# Design of New Topoisomerase II Inhibitors Based upon a Quinobenzoxazine Self-Assembly Model

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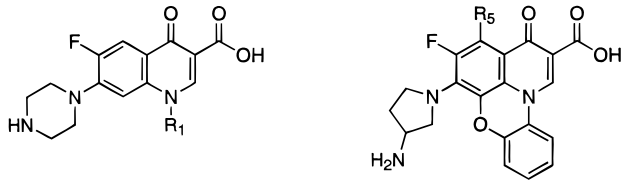
A new class of pyridobenzophenoxazine compounds has been developed as topoisomerase II inhibitors for anticancer chemotherapy. These compounds were designed based on a proposed model of a quinobenzoxazine self-assembly complex on DNA. They showed excellent inhibitory effects on several tumor cell lines with nanomolar $IC_{50}$ values. Their cytotoxic potency correlates with their ability to unwind DNA and inhibit topoisomerase II.

## Introduction

Quinobenzoxazines are synthetic analogues of antibacterial fluoroquinolones, exemplified by norfloxacin and ciprofloxacin.[1−3] As shown in Chart 1, A-62176 (1-(3-aminopyrrolidin-1-yl)-2-fluoro-4-oxo-4*H*-quino[2,3,4-*ij*][1,4]benzoxazine-5-carboxylic acid) and A-85226 (3-amino-1-(3-aminopyrrolidin-1-yl)-2-fluoro-4-oxo-4*H*-quino[2,3,4-*ij*][1,4]benzoxazine-5-carboxylic acid) are two examples of the quinobenzoxazines. Studies have shown that a norvaline prodrug derivative of A-62176 has curative activity against MX-1 and delays tumor growth in other xenographs together with significant delays in solid tumor growth in several murine tumor models.[4] Fluoroquinolones function through inhibition of bacterial DNA topoisomerase II (gyrase) or IV. The cytotoxicity of the fluoroquinolones derives from their ability to shift the cleavage−religation equilibrium required for topoisomerase action toward cleavage, thereby effectively trapping the enzyme on DNA to form the "cleaved complex".[5−8] The quinobenzoxazines, on the other hand, are potent mammalian DNA topoisomerase II inhibitors. It has been proposed that they inhibit the DNA topoisomerase II reaction at a step prior to the formation of the "cleaved complex" intermediate.[9] Our experimental results are in accord with this proposal but also demonstrate that A-62176 stimulates the formation of topoisomerase II-mediated cleavage at certain DNA sequence sites at low concentrations and pH.[10]

DNA binding studies reveal that the antibacterial fluoroquinolones prefer to bind to single-stranded DNA rather than to duplex DNA. They also bind to the DNA−gyrase complex in the presence of $Mg^{2+}$.[8] In contrast to the antibacterial fluoroquinolones, the quinobenzoxazines bind to duplex DNA through an intercalation mechanism. A self-assembly model has been proposed for the quinobenzoxazines on the basis of results of biophysical and biochemical studies.[11,12] In this model, a 2:2 drug−$Mg^{2+}$ dimer binds to DNA, with one drug molecule intercalating between the DNA base pairs and the other drug molecule externally bound through two chelated $Mg^{2+}$ ions. The two magnesium cations link the two drug molecules in a head-to-tail fashion, such that the bidentate ligand of the $\beta$-keto acid moiety and the primary amino group of the aminopyrrolidine side chain are the head and tail, respectively. Each magnesium cation also binds with one phosphate oxygen of the DNA backbone and two water molecules to form an octahedral complex. Norfloxacin, an antibacterial fluoroquinolone that does not intercalate into duplex DNA, shows synergistic effects on the DNA binding of the quinobenzoxazines, suggesting that norfloxacin can replace the externally bound quinobenzoxazine in a DNA-bound 2:2 drug−$Mg^{2+}$ complex.[10−12]

**Chart 1.** Structures of Fluoroquinolones and Quinobenzoxazines




Norfloxacin $R_1$ = ethyl
Ciprofloxacin $R_1$ = cyclopropyl

**Fluoroquinolones**

A-62176 $R_5$ = H
A-85226 $R_5$ = $NH_2$

**Quinobenzoxazines**

On the basis of this model, it can be proposed that there are three different structural motifs within the quinobenzoxazines that determine the ability of these drugs to self-assemble on DNA and interact with topoisomerase II. One motif is its polyaromatic ring that intercalates into DNA base pairs and anchors the whole assembly on DNA, and the other motifs are the $\beta$-keto acid functionality and the 3-aminopyrrolidine substituent that chelate $Mg^{2+}$ through which an external molecule is bound in the DNA minor groove. The external portion of the assembly may be responsible for interacting with topoisomerase II. To develop more potent topoisomerase II inhibitors, the three motifs can be changed systematically by altering either the quinobenzoxazine's intercalation ability or its magnesium binding ability.

