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Transition-Metal-Mediated Cytotoxicity of Quinolones to L1210 Cells

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Transition metals tested, Cu^{2+} , and Ni^{2+} , were found effective in the induction of the cytotoxicity of the quinolones tested, nalidixic acid, oxolinic acid, and piperidic acid, against L1210 leukemia cells *in vitro*, whereas the alkaline earth metal, Mg^{2+} , was not. The differential effect of the metals on the quinolone cytotoxicity can be explained by their different mode of interaction with the quinolones. Our present difference spectroscopic titration data suggest that the transition metals can form DNA-intercalating agents, with the quinolones, which can cause the cytotoxicity.

Introduction

Although the intermediation of certain metal ions in the quinolone interaction with DNA is obvious based on previous reports¹ that 4-oxo-3-carboxylic acid moiety is essential for significant antibacterial activity and that quinolone drugs can act as chelating agents of certain divalent cations², discriminatory chelating behavior of the quinolones toward the metal ions or differential effect of metal ions on the functional activity of quinolones has not yet been extensively investigated.

Previously from our data of competitive binding experiments using ethidium bromide³ demonstrating intercalative binding of nalidixate to calf thymus DNA *via* metal chelate complex formation with Cu^{2+} , we proposed that a trinuclear aromatic chromophore produced from the binding of the nalidixate anion to the metal ion by the 4-oxo and 3-carboxylate groups, will be an intercalating agent with respect to DNA. The trinuclear form of the metal-quinolone complex can have a geometry better suited for the intercalative binding to the DNA double helix than the binuclear free quinolone anion. Through the formation of the metal-drug complex, the quinolone drugs may become competent intercalating agents having a flat aromatic chromophore closer to the size of a base pair and increased positive charge⁴. Divalent transition

metal mediated intercalative binding of quinolone to DNA could be further ascertained previously by our observation that the viscosity of calf thymus DNA solution is increased with increasing concentrations of bound metal-quinolone complexes until the saturation limit binding at a ratio of ~ 0.22 metal ions per DNA phosphate groups^{3,4}.

On the other hand, DNA intercalating drugs can be inhibitors of DNA topoisomerases⁵ and can induce both DNA single-strand breaks and DNA double-strand breaks in mammalian cells^{6,7}. Furthermore there has been a substantial body of evidence indicating that certain classes of intercalating agents cause topoisomerase-mediated DNA breaks and thereby can act as antitumor agents⁸⁻¹⁴.

We were, thereupon, tempted to examine metal-dependent cytotoxicity of quinolones to eukaryotic cells, metal-mediated intercalation reactivity of quinolones to DNA, and stability constant of the metal chelate complexes of the quinolone and the relationship of these metal-dependent properties of quinolones. Here we used L1210 leukemia cells for cytotoxicity test and report that Mg^{2+} , which does not form a stable chelate complex with the quinolone drugs tested, neither induce intercalation reactivity of quinolones to DNA, nor cytotoxicity of quinolones against L1210 cells *in vitro*, whereas the alkaline earth metals, Cu^{2+} , which form stable metal-chelate complex with quinolones, induced the intercalating reac-

Table 1. Apparent Stability Constants of Quinolone Antibacterials Complexed (1 : 1) with Cu^{2+} , Ni^{2+} , and Mg^{2+} , respectively.

Drugs	Stability constant		
	Cu^{2+}	Ni^{2+}	Mg^{2+}
Nalidixic acid	5×10^4	3×10^4	1×10^3
Oxolinic acid	3×10^5	4×10^5	
Pipemidic acid	1×10^4	1×10^4	

tivity and the cytotoxicity of the quinolones.

Experimental Section

Calf thymus DNA (type I : sodium salt, highly polymerized) and quinolone drugs purchased from Sigma Chemical Company were used without further purification. DNA concentrations are stated in terms of nucleotide phosphorus by using the extinction coefficient $\epsilon_{260} = 6600 \text{ M}^{-1}\text{cm}^{-1}$.

