity at 20°C and a Bruker ER 100 D spectrometer (9.3 GHz). All experiments were carried out in triplicate or more.

RESULTS

DNA Strand Scission by the Non-Illuminated Orellanine, Fe^{2+} and O_2 System

The system inflicted significant single-strand breaks in plasmid DNA when orellanine (1.5 mM) and Fe^{2+} (0.5 mM) were successively added to an aerobic DNA solution in 5 mM phosphate buffer pH 7 as shown in Figure 2. Lanes 5 and 6 revealed a minor band corresponding to the intact supercoiled covalently closed-circular plasmid DNA (Form I), and a major band corresponding to nicked-circular form (Form II). Under these rigorously definite conditions, oxy-radicals and Fe(III)Or_3 complex were formed in presence of DNA. On the contrary, when DNA was added last to the preliminary formed complex, no DNA damage was induced.

DNA alone (Figure 2, lanes 1 and 2) or in the presence of 1.5 mM non-illuminated orellanine

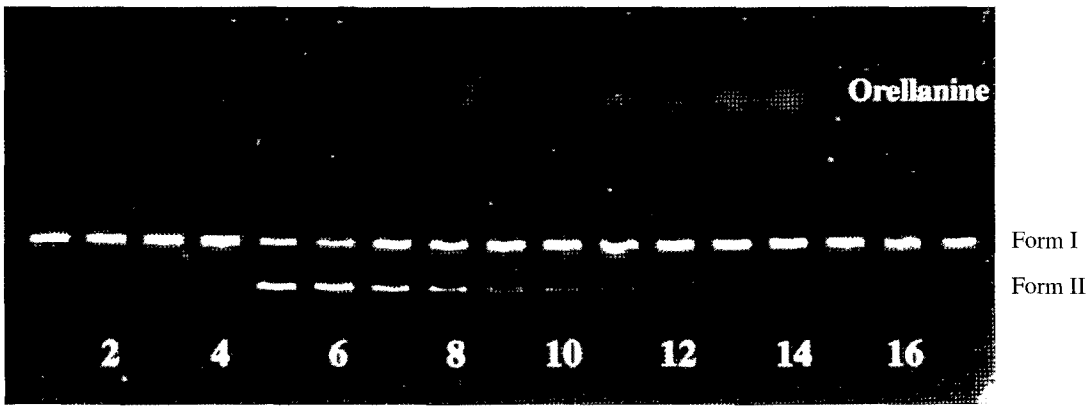


FIGURE 2 Effect of iron–orellanine ratio and oxidation state of iron on DNA cleavage in aerobic buffer pH 7. Cleavage was carried out in the dark at 37°C for 10 min in 5 mM phosphate buffer pH 7. Lanes 1 and 2: pBR322 plasmid DNA alone; lanes 3 and 4: DNA, orellanine (1.5 mM). Lanes 5–14: DNA, fixed concentration of Fe^{2+} (0.5 mM) and various concentrations of orellanine. Lanes 5 and 6: 1.5 mM; lanes 7 and 8: 2 mM; lanes 9 and 10: 2.5 mM; lanes 11 and 12: 3 mM; lanes 13 and 14: 3.5 mM. Lanes 15 and 16: DNA, orellanine (1.5 mM) and Fe^{3+} (0.5 mM); lane 17: DNA, Fe^{3+} (0.5 mM).

(lanes 3 and 4) remained intact after 10 min incubation. Controls with ferrous iron (1 mM) and DNA did not reveal damage as shown in Figure 5, lanes 5 and 6.

Iron chelators such as DTPA and EDTA (1.5 mM) did not prevent DNA damage when they were added to a mixture of DNA and orellanine (1.5 mM) before the addition of Fe^{2+} (0.5 mM). Only Desferal slightly protected the plasmid DNA (data not shown).

Effect of Complex and Ligand Concentrations

In order to evaluate the Fe(III)Or_3 complex concentration which was the most efficient to mediate oxidative DNA-strand breaks at pH 7, varying amounts of Fe^{2+} (5–800 μM) were added to varying amounts of orellanine (15 μM to 2.4 mM). Relaxation of supercoiled DNA linearly increased over the range 0–20 μM of complex ($y = 8.4 + 1.7x$, $r = 0.9988$). The maximal conversion of supercoiled (Form I) DNA to relaxed (Form II) DNA was achieved at 40 μM. For higher concentrations, the damage remained approximately constant. Linear form (Form III) resulting from a double-strand break was never detected.