Since the phenyl ring portion of the quinobenzoxazines is actually inserted between DNA base pairs, its intercalation ability can be increased by extending the

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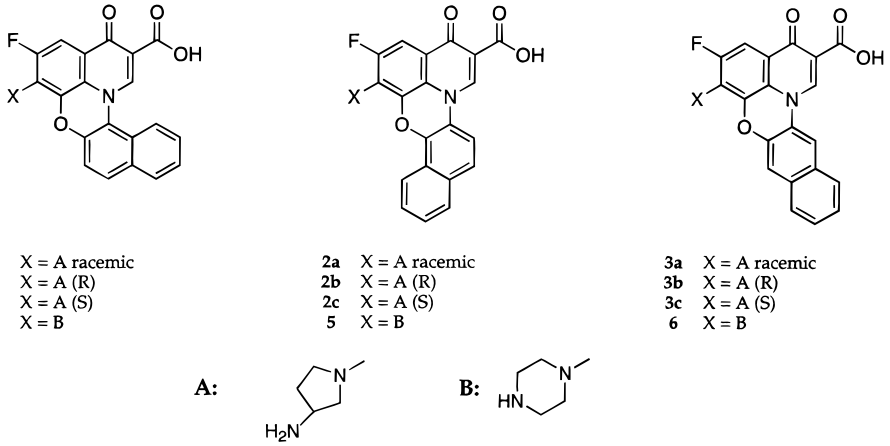
**Chart 2.** Structures of the Pyridobenzophenoxazine Analogues Synthesized in This Study



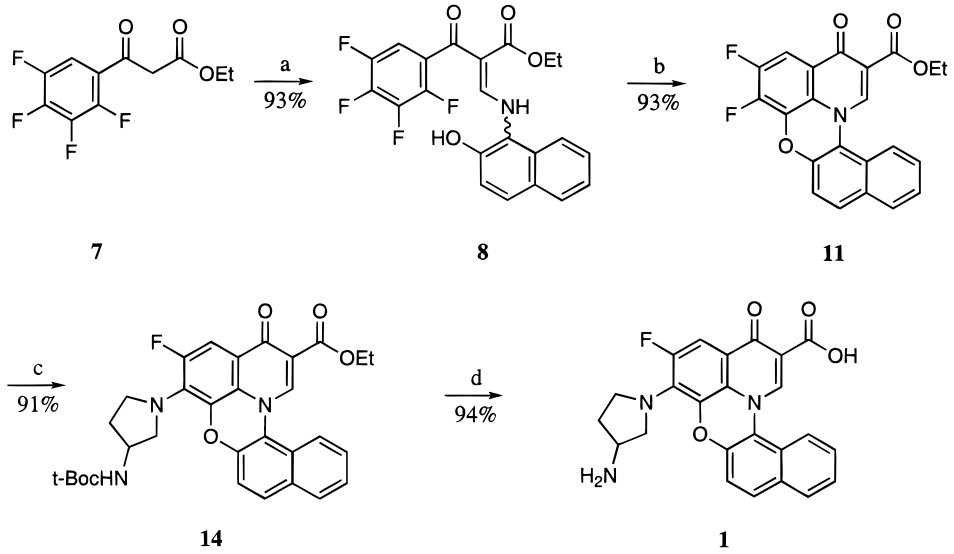
**1a** X = A racemic
**1b** X = A (R)
**1c** X = A (S)
**4** X = B

**2a** X = A racemic
**2b** X = A (R)
**2c** X = A (S)
**5** X = B

**3a** X = A racemic
**3b** X = A (R)
**3c** X = A (S)
**6** X = B

**A:** **B:**

**Scheme 1**[a]



**7** **8** **11** **14** **1**

[a] (a) (1) $(EtO)_3CH$, $Ac_2O$, (2) 1-amino-2-naphthol; (b) NaH; (c) *t*-Boc-3-aminopyrrolidine; (d) $OH^-/H^+$.

phenyl ring to a naphthyl ring. A series of new pyridobenzophenoxazine analogues were thus designed and synthesized. These analogues showed strong cytotoxic effects on MDA-231 breast, H226 non-small-cell lung, HT-29 colon, Rali lymphoma, and DU-145 prostate human cancer cells and B16 murine melanoma cells. The $IC_{50}$ values of these inhibitors were in the range of 4 nM to 2 $\mu$M. They also showed potent inhibitory effects against human topoisomerase II, with $IC_{50}$ values in the micromolar range. Some of these new analogues have a greater DNA unwinding ability in a decatenation assay than A-62176. It appears that for these compounds the higher DNA unwinding ability leads to higher potency in topoisomerase II inhibition and increased cytotoxicity against tumor cells.

## Results

**Design and Synthesis of the Pyridobenzophenoxazines.** A series of pyridobenzophenoxazines has been designed and synthesized as topoisomerase II inhibitors (Chart 2). The design was based on the 2:2 quinobenzoxazine−$Mg^{2+}$ self-assembly model for A-62176. In this model, the benzene ring of A-62176 intercalates into DNA and binds with DNA by $\pi-\pi$ stacking with DNA base pairs. Additional stabilization is achieved by the chelation of two magnesium ions with the $\beta$-keto acid, the amino group of the side chain, and a phosphate oxygen of the DNA backbone. Better intercalation may be achieved by extending the phenyl ring of A-62176 to a naphthalene ring (i.e., A-62176 → **1**, **2**, or **3**). Since the exact orientation of the benzene ring between DNA base pairs is uncertain, all three possible directions of benzo-annulation were explored to achieve the maximum $\pi$-stacking interaction with DNA base pairs. Compounds **4**−**6** have a piperazine ring instead of a 3-aminopyrrolidine ring as side chains. Since the 3-amino group of A-62176 is proposed to chelate magnesium, the change may have some effect on its ability to self-assemble on DNA and, therefore, on topoisomerase II inhibition. For A-62176, the stereochemistry of the 3-aminopyrrolidine side chain affects topoisomerase II inhibition potency; i.e., the *S*-isomer is more potent than the *R*-isomer.[10,12] To study the possible effects of the stereochemistry of the pyridobenzophenoxazines, the compounds were made either racemic or enantiomerically pure.