Examination of the interaction of the quinolones (nalidixic acid, oxolinic acid, pipemidic acid) with metal ions (Cu^{2+} , Ni^{2+} , and Mg^{2+} respectively) was performed at 25°C by difference UV/VIS spectrophotometric titration in 0.1 M NaCl and 0.05 M Tris·HCl, pH 7.5 using double compartment cuvette and UVICON 860 Spectrophotometer, and spectrofluorometric titration with JASCO FP770 Spectrofluorometer. The titration spectra data obtained were analyzed to estimate the molar ratio of metal/quinolone and the stability constant of the metal-quinolone complex as described previously². Binding of metal-quinolone (Mg^{2+} -nalidixate and Cu^{2+} -nalidixate respectively) to DNA was estimated at 25°C by assessing the competitive inhibition of ethidium bromide binding to the DNA by the metal-quinolone in the 0.05 M Tris·HCl buffer, pH 7.5, containing 0.1 M NaCl, following the methods described previously¹⁵. The fluorescence Scatchard plots for the binding of ethidium bromide to calf thymus DNA in the presence of various concentrations of metal-quinolone were constructed. The wavelength of the excitation was 540 nm and the wavelength of the emission was 590 nm.

L1210 leukemia provided by the Korea Research Institute of Chemical Technology was grown in Fischer's medium in accordance with the conditions and protocols described previously¹⁶ to be tested for the *in vitro* inhibition of the leukemia cell growth by the metal-quinolone systems. For the growth studies, tubes were seeded with 5.0 ml of cells (approximately 5×10^4 cells/ml) and then the compounds prepared in whole medium were added under a final volume of 0.1 ml. Tubes were incubated at 37°C for 2 days, and cell numbers were then determined with a hemacytometer. To express the drug effect, the values of ED_{50} (median effective dose) were obtained according to the protocols described by the National Cancer Institute (1972)¹⁷.

Results and Discussion

In the metal-quinolone complex formation, $\text{M}^{2+} + \text{Q}^- \rightleftharpoons \text{M}^{2+}\text{Q}^-$, where Lewis acid is the metal ion (M^{2+}) and the Lewis base is the quinolone anion ligand (Q^-), the equilibrium constant $K_{\text{MQ}} (= [\text{M}^{2+}\text{Q}^-]/[\text{M}^{2+}][\text{Q}^-])$, known as sta-

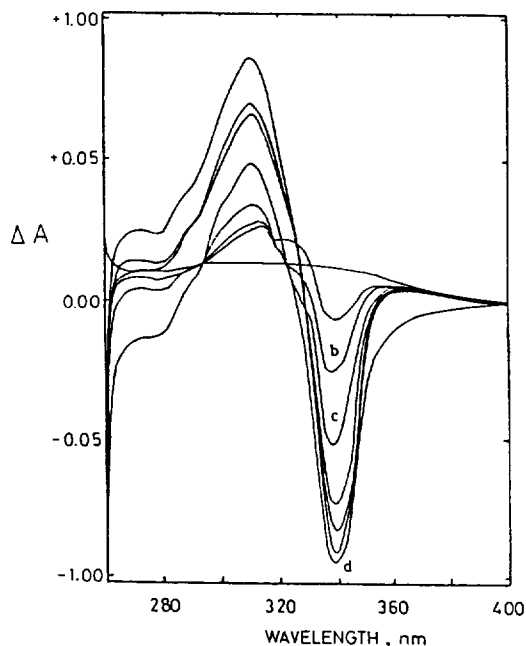


Figure 1. Difference spectra of nalidixic acid in various concentration of Cu^{2+} . Nalidixic acid at constant concentration of $2 \times 10^{-5} \text{ M}$ was titrated with Cu^{2+} of varying concentrations: (a) zero M, (b) $1 \times 10^{-5} \text{ M}$, (c) $2 \times 10^{-5} \text{ M}$, (d) $3 \times 10^{-5} \text{ M}$.

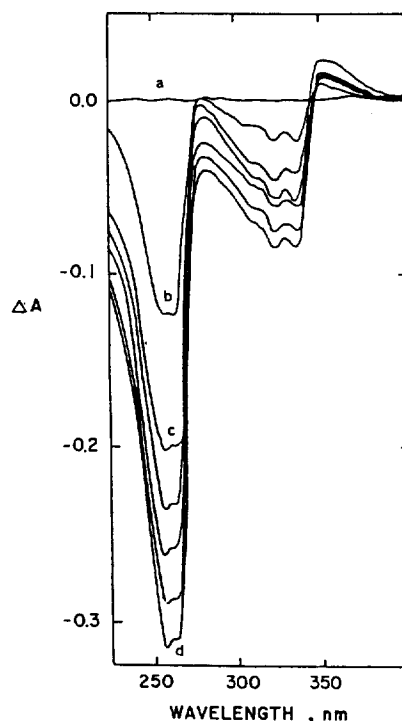


Figure 2. Difference spectra of oxolinic acid in various concentration of Cu^{2+} . Oxolinic acid at constant concentration of $2 \times 10^{-5} \text{ M}$ was titrated with Cu^{2+} of varying concentrations: (a) zero M, (b) $3 \times 10^{-6} \text{ M}$, (c) $4 \times 10^{-6} \text{ M}$, (d) $1 \times 10^{-5} \text{ M}$.