In order to evaluate the effect of an excess of ligand with regard to the stoichiometry 1 : 3 metal to ligand, a fixed amount of Fe^{2+} (0.5 mM) was added to varying amounts of orellanine (0.5–3.5 mM). The maximal damage was obtained with a ratio 1 : 3 metal to ligand (Figure 2, lanes 5 and 6). In contrast, the percentage of Form II decreased in presence of an excess of ligand (Figure 2, lanes 7–14). Under UV light, the excess of orellanine was easily visualized in the gel by a delayed blue fluorescence of its spot (Figure 2, lanes 7–14). Note that, for the proposed 1 : 3 stoichiometry no spot characteristic of free orellanine was observed (Figure 2, lanes 5 and 6). Orellanine had totally chelated iron giving the wine-red Fe(III)Or_3 complex visible under daylight.

Orellanine, bearing six acido-basic functions, has three apparent pK values ($pK_1 = 1.5$; $pK_2 = 5.8$; $pK_3 = 11.0$).[7] Consequently, an anodic migration of the dianionic forms of the toxin occurred in TAE buffer pH 7.8. The ternary complex possessed an anodic migration and a development distance close to the one of the ligand.

Effect of Oxidation State of Iron and of Dioxygen Content

A mixture of 1.5 mM orellanine and 0.5 mM Fe^{3+} under air did not induce DNA damage in the reductant-less medium (Figure 2, lane 15). Nevertheless, the wine-red complex was observed on the gel. Also free ferric ions did not induce damage in the same medium (Figure 2, lane 17).

When the dissolved dioxygen was partly removed by bubbling with nitrogen for 10 min, the percentage of nicked-circular Form II induced by the Fe^{2+}–orellanine system was lowered compared to the one obtained under ambient oxygen pressure (data not shown). Under anaerobic conditions (under argon) no DNA damage was observed with the mixture remaining colourless. It clearly appears that DNA degradation proceeds from the concomitant oxy-radicals and Fe(III)Or_3 complex formation which was prevented under anaerobic conditions.

Effect of Incubation Time and pH

DNA-strand breakage was incubation time independent at 37°C for a duration of 1–50 min. Approximately equal amounts of Form II were formed showing the high level of oxidability of the Fe^{2+}–orellanine system (data not shown).

DNA damage was pH dependent in a range of pH 5–9, conditions in which Fe(III)Or_3 remained soluble. DNA-strand breaks significantly increased with decreasing pH (Figure 3(a)).

Effect of SOD, Catalase and DMSO

SOD (0.05 mg ml^{-1}) had no significant effect on DNA cleavage at pH 7. In contrast, the cleavage decreased in the presence of 0.5 mg ml^{-1} catalase.

