Bioorganic Chemistry **27,** 326–337 (1999)
Article ID bioo.1999.1138, available online at http://www.idealibrary.com on IDEAL®

PRELIMINARY COMMUNICATION

The Novel Bis(benzoxazole) Cytotoxic Natural Product UK-1 Is a Magnesium Ion-Dependent DNA Binding Agent and Inhibitor of Human Topoisomerase II

Michael B. Reynolds, Mark R. DeLuca, and Sean M. Kerwin[1]

Division of Medicinal Chemistry, College of Pharmacy, and Institute for Cellular and Molecular Biology, University of Texas, Austin, Texas 78712

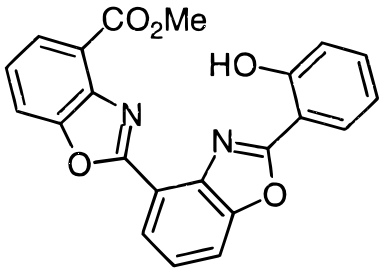
Received September 30, 1998

UK-1 is a novel, bis(benzoxazole) metabolite of *Streptomyces* sp. 517-02. UK-1 is cytotoxic to a variety of cancer cell lines, although it displays no antibacterial effects. We have investigated the metal ion coordination by UK-1 and find that UK-1 binds Zn^{2+} and Mg^{2+} to form stable complexes of overall 1:1 stoichiometry. Fe^{3+} metal ions are bound slightly less well by UK-1, and Ca^{2+} ions form weak complexes with this ligand. In contrast, potassium ions are not bound. The fluorescence spectrum of UK-1 displays a large Stokes shift, presumably as a result of the 2-(2′-hydroxyphenyl)benzoxazole chromophore present in this natural product. The intrinsic fluorescence of UK-1 was exploited in studying the interaction of this compound with DNA, along with changes in the UV/Vis spectrum of the compound and DNA melting studies. In the presence of Mg^{2+}, UK-1 binds to double-stranded DNA 10 times more tightly than in the absence of Mg^{2+}. Like the antitumor quinobenzoxazines, which also bind Mg^{2+} and bind to DNA in a magnesium ion-dependent fashion, UK-1 is an inhibitor of human topoisomerase II. Although these studies do not directly address the issue of the cellular target or targets responsible for UK-1 selective cytotoxicity, the similarities between UK-1 and the synthetic antitumor quinobenzoxazines indicate that UK-1 may exert its cytotoxic potential by targeting topoisomerase II.

UK-1 is a structurally unique bis(benzoxazole) metabolite produced by *Streptomyces* sp. 517-02 (*1,2*) (Scheme 1). During the structural elucidation of UK-1, Taniguchi and co-workers prepared two UK-1 derivatives: the hydrolysis product desmethyl UK-1 (DMUK-1) and the methyl ether of UK-1 (MUK-1) (*2*). Recently, Taniguchi and co-workers described studies on the antibacterial effects of DMUK-1 (*3*) and the antifungal action of MUK-1 (*4*). UK-1 has significant cytotoxic activity against B16, HeLa, and P338 cells, but does not inhibit the growth of gram-positive or gram-negative bacteria, yeast, or fungi (*1*). The origin of the selective cytotoxicity of UK-1 has not been previously investigated. We have recently reported a synthetic route

[1] To whom correspondence should be addressed. Fax: (512) 232-2606. E-mail: skerwin@mail.utexas.edu.

0045-2068/99 $30.00



UK-1

SCHEME 1

for the preparation of UK-1 (*5*). Here we present studies of the metal ion coordination chemistry, metal ion-dependent DNA binding, and human topoisomerase II inhibition by synthetic UK-1. These studies indicate that UK-1 is capable of binding a variety of biologically important metal ions, particularly Mg^{2+} ions. Like the Mg^{2+}-binding aureolic acid group of antitumor antibiotics and synthetic antitumor quinobenzoxazines, UK-1 binds DNA in a metal ion-dependent fashion. One consequence of this interaction with DNA is the inhibition of topoisomerase II. While the mechanism of UK-1's potent cytotoxicity remains unknown, these results support the role of the Mg^{2+}–UK-1 complex as the cytotoxic species. The DNA binding and topoisomerase II inhibition by this species could be involved in the mechanism by which UK-1 exerts its cytotoxic potential, in a manner similar to the Mg^{2+}-dependent DNA-binding antitumor quinobenzoxazines (*6*).