bility constant (see Hartley *et al.*, 1980)¹⁸ and the compositional molar ratio of metal : quinolone (= 1 : 1) estimated from the difference spectra of the spectrophotometric titrations

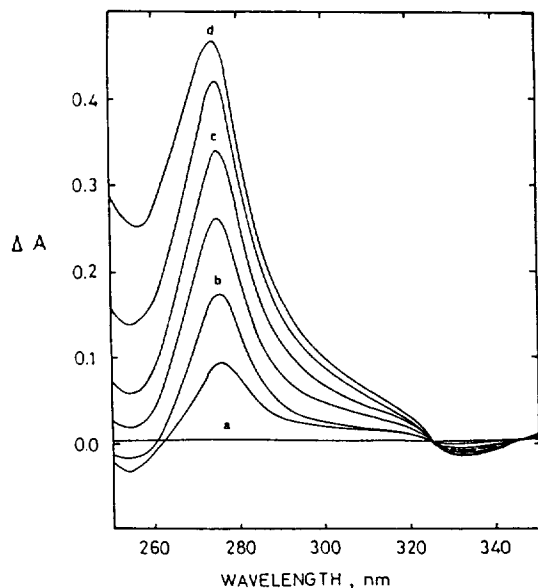


Figure 3. Difference spectra of pipemic acid in various concentration of Cu^{2+} . Pipemic acid at constant concentration of 2×10^{-5} M was titrated with Cu^{2+} of varying concentrations: (a) zero M, (b) 1×10^{-5} M, (c) 2×10^{-5} M, (d) 3×10^{-5} M.

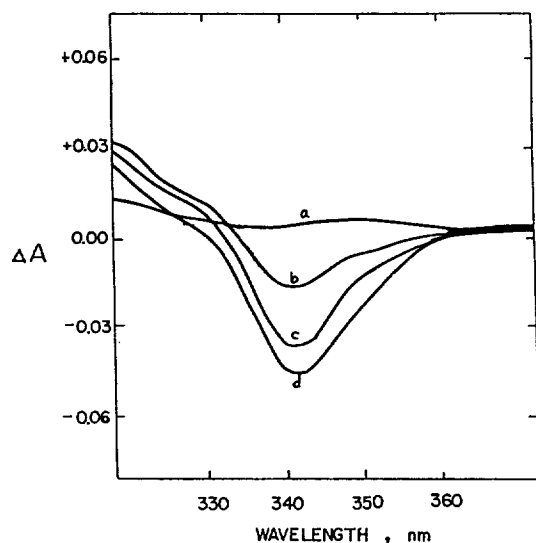


Figure 4. Difference spectra of nalidixic acid in various concentration of Ni^{2+} . Nalidixic acid at constant concentration of 1×10^{-5} M was titrated with Ni^{2+} of varying concentrations: (a) zero M, (b) 1×10^{-4} M, (c) 2×10^{-4} M, (d) 8×10^{-4} M.

in accordance with the method described previously² are listed in Table 1. Figures (Figure 1 to Figure 6) show the difference spectra of the titration of the quinolones (nalidixic acid, oxolinic acid, and pipemic acid) with Cu^{2+} and Ni^{2+} respectively. Figure 7 and Figure 8 show the absorption spectra of nalidixic acid at constant concentration of 2×10^{-5} M titrated with various concentrations of Cu^{2+} and Mg^{2+} respectively. Figures (Figure 9 to Figure 14) show the variations of the fractional saturation (θ) of the quinolone *i.e.*, the proportion of total quinolone that is metal-chelated, with metal concentration (each plot of the figures are constituted

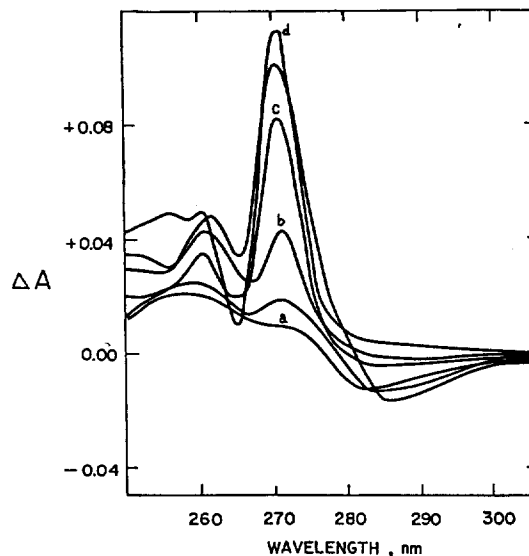


Figure 5. Difference spectra of oxolinic acid in various concentration of Ni^{2+} . Oxolinic acid at constant concentration of 2×10^{-5} M was titrated with Ni^{2+} of varying concentrations: (a) zero M, (b) 4×10^{-6} M, (c) 4×10^{-5} M, (d) 4×10^{-5} M.