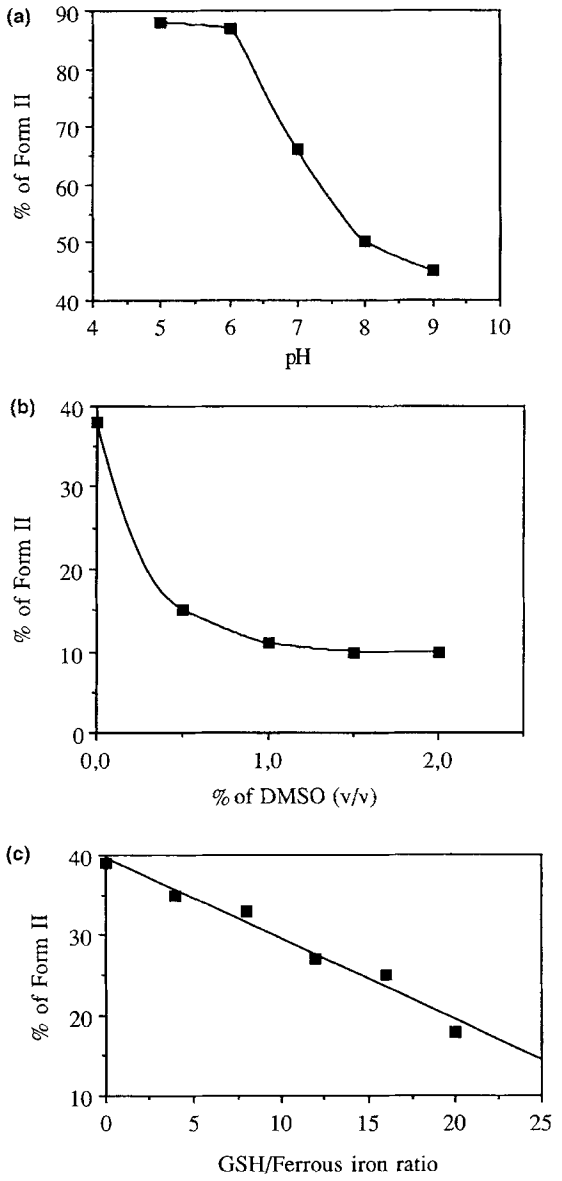


FIGURE 3 Effect of pH, DMSO and GSH on DNA cleavage by orellanine, Fe^{2+} and O_2 system. Reaction mixtures: pBR322 plasmid DNA, orellanine (1.5 mM) and Fe^{2+} (0.5 mM) in 5 mM phosphate buffer. (a) pH varying from 5 to 9; (b) percentages of DMSO varying from 0% to 2% v/v (pH 7); (c) GSH to give reductant/ferrous iron ratio varying from 1.5 to 20 (pH 7).

A more efficient decrease was noticed in the presence of both enzymes (data not shown).

DMSO protected DNA from iron–orellanine-mediated damage. The breakage decreased with increasing DMSO concentration up to 1% v/v and remains approximately constant for higher concentrations (Figure 3(b)).

Effect of Thiols

As shown in Figure 3(c), the number of DNA-strand breaks linearly decreased with increasing GSH concentration (1.5–10 mM), the reductant/ferrous iron ratio varying from 1.5 to 20. Another thiol, L-cysteine (orellanine 1.5 mM, Fe^{2+} 0.5 mM and cysteine 1.5 mM), induced an interesting response. The wine-red mixture slowly turned colourless. At a reductant/ferrous iron ratio of 3, this thiol induced stronger damage in presence of orellanine than in blanks (Fe^{2+} 0.5 mM as well as Fe^{2+} 0.5 mM and cysteine 1.5 mM). In order to check if oxy and thiyl radicals were generated, controls containing a mixture of ferrous iron, cysteine and DMPO incubated for 10 min were studied with the help of ESR spin-trapping measurements. They were ESR-silent under our experimental conditions (data not shown). The DNA damage reflects the reducing effect of cysteine on the Fe(III)Or_3 complex.

Study of the Orelline, Fe^{2+} and O_2 System

Taking into account the presence of two *ortho*-dihydroxylated groups on the bipyridine structure of orelline, a ratio of 3 mol of orelline for 1 iron atom was chosen for the experiments in order to compare the results to those obtained with the nephrotoxin.

Interestingly, orelline and its ferric complex were soluble in alkaline media but slightly soluble at pH 7. Consequently, the system was also studied at pH 10.6 with high concentrations to complete the following spectrometric studies.

UV–Visible Spectrophotometry

When Fe^{2+} was added to a pale-yellow orelline solution in aerated buffer pH 10.6, a red-brown solution was instantaneously obtained. Moreover, with ferric ions, a soluble red-brown

compound was also observed under aerobic or anaerobic conditions. The UV–Visible spectra of the solutions obtained by mixing 12 mM orelline and 4 mM Fe^{2+} or Fe^{3+} in 100 mM aerated phosphate buffer pH 10.6 presented intense bands at 243 (shoulder at 255 nm), 382 and 403 nm and a broad absorption band at 473 nm (shoulders at 550 and 598 nm). The UV bands were similar to those of orelline at the same pH. These data were consistent with a dihydroxylate-type coordination. Thus orelline was able to ligate iron giving a ferric ternary complex and to induce instantaneous autoxidation of Fe^{2+}.