The synthetic procedure was based on a published method for the synthesis of the quinobenzoxazines, with some modification.[1] The synthesis of compound **1** is shown in Scheme 1. The synthesis was started with ethyl 2,3,4,5-tetrafluorobenzoylacetate, which was prepared according to the published procedure.[1] Treatment

**Table 1.** Decatenation Inhibition, DNA Unwinding, and Cytotoxicity Data for the Pyridobenzophenoxazines

| compd | topoisomerase II inhibition $IC_{50}$ ($\mu$M)[a] | DNA unwinding concn ($\mu$M)[b] | $IC_{50}$ (nM) B16 melanoma | MDA-231 breast | H226 non-small-cell lung | HT-29 colon | Raji lymphoma | DU-145 prostate |
|---|---|---|---|---|---|---|---|---|
| A-62176 (*S*) | 0.51 | 50 | 170 ± 10 | 160 ± 20 | 90 ± 3 | 160 ± 10 | 50 ± 6 | 160 ± 30 |
| **1a** (racemic) | 0.77 | | 440 ± 60 | 150 ± 40 | 140 ± 10 | 180 ± 10 | 86 ± 6 | 200 ± 30 |
| **1b** (*R*) | 1.22 | 35 | 680 ± 50 | 350 ± 60 | 2400 ± 100 | 360 ± 40 | 160 ± 70 | 360 ± 120 |
| **1c** (*S*) | 0.72 | 19 | 130 ± 20 | 190 ± 40 | 130 ± 10 | 270 ± 40 | 82 ± 8 | 140 ± 10 |
| **2a** (racemic) | 0.55 | | 40 ± 4 | 27 ± 2 | 22 ± 2 | 30 ± 3 | 18 ± 1 | 17 ± 7 |
| **2b** (*R*) | 0.24 | 3 | 20 ± 2 | 5 ± 1 | 13 ± 5 | 26 ± 1 | 34 ± 2 | 30 ± 10 |
| **2c** (*S*) | 0.49 | 10 | 180 ± 20 | 84 ± 7 | 27 ± 4 | 51 ± 6 | 20 ± 4 | 64 ± 7 |
| **3a** (racemic) | 0.77 | | 100 ± 10 | 23 ± 4 | 4 ± 2 | 28 ± 5 | 38 ± 6 | 25 ± 9 |
| **3b** (*R*) | 0.42 | 15 | 140 ± 15 | 21 ± 2 | 22 ± 15 | 22 ± 3 | 32 ± 5 | 48 ± 8 |
| **3c** (*S*) | 0.44 | 13 | 20 ± 6 | 10 ± 2 | 14 ± 2 | 23 ± 3 | 29 ± 5 | 30 ± 6 |
| **4** | 9.6 | >100 | 2100 ± 300 | 2200 ± 300 | 2100 ± 200 | 2000 ± 200 | 800 ± 50 | 840 ± 270 |
| **5** | 1.84 | >100 | 650 ± 60 | 380 ± 50 | 200 ± 30 | 360 ± 90 | 210 ± 40 | 140 ± 40 |
| **6** | 0.64 | >100 | 45 ± 48 | $>10^6$ | $>10^5$ | 6800 ± 1800 | 7.2 ± 7.6 | 75 ± 32 |

[a] Drug concentration that inhibits 50% of the conversion of catenated to decatenated KDNA by human topoisomerase II. [b] Drug concentration that causes a shift of the topoisomers from the original to the middle position between the relaxed and the supercoiled bands.

of 2,3,4,5-tetrafluorobenzoylacetate with triethyl orthofomate in acetic anhydride followed by various aminonaphthols generated a small library of enamino keto acid intermediates. These intermediates were treated with sodium hydride at −78 °C and then heated to reflux to complete the double annulation. The double annulation allowed us to avoid hydroxyl protection and deprotection, which was necessary in the synthesis of the quinobenzoxazines.[1] As a result, double annulation shortened the synthesis by three steps and improved the overall yields. Regioselective nucleophilic substitution of fluoride with racemic or optically pure 3-(*tert*-butoxycarbonylamino)pyrrolidine followed by hydrolysis of the carboxylate and deprotection of the amino group produced the final products (compounds **1**−**3**). Compounds **4**−**6** were obtained by using *tert*-butyl-1-piperazinecarboxylate for the nucleophilic aromatic substitution. The yields of nucleophilic substitutions with *tert*-butyl-1-piperazinecarboxylate were lower than with 3-(*tert*-butoxycarbonylamino)pyrrolidine.

**Inhibition of Topoisomerase II by the Pyridobenzophenoxazines.** The inhibitory effect of these compounds against human topoisomerase II was measured by kinetoplast DNA (KDNA) decatenation assays in which a catenated network of DNA rings was decatenated by topoisomerase II to generate the monomeric DNA. Decatenation is a highly specific assay for topoisomerase II activity[13] and can be used to screen for inhibitors that block the catalytic activity of topoisomerase II. The $IC_{50}$ values for decatenation are shown in Table 1 and are based on three parallel experiments. Compounds **2**, **3**, and **6** show comparable or slightly higher activities than A-62176, compounds **1** and **5** are slightly less active than A-62176, and compound **4** was about 20-fold less active than A-62176.