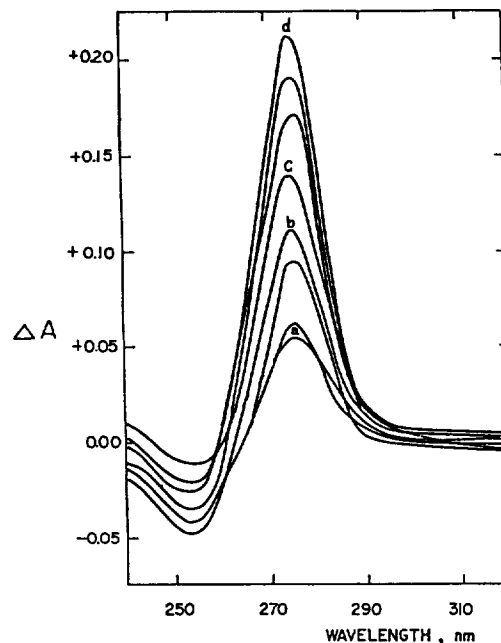


Figure 6. Difference spectra of pipemic acid in various concentration of Ni^{2+} . Pipemic acid at constant concentration of 1×10^{-5} M was titrated with Ni^{2+} of varying concentrations: (a) 2×10^{-5} M, (b) 8×10^{-5} M, (c) 2×10^{-4} M, (d) 1×10^{-3} M.

from the spectra data of corresponding each figure of Figure 1-Figure 6).

From the values of the stability constant of the metal-quinolone complexes listed in Table 1, we can notice that both Cu^{2+} and Ni^{2+} which are positioned side by side in Irving-William series have similar stability constants which are much greater than those of Mg^{2+} .

On examination of Figure 7 and Figure 8, which exhibit UV-VIS absorption spectra of nalidixic acid in various con-

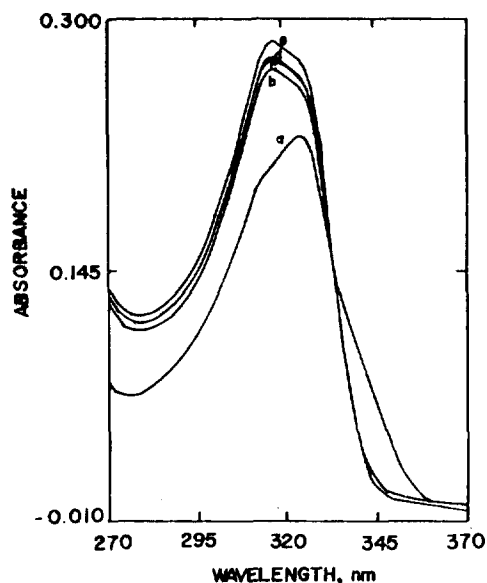


Figure 7. UV-Visible absorption spectra of nalidixic acid in various concentration of Cu^{2+} . Nalidixic acid at constant concentration of 2×10^{-5} M was titrated with Mg^{2+} of various concentrations: (a) zero M, (b) 1×10^{-4} M, (c) 2×10^{-4} M, (d) 4×10^{-4} M, (e) 1×10^{-3} M.

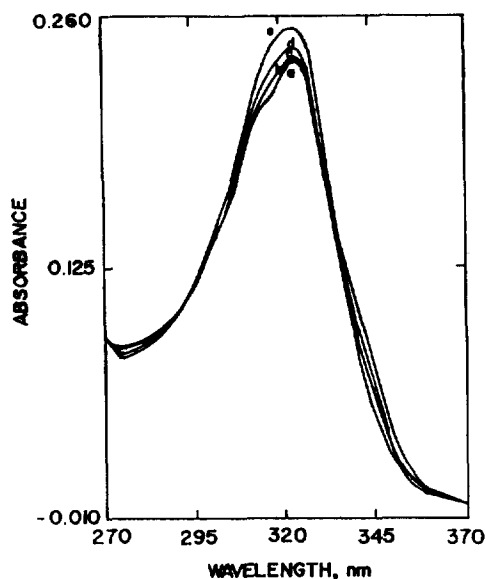


Figure 8. UV-Visible absorption spectra of nalidixic acid in various concentration of Mg^{2+} . Nalidixic acid at constant concentration of 2×10^{-5} M was titrated with Mg^{2+} of various concentrations: (a) zero M, (b) 1×10^{-4} M, (c) 2×10^{-4} M, (d) 4×10^{-4} M, (e) 1×10^{-3} M.