ESR Spectroscopy and Spin-Trapping Experiments

In order to confirm the oxidation-state of iron in the complex, ESR spectra of mixtures of orelline (10.8 mM), Fe^{2+} (3.6 mM) were recorded at −174°C in 100 mM aerated phosphate buffer pH 7 or 10.6. A broad absorption band centred near a $g = 4.35$ was observed when the red-brown mixture was immediately frozen. Under rigorously anaerobic conditions no ESR signal was obtained (data not shown). If the anaerobic ESR-silent sample was exposed to air after thawing and refrozen, the characteristic ESR signal was instantaneously recorded. Ferrous blanks were ESR-silent.[3] The same broad band attributed to high spin ferric iron, was obtained with a mixture of orelline and Fe^{3+} under aerobic or anaerobic conditions.

Spectrophotometric and ESR studies reveal that the same complex is formed by the autoxidation of Fe^{2+}–orelline system or by addition of Fe^{3+} to orelline. These results demonstrated the role of dioxygen in the formation of a ferric complex when orelline ligated ferrous iron.

In the presence of DMPO (300 mM), aerobic solutions of orelline (1 mM) and Fe^{2+} (0.33 mM) in 100 mM phosphate buffer pH 7 exhibited (Figure 4) the typical four line ESR spectrum of $^{\bullet}OH$ radical (intensity ratio 1:2:2:1; $a_N = a_H = 1.49$ mT), while the complex slowly precipitated.

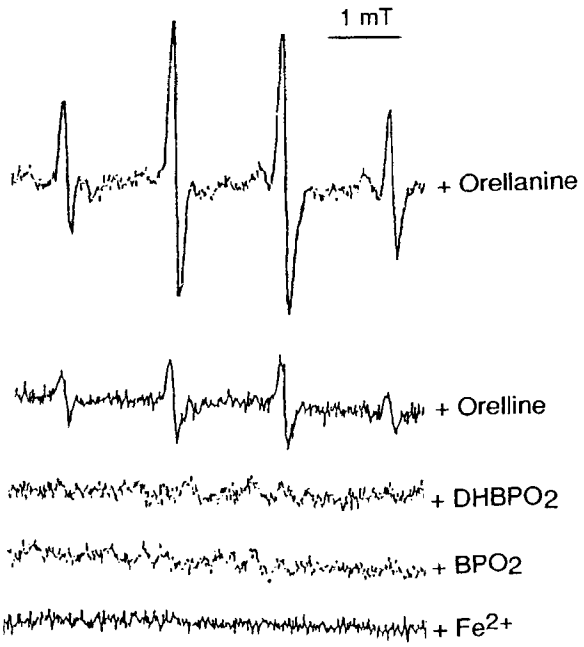


FIGURE 4 ESR spectra of DMPO–$^{\bullet}OH$ spin adduct produced by orellanine or related compounds in presence of ferrous iron in aerobic buffer pH 7. Samples: bipyridinic compound (1 mM), Fe^{2+} (0.33 mM), DMPO (300 mM) in 100 mM phosphate buffer pH 7 under normal aerobic conditions. Control: Fe^{2+} (0.33 mM) and DMPO (300 mM). Spectrometer settings: cell temperature, 20°C; microwave power, 20 mW; modulation amplitude, 0.16 mT; modulation frequency, 100 kHz; time constant, 1 s; scan time, 100 s; scan range, 10 mT.

A poor oxy-radical production due to the lack of solubility of this system was evidenced. In contrast, at pH 10.6 the complex remained soluble and a DMPO–$^{\bullet}OH$ signal approximately as intense as the one observed with orellanine at pH 7 was recorded (Figure 4).

The DMPO–$^{\bullet}OH$ signal intensity was strongly lowered when the dissolved dioxygen was partly removed and under rigorously anaerobic conditions, no signal was observed, the initial colour of the medium remaining unchanged. The role of dioxygen in the generation of oxy-radicals was unambiguously confirmed.