**Unwinding of DNA by the Pyridobenzophenoxazines.** Unwinding of duplex DNA is a hallmark feature of intercalating agents such as chloroquine, ethidium bromide, and *m*-AMSA.[14] The antineoplastic activity of the quinobenzoxazines was shown to correlate with their ability to unwind DNA in a decatenation assay.[9,11] The DNA unwinding effect of the pyridobenzophenoxazines was investigated, and the concentrations of compounds required to produce a 50% conversion of catenated to decatenated KDNA are shown in Table 1. Compounds **2** and **3** showed the highest ability to unwind DNA, with unwinding concentrations in the 3−15 $\mu$M range. The unwinding ability of compound **1** (19−35 $\mu$M) was lower than that of **2** and **3** but higher than that of A-62176 (50 $\mu$M). Compounds **4**−**6** showed no significant unwinding effect, even at concentrations higher than 100 $\mu$M.

**Cytotoxic Potency of the Pyridobenzophenoxazines.** The cytotoxicity of each of these compounds against MDA-231 breast, H226 non-small-cell lung, HT-29 colon, Raji lymphoma, and DU-145 prostate human cancer cells and B16 murine melanoma cells was measured and is shown in Table 1. In general, compounds **2** and **3** are the most potent derivatives, with $IC_{50}$ values in the 10−50 nM range. The *R*-enantiomer of compound **2** shows selectivity against MDA-231 breast cancer cell lines, with an $IC_{50}$ value of 5 nM. Compounds **1** and **5** and A-62176 are in the same range of potency against all tested cell lines. They are about 10−100-fold less potent than compounds **2** and **3**. Compound **6** shows some selective cytotoxicity, with $IC_{50}$ values of 7 nM against lymphoma, 75 nM against DU-145 prostate, and 50 nM against B16 murine melanoma cells. Compound **4** is the least potent derivative, with $IC_{50}$ values in the 0.8−2 $\mu$M range.

## Discussion

The pyridobenzophenoxazines were designed and synthesized based on a quinobenzoxazine self-assembly model[11] with the premise that increasing the intercalation ability of this class of molecules will increase their potency against topoisomerase II and, therefore, their antineoplastic activity. Our experimental results are consistent with this premise. Compounds **2** and **3** showed a stronger DNA unwinding ability than A-62176. They also showed higher potency than A-62176 in both the cell-free topoisomerase II inhibition assay and the in vitro cytotoxicity assay using a range of cell lines. Because of the steric hindrance between the hydrogens on the pyrido and benzo rings, the naphthyl and quinolone portions of compound **1** are not coplanar. This may have a negative effect on its DNA intercalation ability. Therefore, compound **1** was less potent than compounds **2** and **3** and A-62176. Compounds **4**−**6** have a piperazine ring as side chains instead of an aminopyrrolidine ring, as found in A-62176 and compounds **1**−**3**. Compounds **4**−**6** showed the least activity in the

cell-free and in vitro assays. This may be because the secondary amino nitrogen of the piperazine side chain is in a less optimal position to chelate $Mg^{2+}$ and to form a 2:2 self-assembly ternary complex in the DNA minor groove. The stereochemistry of the 3-aminopyrrolidine side chain of compounds **1**−**3** may play some role in their potency, and the 2:2 model would allow racemic mixtures to have different potencies than an average of the *R*- and *S*-isomers. However, the relationship between the stereochemistry of these compounds and their biological activity remains to be determined. In summary, it appears that there is a correlation among DNA unwinding, topoisomerase II inhibition, and cytotoxicity with these pyridobenzophenoxazine derivatives.

In conclusion, a novel group of pyridobenzophenoxazine compounds has been designed and synthesized based upon the quinobenzoxazine self-assembly model on DNA. These compounds produce potent unwinding of duplex DNA, inhibit human topoisomerase II catalytic activity, and show potent inhibitory effects on several tumor cell lines with $IC_{50}$ values ranging from subnanomolar to micromolar.

## Experimental Section

**General Methods for Chemistry.** All melting points were recorded on a Thomas-Hoover capillary melting point apparatus and are uncorrected. $^1H$ and $^{19}F$ NMR data were obtained on a Varian Unity 300-MHz NMR spectrometer. $^{13}C$ NMR data were obtained on a Varian Unity 500-MHz NMR spectrometer. The chemical shifts are relative to the trace proton, carbon, or fluorine signals of the deuterated solvent. Coupling constants, *J*, are reported in Hz and refer to apparent peak multiplicity and not true coupling constants. Mass spectroscopic experiments were performed by the Mass Spectroscopy Center at The University of Texas at Austin. Elemental analysis of C, H, N was done by Quantitative Technologies Inc., White House, NJ. Flash column chromatography was performed on silica gel 60, 230−400 mesh, purchased from Spectrum. All starting materials were obtained from commercial sources unless otherwise specified.

**Ethyl 3-((2-Hydroxynaphthyl)amino)-2-((2,3,4,5-tetrafluorophenyl)carbonyl)prop-2-enoate (8).** A solution of ethyl 2,3,4,5-tetrafluorobenzoylacetate (1100 mg, 4.18 mmol) in triethyl orthoformate (1 mL, 6.06 mmol) and acetic anhydride (1.8 mL, 19 mmol) was heated to 130 °C and stirred for 4 h. During the reaction, ethyl acetate was formed and then removed by distillation. The resulting reaction mixture was distilled under vacuum to yield an orange oil, which was dissolved in 30 mL of dichloromethane. A solution of 1-aminonaphthalen-2-ol hydrogen chloride (1360 mg, 6.26 mmol), in 2 equiv of pyridine (1.0 mL, 12.4 mmol), was added to the dichloromethane solution, and the mixture was stirred overnight at 22 °C. After the solvent was removed under reduced pressure, the remaining residue was purified by silica gel chromatography (gradient ethyl acetate/hexanes = 8:1 to 4:1), yielding a bright-yellow powder (1690 mg, 93%): $^1H$ NMR ($CDCl_3$) $\delta$ 1.02 (t, $J$ = 7.2 Hz, 3H), 4.01 (q, $J$ = 7.2 Hz, 2H), 4.10 (m, 1H), 7.34 (d, $J$ = 9.0 Hz, 1H), 7.41 (m, 1H), 7.60 (m, 2H), 7.82 (8, 2H), 7.91 (d, $J$ = 8.1 Hz, 1H), 8.54 (br, 1H).