centrations of Cu^{2+} and Mg^{2+} respectively, we can notice that the absorption peak of nalidixic acid at 330 nm wavelength has a shoulder at the left side of the peak. In Figure 7, as the concentration of Cu^{2+} is increased, the shoulder grows up and outweighs the 330 nm original major peak. The spectra of nalidixic acid are more distinctively characterized by the two absorption subpeaks in the wavelength range 310-330 nm in organic solvents (unpublished data but

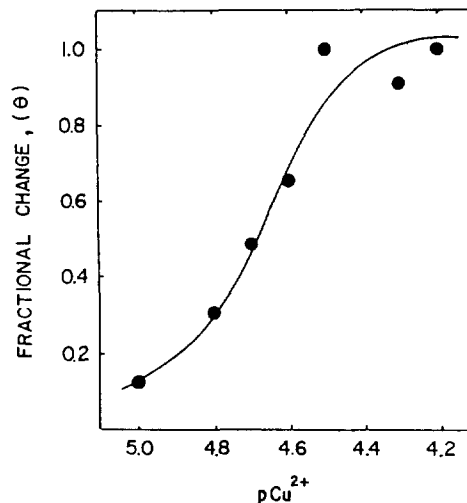


Figure 9. Variation of fraction change (θ) in absorbance at 340 nm of nalidixic acid with concentration of Cu^{2+} .

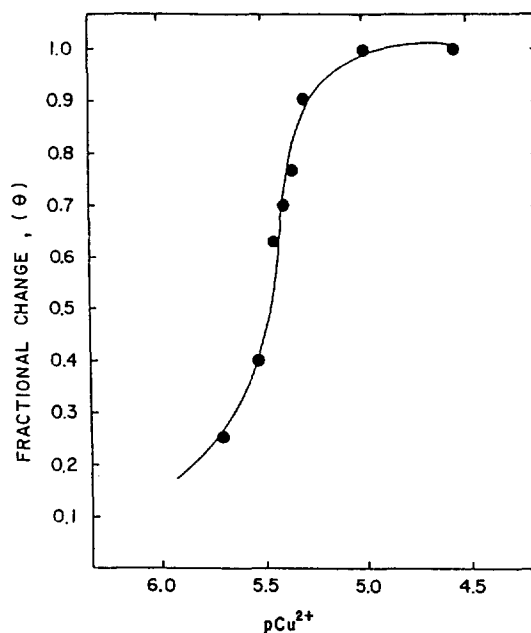


Figure 10. Variation of fractional change (θ) in absorbance at 260 nm of oxolinic acid with concentration of Cu^{2+} .

presented at the 67th Korean Chemical Society Meeting). The structural species corresponding to the left subpeak (*i.e.*, of shorter wavelength) and the right subpeak (*i.e.*, of longer wavelength) were postulated previously¹⁹ to be binuclear form of free quinolone anion and trinuclear form of Cu^{2+} -chelate complex of the quinolone, respectively in equilibrium as shown in Scheme 1.

In Figure 8 we can notice that Mg^{2+} has not significant effect on nalidixic acid spectra in contrast with Cu^{2+} which has significant effect as shown in Figure 7. In another respect the effects of Mg^{2+} and Cu^{2+} are different: the increase of Mg^{2+} concentration does not cause the growth of left-side shoulder over the peak, *i.e.*, the overall original peak shape is maintained even at very high Mg^{2+} concentration (cf. Figure 7 and Figure 8). In view of these spectroscopic observa-