When DMSO (1% v/v) was added to a mixture of DMPO (300 mM), orelline (1 mM) and Fe^{2+} (0.33 mM added last to initiate the reaction), the emergence of a DMPO–$^{\bullet}CH_3$ spin-adduct ($a_N = 1.53$ mT and $a_H = 2.2$ mT) superimposed to a

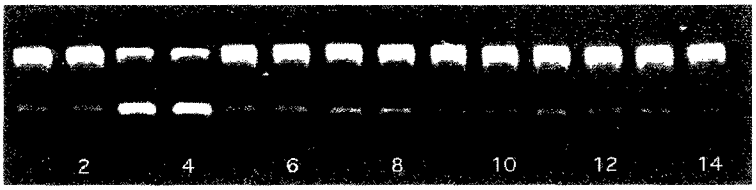


FIGURE 5 Effect of $DHBPO_2$ or BPO_2, Fe^{2+} systems on DNA cleavage in aerobic buffer pH 7. Cleavage carried out as Figure 2. Controls: lanes 1 and 2: pBR322 plasmid DNA alone; lane 3 and 4: DNA, orellanine (1.5 mM), Fe^{2+} (0.5 mM): lanes 5 and 6: DNA, Fe^{2+} (1 mM). Assays: lanes 7 and 8: DNA, $DHBPO_2$ (3 mM); lanes 9 and 10: DNA, $DHBPO_2$ (3 mM), Fe^{2+} (1 mM); lanes 11 and 12: DNA, BPO_2 (3 mM); lanes 13 and 14: DNA, BPO_2 (3 mM), Fe^{2+} (1 mM).

DMPO–•OH adduct was recorded (data not shown). The line height of the DMPO–$^{\bullet}CH_3$ signal increased at the expense of that of DMPO–•OH signal, a fact that confirmed the production of •OH radical.

DNA Strand Scission Experiments

Orelline is visualized in the gel by its native blue fluorescence under UV light. The Tris ferric complex is detected as a red-brown spot under visible light. These two molecules possessed an anodic migration. The presence of negative charges on both compounds at pH 7 induced this anodic mobility as observed for orellanine and its complex. At pH 7, the Fe^{2+}–orelline system did not mediate significant oxidative DNA strand scission (data not shown). This result was consistent with the one obtained by spin-trapping exhibiting a poor •OH generation as shown in Figure 4. Indeed, a high concentration of •OH radical in solution is expected to induce DNA damage.

Study of $DHBPO_2$ or BPO_2, Fe^{2+} and O_2 Systems

The chelating properties of two other synthetic diaminoxidized compounds, which differ from orellanine in the number of hydroxyl groups, was investigated in presence of ferrous iron. Addition of Fe^{2+} (0.33 mM) to $DHBPO_2$ or BPO_2 solutions (1 mM) in aerated phosphate buffer pH 7 did not induce any change in colouration and in the UV–Visible spectra. Thus, both compounds were unable to ligate iron and to produce DMPO–•OH spin-adduct (Figure 4). Parallel to these spectroscopic experiments was run a set of DNA cleaving assays at pH 7. No significant DNA damage was observed as shown in Figure 5, lanes 9 and 10 (Fe^{2+}–$DHBPO_2$ system) and lanes 13 and 14 (Fe^{2+}–BPO_2). Controls with Fe^{2+} (lanes 5 and 6), $DHBPO_2$ (lanes 7 and 8) and BPO_2 (lanes 11 and 12) were also presented.

DISCUSSION

Here, we report highly efficient DNA cleavage mediated by the orellanine, Fe^{2+} and O_2 system at physiological pH. The $O_2^{\bullet-}$ and •OH generation during the instantaneous oxidation of Fe^{2+} in the presence of the dianionic form of orellanine at pH 7 mediates DNA-strand breaks under air. Oxy-radicals are formed in close proximity to the plasmid DNA, condition required to induce strand scission (Figure 2). Indeed oxy-radicals have short life times ($t_{1/2}$ at 37°C: 10^{-5} s for $O_2^{\bullet-}$ and 10^{-9} s for •OH) and hydroxyl radicals possess very short diffusion path.[8] In addition the high level of oxidability of the Fe^{2+}–orellanine system requires that oxy-radicals are generated in a medium containing DNA previously added to induce plasmid damage.

The DNA breakage increases with increasing the amounts of Fe^{2+} and orellanine. On the contrary, an excess of ligand with regard to the stoichiometry of the ternary complex ($Fe(III)Or_3$) induces a decrease of damage as well as a decrease of hydroxyl radical production probably due to the ability of orellanine to scavenge oxy-radical

as previously demonstrated in our spin-trapping study.[3] The role of oxy-radicals on DNA-breakage reaction is also supported by the protective effect of catalase and of an hydroxyl radical scavenger such as DMSO.