EXPERIMENTAL PROCEDURES

Materials. UK-1 was prepared as described previously (*5*). All reagents and solvents were purchased from Aldrich Chemical Company. Human topoisomerase II (p170 form), KDNA, and topoisomerase assay buffer were purchased from TopoGen, Inc. Calf thymus DNA was purchased from Sigma.

General methods. UV/Vis spectra for the continuous variation plots were determined on a Varian Cary 3E spectrophotometer. DNA melting curves were determined on a Gilford Model 2600 spectrophotometer with thermal programmer. Fluorescence spectra were obtained on a Hitachi Model F-2000 spectrophotometer.

Continuous variation plots. A 100 μM solution of UK-1 and 100 μM solutions of $Ca(NO_3)_2$, $Mg(NO_3)_2$, $Zn(NO_3)_2$, $Fe(NO_3)_3$, or KCl were prepared in methanol. From these stock solutions, 15 samples (1 mL) of varying mole fraction of the metal ion were prepared at a constant, combined concentration of 30 μM for UK-1 and the metal ion. The change in absorbance was monitored from 500 to 200 nm for each sample using as a reference a 1-mL solution containing the same concentration of UK-1 as the sample being measured but without the metal ion. The maximum absorbance change was typically around 418 nm but varied somewhat for each metal ion. The absorbance at 418 nm was plotted as a function of the mole fraction of the metal ion and the maximum absorbance change was determined. The absorbance of

a sample containing the metal ion concentration corresponding to this maximum change and a 15-fold excess of UK-1 at this metal ion concentration, subtracted from a reference containing the same concentration of UK-1 but no metal ion, was used to normalize the curve and obtain the conditional formation constant as described (*7*).

Fluorescence titrations. The fluorescence of solutions (1.0 mL) of UK-1 (2 μM) in Tris buffer (pH 8.0) with or without added $Mg(NO_3)_2$ (5 mM) was measured (λ_{ex} 335 nm) by scanning the emission spectrum from 500 to 600 nm and noting the fluorescence intensity at 550 nm. Small volumes ($\sim$10 μL) of calf thymus DNA (3 mM base pairs) were added, and the solution was mixed well and allowed to equilibrate for 5 min before the fluorescence intensity was measured again.

UV/Vis titrations. The UV/Vis spectra of solutions (1.0 mL) of UK-1 (10 μM) in 20 mM sodium phosphate buffer (pH 7.0) with or without added $Mg(NO_3)_2$ (1 mM) were measured before and after the addition of an excess of calf thymus DNA (3 mM base pairs stock solution). In a separate experiment, the A_{340nm} of solutions (1.0 mL) of l0 μM UK-1, either in the absence or in the presence of 1 mM $Mg(NO_3)_2$, was determined before and after the addition of small volumes (10 μL) of stock DNA solution. The concentration of bound UK-1 after the addition of DNA was estimated by taking the ratio of the A_{340nm} to the A_{340nm} observed upon addition of excess DNA. Using these values, and the McGee–von Hipple equation (*7*), the concentration of free DNA was calculated assuming a binding site size of 2 bp. An estimate of the DNA binding constant was obtained from the resulting *x*-reciprocal plot. This estimated binding constant was used to recalculate the bound UK-1 and the process repeated until convergence was reached (three or four iterations).

DNA melting studies. Solutions of calf thymus DNA (10 μM base pairs) in 20 mM phosphate buffer, pH 6.8, or in 20 mM phosphate buffer containing 2 mM $Mg(NO_3)_2$, pH 6.8, in the presence or absence of UK-1 (5 μM) were denatured by heating from 50 to 90°C at a rate of 1°C/min with continual monitoring of the absorbance at 260 nm (A_{260}). The derivatives of the A_{260} vs temperature curves were calculated using a four-point sliding window and smoothing.