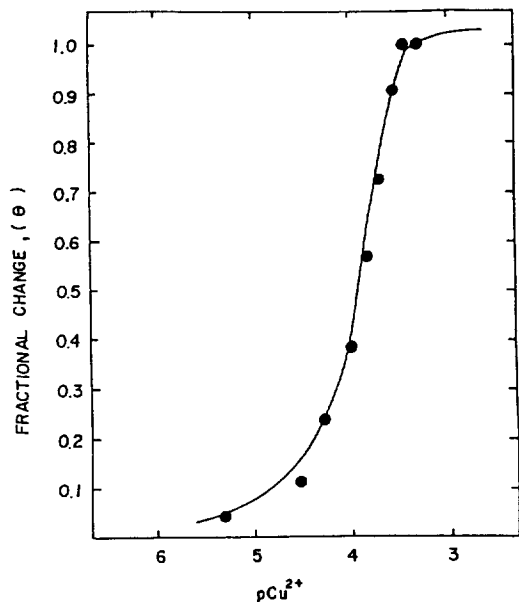


Figure 11. Variation of fractional change (θ) in absorbance at 275 nm of pipemidic acid with concentration of Cu^{2+} .

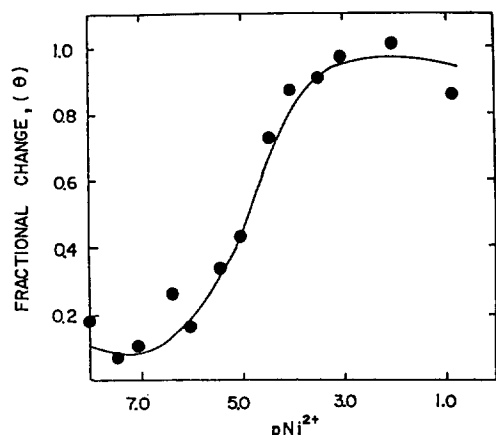


Figure 12. Variation of fractional change (θ) in absorbance at 340 nm of nalidixic acid with concentration of Ni^{2+} .

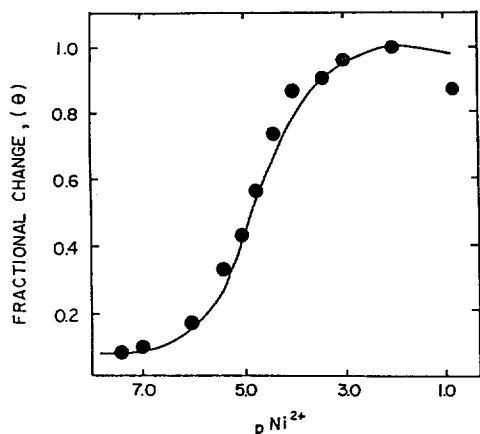


Figure 13. Variation of fractional change (θ) in absorbance at 265 nm of oxolinic acid with concentration of Ni^{2+} .

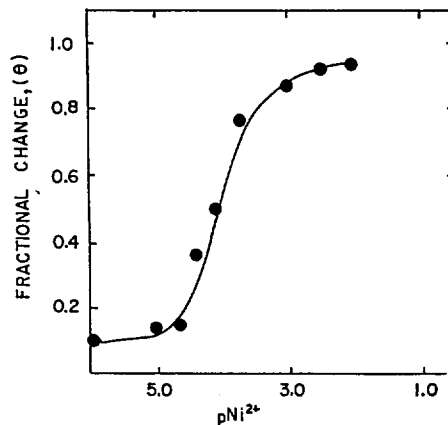
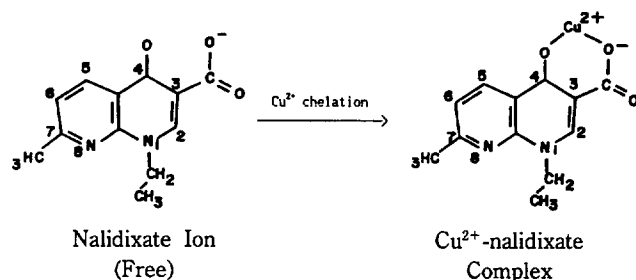


Figure 14. Variation of fractional change (θ) in absorbance at 275 nm of pipemidic acid with concentration of Ni^{2+} .