In conditions that inhibits ferrous oxidation, the Fe^{2+}–orellanine system is not able to induce DNA-nicking. The same result is obtained with ferric ions added to orellanine in a reductant-less medium. So it confirmed that oxidation of iron is essential in the present mechanism of toxicity of orellanine.

The absence of significant protecting effect of iron chelators is consistent with the formation of a strong and highly reactive complex between iron and orellanine.

A decrease of pH induces an increase in DNA damage (Figure 3(a)). A similar effect has been underlined with a synthetic analogue of bleomycin in presence of copper.[9] In general, the damage done to cells by $O_2^{\bullet -}$ and H_2O_2 is due to their conversion into more highly reactive species among which hydroperoxyl radicals ($HO_2^{\bullet}$). Protonation of $O_2^{\bullet -}$ yields the hydroperoxyl radical in acidic medium (pK_a of $HO_2^{\bullet}$ is 4.7).[10] In close proximity to membrane of tubular cells, pH is lower than the physiological one, thus a considerable amount of $O_2^{\bullet -}$ generated by the studied system may exist as the more reactive $HO_2^{\bullet}$ radical. The stronger DNA cleavage capacity of the Fe^{2+}–orellanine system at lower pH could partly explain the severe necrosis observed in the slightly acidic medium of renal tubules as described in the past.[11]

GSH protected DNA by acting as a scavenger of reactive oxygen species. On the contrary, a more reducing thiol, L-cysteine ($E^{0\prime}$: −340 mV), significantly increased DNA damage, acting as a reductant, involving orellanine, Fe^{2+} and $Fe(III)Or_3$ in a redox cycling process. Indeed, an $E^{0\prime}$ value of 280 mV vs NHE (pH 7) has been previously calculated for the $Fe(III)Or_3/Fe(II)Or_3$ redox couple.[3] Thus, the level of reductant is likely to modulate iron reactivity also in biological systems. A dramatic depletion of renal glutathione levels in rats treated by *orellanus* mushroom extracts has been reported.[12] This *in vivo* observation is in agreement with our results.

Orelline, a bipyridine derivative with catechol-like binding subunits possesses the ability to chelate iron, like the parent toxin. This metabolite can induce instantaneous autoxidation of Fe^{2+}, producing reactive oxygen species under air in neutral and alkaline media. At pH 7, an absence of DNA damage is observed which can be related to a minor $^{\bullet}OH$ radical production as demonstrated in our spin-trapping study. Oxy-radical generation by the orelline system could be correlated with its solubility varying with pH. The mechanism of oxy-radical formation by the orelline system was identical with that proposed in the case of the orellanine system. On the other hand, oxy-radical generation takes place at different pH with the toxin or with the metabolite. Physiological and slightly acidic pH compatible with cell function are favourable to the toxin activity and essentially alkaline one to its non-toxic metabolite. This fact can be attributed to the aminoxide functionalities which allow the *ortho*-dihydroxylated groups to be more easily ionizable in the orellanine molecule.

Two other orellanine-related molecules $DHBPO_2$ or BPO_2 were also investigated. The diaminoxide functionalities are present on the bipyridine structure. In presence of Fe^{2+} and O_2, they are not able to generate oxy-radical and DNA damage. This result could be related to the absence of iron oxidation under air due to the lack of the identified catecholic metal binding sites. In addition, these last compounds are known to be very slightly toxic.[13]

Orellanine has to be altered prior to expressing its toxicity as demonstrated *in vitro* for the inhibition of protein synthesis[14] and as suggested *in vivo* by a characteristic lag period observed in all cases of poisoning before renal failure occurs. One mechanism of action of the toxin can result from oxidation of Fe^{2+} by dioxygen and coupling this oxidation to the generation of reactive free

radicals able to induce cellular molecules and DNA damage. Cleavage activity of orellanine can consequently cause cell death.