**Ethyl 3-((1-Hydroxy-2-naphthyl)amino)-2-((2,3,4,5-tetrafluorophenyl)carbonyl)prop-2-enoate (9).** The synthesis of compound **9** was accomplished in a manner similar to compound **8** and yielded a yellow amorphous solid (90%): $^1H$ NMR ($CDCl_3$) $\delta$ 1.14 (t, $J$ = 7.2 Hz, 3H), 4.13 (q, $J$ = 7.2 Hz, 2H), 6.86 (m, 1H), 7.40−7.64 (m, 4H), 7.80 (m, 1H), 7.93 (d, $J$ = 8.4 Hz, 1H), 8.69−8.79 (m, 1H).

**Ethyl 3-((3-Hydroxy-2-naphthyl)amino)-2-((2,3,4,5-tetrafluorophenyl)carbonyl)prop-2-enoate (10).** The synthesis of compound **10** was accomplished in a manner similar to compound **8** and yielded a yellow amorphous solid (90%): $^1H$ NMR ($CDCl_3$) $\delta$ 1.08 (t, $J$ = 7.2 Hz, 3H), 4.07 (q, $J$ = 7.2 Hz, 2H), 7.30−7.40 (m, 3H), 7.51 (m, 1H), 7.71 (d, $J$ = 7.8 Hz, 1H), 7.85 (d, 7.0 Hz, 1H), 8.20 (br, 1H), 8.87 (br, 1H).

**Ethyl 1,2-Difluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]benzo[*h*]-phenoxazine-5-carboxylate (11).** Ethyl 3-((2-hydroxynaphthyl)amino)-2-((2,3,4,5-tetrafluorophenyl)carbonyl)prop-2-enoate (110 mg, 0.25 mmol) and NaH (60% in mineral oil) (23.2 mg, 0.58 mmol) were mixed with freshly distilled THF and stirred at −78 °C for 15 min. The mixture was gradually warmed to room temperature and then heated at 65 °C for 30 min. The excess NaH was quenched by addition of methanol (30 mL). The solution was evaporated to dryness, and the product was purified by silica gel chromatography (hexanes/ethyl acetate = 3:1), yielding a yellow powder (94 mg, 93%): $^1H$ NMR ($CDCl_3$) $\delta$ 1.42 (t, $J$ = 7.2 Hz, 3H), 4.42 (q, $J$ = 7.2 Hz, 2H), 7.35 (d, $J$ = 8.7 Hz, 1H), 7.53 (t, $J$ = 7.2 Hz, 1H), 7.64 (t, $J$ = 9.0 Hz, 1H), 7.76 (dd, $J_{F-H}$ = 7.5, 9.9 Hz, 1H), 7.80 (d, $J$ = 8.7 Hz, 1H), 7.89 (d, $J$ = 7.5 Hz, 1H), 8.20 (d, $J$ = 8.7 Hz, 1H), 8.36 (s, 1H).

**Ethyl 1,2-Difluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]benzo[*f*]-phenoxazine-5-carboxylate (12).** The synthesis of compound **12** was accomplished in a manner similar to compound **11** and yielded a yellow amorphous solid (60%): $^1H$ NMR ($CDCl_3$) $\delta$ 1.46 (t, $J$ = 7.2 Hz, 3H), 4.45 (q, $J$ = 7.2 Hz, 1H), 7.50 (d, $J$ = 9.1 Hz, 1H), 7.54 (t, $J$ = 7.5 Hz, 1H), 7.60 (t, $J$ = 7.5 Hz, 1H), 7.66 (d, $J$ = 9.1 Hz, 1H), 7.74 (dd, $J_{F-H}$ = 7.5, 10.8 Hz, 1H), 7.77 (d, $J$ = 8.4 Hz, 1H), 8.18 (d, $J$ = 8.4 Hz, 1H), 8.93 (s, 1H).

**Ethyl 1,2-Difluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]benzo[*g*]-phenoxazine-5-carboxylate (13).** The synthesis of compound **13** was accomplished in a manner similar to compound **11** and yielded a light-yellow amorphous solid (52%): $^1H$ NMR ($CDCl_3$) $\delta$ 1.48 (t, $J$ = 7.2 Hz, 3H), 4.49 (q, $J$ = 7.2 Hz, 2H), 7.51 (m, 2H), 7.62 (s, 1H), 7.75 (m, 1H), 7.79−7.85 (m, 2H), 7.89 (s, 1H), 9.19 (s, 1H).