Topoisomerase assay. Stock solutions of UK-1 (3202, 2500, 1300, 500, 100, and 20 μM) were prepared in dimethyl formamide (DMF). A master solution of catenated kinetoplast DNA (KDNA) (TopoGen, Inc.) was prepared in Topo II assay buffer (TopoGen, Inc.). For each assay, an 18-μL aliquot (200 ng KDNA/18-μL aliquot) of the master solution was added to a microfuge tube to which was added 1 μL of the appropriate UK-1 stock solution or 1 μL of DMF. The reaction mixtures were mixed and placed on ice. The reactions were initiated by the addition of 1 μL of human topoisomerase II (p170 form) (2 units/μL) (TopoGen, Inc.) followed by incubation at 37 °C in a circulating water bath for 30 min. The reactions were stopped by the addition of 5 μL of 5$\times$ stop buffer (TopoGen). Each reaction mixture was loaded on to a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide, and the gel was subjected to electrophoresis at 80 V for 1 h in 1$\times$ TAE buffer containing 0.5 μg/ml ethidium bromide. The gel was destained for 15 min in deionized water and imaged on a Molecular Dynamics Fluorimager. The amount of decatenated DNA for each reaction was quantified using ImageQuant software (Molecular Dynamics Version 4.2, Build 11) and normalized to the amount of decatenated DNA produced in the presence of DMF only. Assays were run on each UK-1 concentration in triplicate,

and the results are reported as the mean normalized percentages of decatenated DNA ± the standard deviation. Using the computer program EZ-ED_{50}, the resulting data were fitted to a logarithmic equation,

$$Y = \frac{A_{max} - A_{min}}{(1 + X/EC_{50})^n} + A_{min},$$

where A_{max} is the maximal percentage decatenation, A_{min} is the percentage decatenation in the presence of the highest concentration of UK-1, X is the input concentration of UK-1, Y is the observed percentage decatenation, and n is the slope.

RESULTS AND DISCUSSION

The 2-(2′-hydroxyphenyl)benzoxazole moiety present in UK-1 is also present in a number of synthetic metal ion chelators (*8*) and is analogous to the 2-(2′-hydroxyphenyl)oxazole moiety present in a class of microbial siderophores (*9*). We considered the possibility that UK-1 might bind metal ions and that this metal ion binding might be involved in the mode of action of UK-1. The UV/Vis spectrum of a methanolic solution of UK-1 changes significantly upon the addition of $Mg(NO_3)_2$, the most pronounced effect being a prominent hyperchromicity in the 380–480 nm region (Fig. 1). As a result, methanolic solutions of UK-1 containing Mg^{2+} ions are noticeably yellow, as are the corresponding aqueous solutions, indicating that UK-1 binds Mg^{2+} ions in both polar organic and aqueous media. Similar changes in the spectra of methanolic solutions of UK-1 are observed upon the addition of $CuCl_2$, $Fe(NO_3)_3$, or $ZnCl_2$. In contrast, addition of KCl does not affect the spectrum of UK-1.