Acknowledgements

We are grateful to Dr. J.-F. Constant and Dr. J.-L. Décout for their helpful discussions. Thanks are due to Drs. J. Lhomme, A. Jeunet, P. Block for allowing us to use some of their equipment and to J. de Gabrielli for her contribution to the improvement of the text. We are grateful to Dr. G. Quéguiner for the gift of synthetic orelline.

References

[1] H. Oubrahim, J.-M. Richard, D. Cantin-Esnault, F. Seigle-Murandi and F. Trécourt (1997) Novel methods for identification and quantification of the mushroom nephrotoxin orellanine. Thin-layer chromatography and electrophoresis screening of mushrooms with electron spin resonance determination of the toxin. *Journal of Chromatography A*, **758**, 145–157.

[2] J.-M. Richard, D. Cantin-Esnault and A. Jeunet (1995) First electron spin resonance evidence for the production of semiquinone and oxygen free radicals from orellanine, a mushroom nephrotoxin. *Free Radical Biology and Medicine*, **19**, 417–429.

[3] D. Cantin-Esnault, J.-M. Richard and A. Jeunet (1997) Generation of oxygen radicals from iron complex of orellanine, a mushroom nephrotoxin; preliminary ESR and spin-trapping studies. *Free Radical Research*, **28**, 45–58.

[4] D. Cantin, J.-M. Richard and J. Alary (1989) Chromatographic behaviour and determination of orellanine, a toxin from the mushroom *Cortinarius orellanus*. *Journal of Chromatography*, **478**, 231–237.

[5] F. Trécourt, M. Mallet, O. Mongin, B. Gervais and G. Quéguiner (1993) New synthesis of orelline by metalation of methoxypyridines. *Tetrahedron*, **49**, 8373–8380.

[6] D. Cantin, J.-M. Richard, J. Alary and D. Serve (1988) Electrochemical study of the mushroom toxin orellanine and of related pyridine-1-oxides: 1. Reduction. *Electrochimica Acta*, **33**, 1047–1059.

[7] J.-M. Richard, D. Cantin-Esnault, C. Dumont and J.-L. Benoit-Guyod (1991) Détermination des pK et des équilibres acido-basiques d'une toxine d'origine fongique, l'orellanine. *Analusis*, **19**, 236–243.

[8] W.A. Pry (1994) Oxidants and antioxidants: Free radicals and lipid peroxidation: What they are and how they got that way. In *Natural Antioxidants in Human Health and Disease* (Ed. B. Frei), Academic Press, San Diego, pp. 1–24.

[9] S.E. Hudson and P.K. Mascharak (1989) Radical-induced DNA damage by a synthetic analogue of copper(II)-bleomycin. *Chemical Research in Toxicology*, **2**, 411–415.

[10] B. Halliwell and J.M.C. Gutteridge (1990) Role of free radicals and catalytic metal ions in human disease: An overview. In *Methods in Enzymology* (Eds. L. Packer and A.N. Glazer), Vol. 186, Academic Press, New York, pp. 1–85.

[11] J.-M. Richard, J. Louis and D. Cantin (1988) Nephrotoxicity of orellanine, a toxin from the mushroom *Cortinarius orellanus*. *Archives of Toxicology*, **62**, 242–245.

[12] W. Pfaller, G. Gstraunthaler, H. Prast, L. Rupp, C. Ruedl, S. Michelitsch and M. Moser (1991) Effects of the fungal toxin orellanine on renal epithelium. In *Nephrotoxicity: Mechanisms, Early Diagnosis and Therapeutic Management* (Eds. P.H. Bach, N.T. Gregg, M.F. Wilks and L. Delacruz), Marcel Dekker, New York, pp. 63–69.

[13] J.-M. Richard, G. Taillandier and J.-L. Benoit-Guyod (1985) A quantitative structure–activity relationship study on substituted pyridines as a contribution to the knowledge of the toxic effects of orellanine, a toxin from the mushroom *Cortinarius orellanus*. *Toxicon*, **23**, 815–824.

[14] J.-M. Richard, E.E. Creppy, J.-L. Benoit-Guyod and G. Dirheimer (1991) Orellanine inhibits protein synthesis in Madin–Darby canine kidney cells, in rat liver mitochondria and *in vitro*: indication for its activation prior to *in vitro* inhibition. *Toxicology*, **67**, 53–62.