**Ethyl 1-(3-(*tert*-Butoxycarbonylamino)pyrrolidin-1-yl)-2-fluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]benzo[*h*]phenoxazine-5-carboxylate (14a).** Ethyl 1, 2-difluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]benzo[*h*]phenoxazine-5-carboxylate (**11**) (100 mg, 0.25 mmol) and racemic 3-(*tert*-butoxycarbonylamino)-pyrrolidine (140 mg, 0.77 mmol) were dissolved in pyridine (15 mL), and the mixture was stirred under argon at 110 °C for 36 h. After the pyridine was removed, the residue was purified by silica gel chromatography (dichloromethane/ethyl acetate = 2:3), yielding a yellow powder (120 mg, 91%): $^1H$ NMR ($CDCl_3$) $\delta$ 1.41 (t, $J$ = 7.2 Hz, 3H), 1.46 (s, 9H), 1.94 (m, 1H), 2.20 (m, 1H), 3.52 (br, 1H), 3.65 (m, 1H), 3.81 (m, 1H), 3.92 (m, 1H), 4.33 (br, 1H), 4.40 (q, $J$ = 7.2 Hz, 2H), 5.05 (br, 1H), 7.18 (d, $J$ = 8.7 Hz, 1H), 7.45 (t, $J$ = 7.8 Hz, 1H), 7.54 (t, $J$ = 7.8 Hz, 1H), 7.56 (d, $J_{F-H}$ = 13.5 Hz, 1H), 7.69 (d, $J$ = 8.7 Hz, 1H), 7.83 (d, $J$ = 8.7 Hz, 1H), 8.09 (d, $J$ = 8.7 Hz, 1H), 9.12 (s, 1H).

**Ethyl 1-(3-(*tert*-Butoxycarbonylamino)pyrrolidin-1-yl)-2-fluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]benzo[*f*]phenoxazine-5-carboxylate (15).** The synthesis of compound **15** was accomplished in a manner similar to compound **14a** and yielded a yellow amorphous solid (18 mg, 90%): $^1H$ NMR ($CDCl_3$) $\delta$ 1.42 (t, $J$ = 7.2 Hz, 3H), 1.48 (s, 9H), 2.02 (m, 1H), 2.28 (m, 1H), 3.55 (br, 1H), 3.65 (m, 1H), 3.87 (br, 1H), 3.92 (m, 1H), 4.33 (m, 1H), 4.39 (q, $J$ = 7.2 Hz, 2H), 5.24 (m, 1H), 7.37 (d, $J$ = 9.0 Hz, 1H), 7.51−7.61 (m, 3H), 7.78 (d, $J$ = 7.8 Hz, 1H), 7.99 (d, $J$ = 7.8 Hz, 1H), 8.71 (s, 1H).

**Ethyl 1-(3-(*tert*-Butoxycarbonylamino)pyrrolidin-1-yl)-2-fluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]benzo[*g*]phenoxazine-5-carboxylate (16).** The synthesis of compound **16** was accomplished in a manner similar to compound **14a** and yielded a yellow amorphous solid (33 mg, 45%): $^1H$ NMR ($CDCl_3$) $\delta$ 1.45 (t, $J$ = 7.2 Hz, 3H), 1.48 (s, 9H), 1.96 (m, 1H), 2.22 (m, 1H), 3.54−3.70 (m, 2H), 3.86−4.02 (m, 2H), 4.33 (m, 1H), 4.44 (q, $J$ = 7.2 Hz, 2H), 5.22 (br, 1H), 7.34 (s, 1H), 7.40−7.48 (m, 2H), 7.55 (d, $J_{F-H}$ = 13.5 Hz, 1H), 7.65 (d, $J$ = 7.8 Hz, 1H), 7.67 (s, 1H), 7.72 (d, $J$ = 7.8 Hz, 1H), 8.93 (s, 1H).

**1-(3-Aminopyrrolidin-1-yl)-2-fluoro-4-oxo-4*H*-pyrido-[3,2,1-*kl*]benzo[*h*]phenoxazine-5-carboxylic Acid Hydrogen Chloride Salt (1a).** Ethyl 1-(3-(*tert*-butoxycarbonyl-

amino)pyrrolidin-1-yl)-2-fluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]benzo-[*h*]phenoxazine-5-carboxylate (**14a**) (77 mg, 0.138 mmol) was mixed with ethanol (3 mL) and 1 N KOH (1 mL). This mixture was refluxed for 30 min; 2 N HCl (3 mL) and ethanol (2 mL) were added to the mixture, and the resulting mixture was refluxed for 4 h. After the reaction mixture was slowly cooled, a yellow powder was isolated by filtration. The powder was washed with water and ethanol and then dried in vacuo (65 mg, 94%): mp >225 °C dec; $^1$H NMR ($CF_3COOD$) $\delta$ 2.65 (m, 2H), 4.10 (m, 1H), 4.37−4.54 (m, 4H), 7.44 (d, $J$ = 9.0 Hz, 1H), 7.66 (t, $J$ = 7.5 Hz, 1H), 7.78 (t, $J$ = 7.5 Hz, 1H), 7.90 (d, $J$ = 13.0 Hz, 1H), 8.03 (d, $J$ = 8.5 Hz, 1H), 8.08 (d, $J$ = 9.0 Hz, 1H), 8.20 (d, $J$ = 8.5 Hz, 1H), 9.90 (s, 1H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 29.3, 49.0, 49.1, 54.3, 104.8 (d, $J_{C-F}$ = 24.6 Hz), 106.8, 114.1, 116.6 (d, $J_{C-F}$ = 9.2 Hz), 117.0, 118.5, 120.1, 123.8, 125.8, 128.5, 128.8 (d, $J_{C-F}$ = 14.2 Hz), 129.4, 130.4 (d, $J_{C-F}$ = 20.1 Hz), 131.9, 134.3 (d, $J_{C-F}$ = 9.2 Hz), 143.7, 145.4, 153.0 (d, $J_{C-F}$ = 248 Hz), 165.4, 175.9, 180.2; $^{19}$F NMR ($CF_3COOD$) $\delta$ −113.2 (d, $J_{F-H}$ = 12.7 Hz); HRMS calcd for $C_{24}H_{19}FN_3O_4$ 432.1360, found 432.1375. **1a**: Anal. ($C_{24}H_{18}FN_3O_4\cdot HCl\cdot {}^1/_3H_2O$) C, H, N. **1b**: Anal. ($C_{24}H_{18}FN_3O_4\cdot HCl\cdot {}^4/_3H_2O$) C, H, N. **1c**: Anal. ($C_{24}H_{18}FN_3O_4\cdot HCl\cdot {}^3/_2H_2O$) C, H, N.