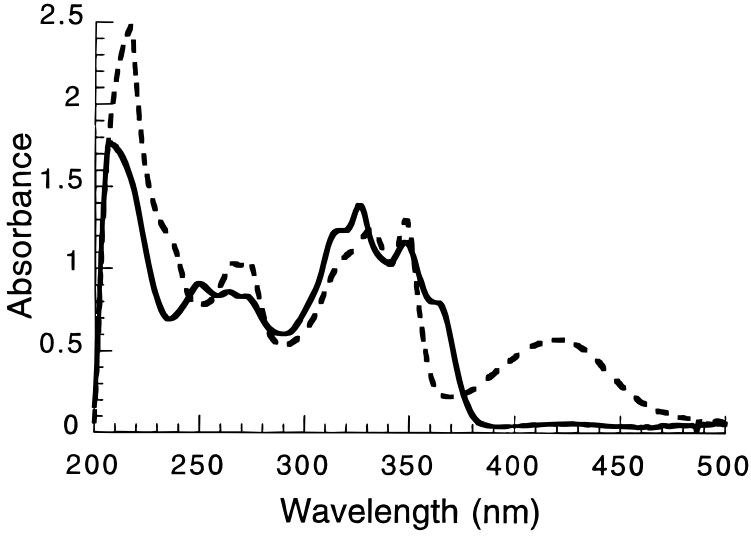


FIG. 1. Changes in the UV/Vis spectrum of UK-1 (10 μM in methanol) upon the addition of increasing concentrations of $Mg(NO_3)_2$. The solid line is the spectrum of UK-1 in the absence of $Mg(NO_3)_2$, and the dashed line is the spectrum of UK-1 in the presence of 2 equivalents of $Mg(NO_3)_2$.

Using the method of continuous variations, (*10*) we further investigated the binding of selected metal ions by UK-1 in more detail. These determinations were done in methanolic solution rather than water, due to the extremely low aqueous solubility of UK-1. The normalized absorbance at 418 nm of methanolic solutions of varying ratios of UK-1 and $Mg(NO_3)_2$ at a combined concentration of 30 μM were measured. As shown in Fig. 2, this continuous variation plot indicates formation of a specific Mg^{2+}–UK-1 complex with an overall stoichiometry of nearly 1:1 (inflection point at 0.45 mole fraction Mg^{2+}). Using the method of Likussar and Boltz, (*7*) the conditional formation constant (log K_f') for the 1:1 Mg^{2+}–UK-1 complex in methanol was determined to be 6.32. Assuming that the affinity for Mg^{2+} ions by UK-1 is similar in water, this relatively high binding constant indicates that under conditions of physiological magnesium ion concentration, UK-1 exists predominantly as the Mg^{2+}–UK-1 complex.

We also investigated the complexation of other metal ions by UK-1, using the above method, and found that Zn^{2+} ions were also tightly bound in methanol with an overall 1:1 stoichiometry and a conditional formation constant of 6.40. In contrast, the interaction of Fe^{3+} ions with UK-1 is much weaker; the 1:1 Fe^{3+}–UK-1 conditional formation constant is only 5.40. Continuous variation plots of UK-1 and $Ca(NO_3)_2$ solutions produce a weak, broad plateau from 0.25 to 0.75 mole fraction Ca^{2+}, indicating the formation of a mixture of weak Ca^{2+}–UK-1 complexes.

Our metal ion binding studies indicate that magnesium binding by UK-1 may be

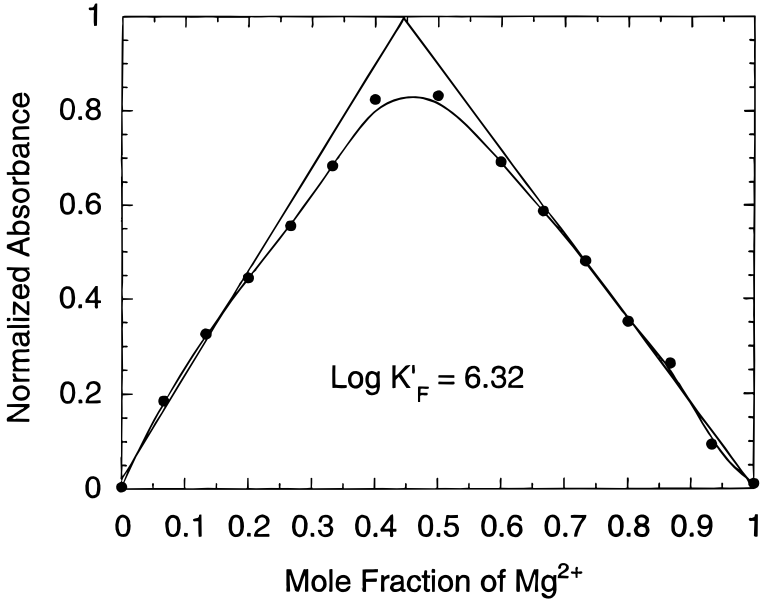


FIG. 2. A normalized continuous variations plot of the Mg^{2+}–UK-1 complex. The normalized absorbance change at 418 nm was monitored as a function of the mole fraction of Mg^{2+} to UK-1 (30 μM total concentration) in methanol. The conditional formation constant (log K'_F) was calculated based on a 1:1 complex.