Scheme 1. Through Cu^{2+} coordination to the 4-oxo-3-carboxylate moiety, the quinolone drug will take on a positively charged flat trinuclear chromophore of approximately base-pair size, capable of intercalation to DNA double helix.

tions, one may expect that Mg^{2+} does not coordinate with the nalidixate to form the trinuclear chelate complex as Cu^{2+} does. Thus free nalidixate anion in binuclear form can be transformed into a DNA-intercalating form of trinuclear aromatic chromophore *via* Cu^{2+} chelation as explained in Introduction, whereas the nalidixate anion can not be transformed into the corresponding DNA-intercalating agent in the presence of Mg^{2+} . Here the binding of the (Cu^{2+} , nalidixate) and the (Mg^{2+} , nalidixate) system respectively with the calf thymus DNA was tested by the competition of the (metal, quinolone) system with ethidium bromide for binding sites on calf thymus DNA. The fluorescence Scatchard plots for the binding of ethidium bromide to calf thymus DNA in the presence of (Cu^{2+} , nalidixate) system and (Mg^{2+} , nalidixate) system, respectively, are shown in Figure 15. As seen from Figure 15, the effective number of binding site n , remains the same (0.24) but the effective dissociation constant, K_s , is increased from 6.9×10^{-7} in the absence of the (metal, quinolone) system to 1×10^{-6} in the presence of (Cu^{2+} , nalidixate) system *i.e.*, (Cu^{2+} , nalidixate) system acts as competitive inhibitor for the intercalative binding of ethidium bromide to the DNA. This observation of the competitive inhibition of ethidium bromide binding to the DNA by the Cu^{2+} -nalidixate complex demonstrates that Cu^{2+} -nalidixate complex also binds to DNA in an intercalative mode with the same value of $n=0.24$. In contrast, the fluorescence Scatchard plot for the binding of ethidium bromide to the DNA

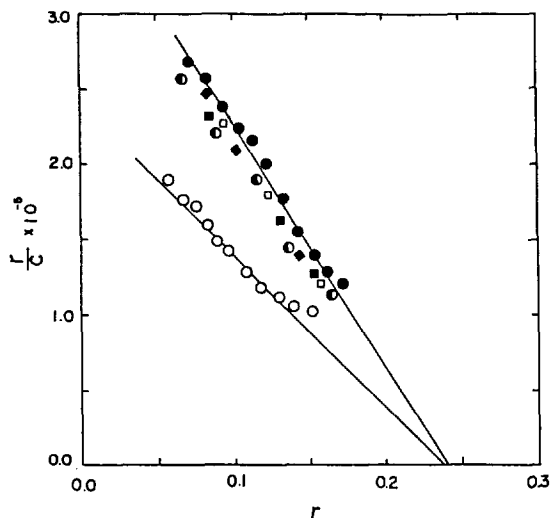


Figure 15. Fluorescence Scatchard plot of ethidium bromide binding to calf thymus DNA under the following conditions: (●) in the absence of (metal quinolone); (○) on the presence of 5×10^{-5} M Cu^{2+} -nalidixate; (□) in the presence of 5×10^{-5} M (Mg^{2+} , nalidixate); (◐) in the presence of 5×10^{-5} M Cu^{2+} ; (■) in the presence of 5×10^{-5} M Mg^{2+} ; (◆) in the presence of 5×10^{-5} M nalidixate. The nucleotide concentration was 3.33×10^{-6} M and the concentration of ethidium bromide added was varied from 1×10^{-7} M to 5×10^{-7} M. Where r is the ratio of bound ethidium bromide per nucleotide phosphate, c is the free ethidium bromide concentration.

Table 2. Effect of Metal Ion Species on the Induction of Cytotoxicity *in vitro* of Quinolones for L1210 Cells

Quinolone species	ED ₅₀ ($\mu\text{g}/\text{ml}$)			
	None	Mg^{2+}	Ni^{2+}	Cu^{2+}
Nalixic acid	Not cytotoxic	Not cytotoxic	11.6	14.3
Oxolinic acid	Not cytotoxic	Not cytotoxic	29.3	31.0
Pipemidic acid	Not cytotoxic	Not cytotoxic	16.2	14.0

Remarks; at the concentrations and under the conditions the metal quinolone complexes were tested, the free metal ions were not cytotoxic.

is not affected by the presence of (Mg^{2+} , nalidixate) system, nor by free metal ions (Cu^{2+} and Mg^{2+} , respectively) and free nalidixate anion as shown in Figure 15. These data of the fluorescence studies are consistent with the UV-Vis absorption spectroscopic data in that the quinolones form stable DNA-intercalating agents with Cu^{2+} but not with Mg^{2+} . Although here the fluorescence studies of the intercalative binding of metal-quinolone chelate complexes to DNA have been done only on Cu^{2+} -nalidixate system, the established data make other divalent transition metal-quinolone systems also interesting as potential intercalators of DNA. It has been established that different anionic quinolones act similarly as

ligand (chelate) of divalent transition metals to form the trinuclear aromatic chromophore of the metal complex.