**1-(3-Aminopyrrolidin-1-yl)-2-fluoro-4-oxo-4*H*-pyrido-[3,2,1-*kl*]benzo[*f*]phenoxazine-5-carboxylic Acid Hydrogen Chloride Salt (2).** The synthesis of compound **2** was accomplished in a manner similar to compound **1a** and yielded a yellow amorphous solid (18 mg, 99%): mp >207 °C dec; $^1$H NMR ($CF_3COOD$) $\delta$ 2.69 (m, 2H), 4.19 (m, 1H), 4.40−4.67 (m, 4H), 7.73−7.81 (m, 3H), 7.87−7.99 (m, 3H), 8.20 (m, 1H), 9.47 (s, 1H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 29.4, 49.0, 49.3, 54.5, 104.6 (d, $J_{C-F}$ = 24.8 Hz), 106.8, 114.1, 116.9 (d, $J_{C-F}$ = 9.2 Hz), 118.8, 121.4, 122.6, 125.2, 126.2, 127.6, 128.0 (d, $J_{C-F}$ = 10.6 Hz), 128.6 (d, $J_{C-F}$ = 14.2 Hz), 132.6, 134.7 (d, $J_{C-F}$ = 8.7 Hz), 137.7, 138.7, 154.0 (d, $J_{C-F}$ = 248 Hz), 165.4, 175.4, 180.2; $^{19}$F NMR ($CF_3COOD$) $\delta$ −111.4 (d, $J_{F-H}$ = 12.1 Hz); HRMS calcd for $C_{24}H_{19}FN_3O_4$ 432.1360, found 432.1355. **2a**: Anal. ($C_{24}H_{18}FN_3O\cdot HCl\cdot H_2O$) C, H, N. **2b**: Anal. ($C_{24}H_{18}FN_3O_4\cdot HCl\cdot {}^4/_3H_2O$) C, H, N. **2c**: Anal. ($C_{24}H_{18}FN_3O_3\cdot HCl\cdot {}^4/_3H_2O$) C, H, N.

**1-(3-Aminopyrrolidin-1-yl)-2-fluoro-4-oxo-4*H*-pyrido-[3,2,1-*kl*]benzo[*g*] phenoxazine-5-carboxylic Acid Hydrogen Chloride Salt (3).** The synthesis of compound **3** was accomplished in a manner similar to compound **1a** and yielded a yellow amorphous solid (29 mg, 99%): mp >220 °C dec; $^1$H NMR ($CF_3COOD$) $\delta$ 2.67 (m, 2H), 4.17 (m, 1H), 4.48−4.60 (m, 4H), 7.60−7.67 (m, 2H), 7.76 (s, 1H), 7.84 (m, 1H), 7.92−7.96 (m, 2H), 8.40 (s, 1H), 9.78 (s, 1H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 29.4, 49.1, 49.2, 54.5, 104.4 (d, $J_{C-F}$ = 24.6 Hz), 108.0, 113.3, 114.9, 116.2 (d, $J_{C-F}$ = 9.0 Hz), 123.1, 125.4, 126.3 (d, $J_{C-F}$ = 15.1 Hz), 127.7, 128.3, 129.0 (d, $J_{C-F}$ = 14.2 Hz), 130.3, 132.3, 133.6 (d, $J_{C-F}$ = 8.7 Hz), 138.6, 141.8, 153.0 (d, $J_{C-F}$ = 248 Hz), 165.4, 175.9, 180.2; $^{19}$F NMR (DMSO-$d_6$) $\delta$ −119.2 (d, $J_{F-H}$ = 14.0 Hz); HRMS calcd for $C_{24}H_{19}FN_3O_4$ 432.1360, found 432.1358. **3a**: Anal. ($C_{24}H_{18}FN_3O_4\cdot HCl\cdot {}^3/_2H_2O$) C, H, N. **3b**: Anal. ($C_{24}H_{18}FN_3O_4\cdot HCl\cdot {}^1/_2H_2O$) C, H, N. **3c**: Anal. ($C_{24}H_{18}FN_3O_4\cdot HCl\cdot {}^1/_2H_2O$) C, H, N.