an important consideration in any model for its mechanism of action. We also rule out a possible biological role of UK-1 as a siderophore for the producing *Steptomyces* organism, as the Fe^{3+} binding by UK-1 is not particularly strong. It appears that UK-1 prefers to bind metal ions of ionic radius 0.8–0.9 Å. The changes in the UK-1 chromophore upon metal ion binding are consistent with formation of a phenolate species; similar spectral changes occur when methanolic solutions of UK-1 are treated with an excess of NaOH. Thus, whereas the ligand itself exists as the neutral species at physiological pH, (*11*) only in the case of di-and trivalent metal ions are the resulting 1:1 M^{2+}–$(UK\text{-}1)^{-1}$ or M^{3+}–$(UK\text{-}1)^{-1}$ complexes cationic. The cationic nature of the UK-1–magnesium complex led us to investigate the interaction between this species and DNA.

Upon excitation at 335 nm, aqueous solutions of UK-1 display a fluorescence emission at 530 nm, presumably due to excited-state intramolecular proton transfer (ESIPT) (*12*) and a much less intense emission band of shorter wavelength (380 nm). In solvents less polar than water, the intensity of the ESIPT emission band decreases, while the shorter wavelength emission band increases in intensity. A similar change is observed when $Mg(NO_3)_2$ is added to aqueous solutions of UK-1. When calf thymus DNA is added to aqueous solutions of UK-1 (2 μM) in Mg^{2+}-containing buffer, a further decrease in intensity of the ESIPT emission band is observed (Fig. 3). In the absence of Mg^{2+}, the more intense fluorescence of UK-1 also decreases upon the addition of DNA, but only at higher concentrations of added DNA. The difference

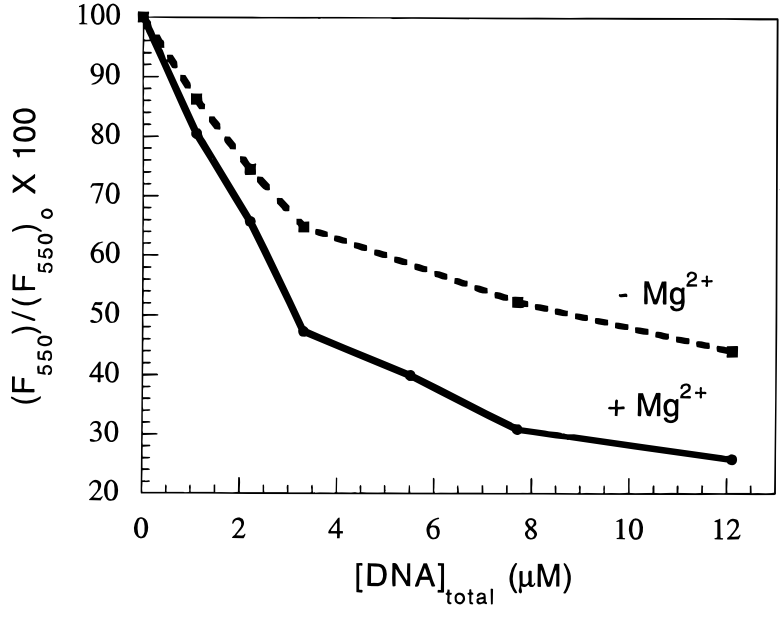


FIG. 3. Changes in the fluorescence of UK-1 upon addition of DNA. Solutions of UK-1 (2 μM) in Tris buffer (pH 8.0) with (solid line) or without (dashed line) added $Mg(NO_3)_2$ (5 mM) were treated with increasing amounts of calf thymus DNA and the changes in the fluorescence emission at 550 nm due to UK-1 were observed (excitation at 335 nm).