In view of recent demonstration with eukaryotic (especially mammalian) cells that protein-associated DNA breaks result from topoisomerase II-mediated action of intercalating agents and that the DNA breaks correlates with cytotoxicity and anti-tumor activity of the intercalating agents (see the commentary of Ross, 1985 and references therein)²⁰, it must be rational to expect that the capability of quinolones to form metal-mediated DNA-intercalators will be linked to metal-mediated cytotoxicity of quinolones toward eukaryotic cells.

Table 2 shows the effect of metals on the cytotoxicity of the quinolone antibacterials against L1210 cells *in vitro*. At the concentrations of the free constituent metal cations (Cu^{2+} and Ni^{2+} respectively), where they alone (without quinolone) don't have significant cytotoxicity to the cells, can endow quinolones with cytotoxicity, as seen in the Table 2. However, alkaline earth metal Mg^{2+} , which has been observed to be incompetent for the formation of stable DNA-intercalating metal-chelate complex with the quinolones, is not effective on the induction of cytotoxicity of quinolones for the L1210 cells *in vitro*.

The present data demonstrate that quinolones which have shown remarkable antibacterial effects²¹⁻²³ can also be endowed with cytotoxicity against L1210 cells *in vitro*, via the formation of divalent transition-metal chelate-complex, which can intercalate to DNA double helix, whereas alkaline earth metal, Mg^{2+} , can not have such functions. Similar cytotoxicity of metal-chelate-quinolone complexes against other eukaryotic cells too is expected. Recently there has been reported new kind of quinolones such as CP-115, 953 to be highly active against eukaryotic topoisomerase II *in vitro* and display potent toxicity against mammalian cells in culture²⁴⁻²⁶. However, in their studies, possibility of the formation of DNA-intercalating metal-chelate complexes with environmental metals has not been eliminated.

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The Synthetic Utilization of 2-Hydroxymethyl-2,5-dihydrothiophene 1,1-Dioxide in the Intramolecular Diels-Alder Reaction

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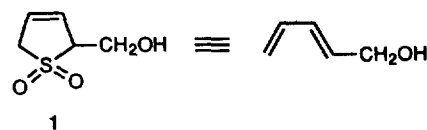
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2-Hydroxymethyl-2,5-dihydrothiophene 1,1-dioxide (1) was prepared from thiophene-2-carboxylic acid by consecutive reactions involving the Birch reduction, esterification, reduction with lithium aluminum hydride, and oxidation with Oxone®. The esterification of alcohol 1 with various unsaturated carboxylic acids provided the precursors 8 for the intramolecular Diels-Alder reaction. The cheletropic expulsion of sulfur dioxide from the esters 8 followed by intramolecular Diels-Alder reaction furnished bicyclic γ - and δ -lactones.

Introduction

The intramolecular Diels-Alder reaction provides a six-membered ring with a fused or a bridged ring of various sizes and has become very useful in organic synthesis.^{1,2} However, one of its limiting factors in the synthetic application is the development of method for the preparation of trienes. As a part of synthetic efforts toward cyclohexyl fragment of tetronasin,² we became interested in the intramolecular Diels-Alder reaction that could lead to bicyclic δ -lactones. In order to carry out the intramolecular Diels-Alder reaction, we had to prepare *s-trans*-penta-2,4-dien-1-ol as a diene for the Diels-Alder reaction. Although several methods for the preparation of *s-trans*-penta-2,4-dien-1-ol were reported in the literatures employing the use of Knoevenagel reaction,⁴ sodium acetylide^{5,6} and dihydropyran,⁶ we found most of them impractical because of difficulties in distillation or storage of product due to its polymerization or decomposition. This fact inspired us to prepare a precursor of 1,3-diene and investigate its application in the intramolecular Diels-Alder reaction.

2,5-Dihydrothiophene 1,1-dioxides (3-sulfolenes) have been utilized as synthetic equivalents of 1,3-dienes because the cheletropic expulsion of sulfur dioxide from those compounds furnishes 1,3-dienes. However, so far there has been no report on the preparation of 2-hydroxymethyl-2,5-dihydrothiophene 1,1-dioxide (1). Herein we would like to report the preparation of 1 and its application to the intramolecular Diels-Alder reaction.



Results and Discussion

Lithiation of 2,5-dihydrothiophene 1,1-dioxides (3-sulfolenes) had been known to furnish 2- and 3-lithio derivatives under the kinetic and thermodynamic reaction conditions, respectively.⁷ The lithiation reaction of sulfolene 2 at -110°C was expected to give 2- or 3-lithiosulfolene. After quenching