**1-(Piperazin-1-yl)-2-fluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]-benzo[*h*]phenoxazine-5-carboxylic Acid Hydrogen Chloride Salt (4).** Ethyl 1,2-difluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]-benzo[*h*]phenoxazine-5-carboxylate (**11**) (77 mg, 0.2 mmol) was treated with *tert*-butyl 1-piperazinecarboxylate (109 mg, 0.6 mmol) in pyridine (5 mL) at 110 °C for 60 h. After removal of the solvent, the residue was purified by chromatography, yielding a yellow solid. The solid was refluxed in a 1:3 mixture of 1 N KOH and ethanol (4 mL) for 30 min. The solution was then mixed with equal volumes of 1:1 2 N HCl (aq) and ethanol (3 mL) and refluxed again for 2 h. After allowing the mixture to cool slowly to room temperature, a yellow amorphous solid was isolated by filtration. This solid was extensively washed with water and ethanol and dried in vacuo (55.6 mg, 60%): mp >230 °C dec; $^1$H NMR ($CF_3COOD$) $\delta$ 3.73 (s, 4H), 3.97 (s, 4H), 7.44 (d, $J$ = 9.0 Hz, 1H), 7.68 (t, $J$ = 7.2 Hz, 1H), 7.80 (t, $J$ = 7.2 Hz, 1H), 7.96 (d, $J_{F-H}$ = 10.8 Hz, 1H), 8.05 (d, $J$ = 7.5 Hz, 1H), 8.11 (d, $J$ = 8.7 Hz, 1H), 8.28 (d, $J$ = 8.7 Hz, 1H), 10.06 (s, 1H); HRMS calcd for $C_{24}H_{19}FN_3O_4$ 432.1360, found 432.1366.

**1-(Piperazin-1-yl)-2-fluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]-benzo[*f*]phenoxazine-5-carboxylic Acid Hydrogen Chloride Salt (5).** The synthesis of compound **5** was accomplished in a manner similar to compound **4** and yielded a yellow amorphous solid (42%): mp >210 °C dec; $^1$H NMR ($CF_3COOD$) $\delta$ 3.81 (s, 4H), 4.07 (s, 4H), 7.80 (m, 3H), 7.97 (m, 3H), 8.30 (br, 1H), 9.65 (s, 1H); HRMS calcd for $C_{24}H_{19}FN_3O_4$ 432.1360, found 432.1351.

**1-(Piperazin-1-yl)-2-fluoro-4-oxo-4*H*-pyrido[3,2,1-*kl*]-benzo[*g*]phenoxazine-5-Carboxylic Acid Hydrogen Chloride Salt (6).** The synthesis of compound **6** was accomplished in a manner similar to compound **4** and yielded a yellow amorphous solid (37%): mp >200 °C dec; $^1$H NMR ($CF_3COOD$) $\delta$ 3.79 (s, 4H), 4.03 (s, 4H), 7.60−7.72 (m, 2H), 7.81 (s, 1H), 7.89 (d, $J$ = 8.7 Hz, 1H), 7.97−8.02 (m, 2H), 8.53 (s, 1H), 9.93 (s, 1H); HRMS calcd for $C_{24}H_{19}FN_3O_4$ 432.1360, found 432.1348.

**Materials for Biochemistry.** KDNA, human topoisomerase II (170-kDa form), and DNA Unwinding Kit including supercoiled pHOT1 DNA, relaxed DNA marker, buffers, and human topoisomerase I were purchased from TopoGEN, Inc. Agarose was purchased from Fisher. Quinobenzoxazine A-62176 (*S*-enatiomer) was provided by Abbott Laboratories or prepared according to published procedures.[1]

**Decatenation Assay.** KDNA (0.25 $\mu$g) was incubated with various concentrations of the quinobenzoxazines in a 20-$\mu$L reaction mixture containing 50 mM Tris-HCl (pH 8), 120 mM KCl, 10 mM $MgCl_2$, and 0.5 mM each of ATP, dithiothreitol, and 30 mg/mL BSA for 10 min. Two units of human topoisomerase II (170-kDa form) was added to the mixture, which was then incubated at 37 °C for 30 min. The reaction was terminated with 0.1 volume of stop buffer (5% sarkosyl, 0.025% bromophenol blue, and 50% glycerol). The decatenation products were analyzed on 1% agarose gels run with 0.5 $\mu$g/mL ethidium bromide. The gels were scanned by FluorImager (Molecular Dynamics) and analyzed by ImageQuaNT software.

**DNA Unwinding Assay.** A 22-$\mu$L mixture of 0.25 $\mu$g of supercoiled pHOT1 DNA and 5 units of topoisomerase I in topoisomerase I buffer (provided with the kit) was incubated at 37 °C for 30 min; 2 $\mu$L of various concentrations of drug was added, and the incubation was continued for another 30 min. The reactions were terminated by addition of SDS to 1%. Proteinase K was added to the reaction mixtures to ca. 50 $\mu$g/mL, and the reaction mixtures were incubated for 15−20 min at 56 °C. After adding 0.1 volume of 10X gel loading buffer, the reaction mixtures were extracted with an equal volume of CIA (chloroform−isoamyl alcohol, 24:1). The samples were loaded on a 1% agarose gel that was cast in TPE buffer (36 mM Tris-HCl, pH 7.8, 1 mM EDTA, 30 mM $NaH_2PO_4$) and 0.2 $\mu$g/mL chloroquine. The gel was run in TPE buffer at room temperature at 10 V for 20 h, destained in water for 15 min, stained in 0.05 $\mu$g/mL ethidium bromide for 15 min, and destained again in water for 15 min. The gel was then photographed, scanned by FluorImager (Molecular Dynamics), and analyzed by ImageQuaNT software.

**Assay for Inhibition of Cell Growth.** Exponentially multiplying cells in 0.1 mL of medium were seeded on day 0 in 96-well microtiter plates. On day 1, 0.1-mL aliquots of medium containing graded concentrations of test agent were added in duplicate to the cell plates. After incubation at 37 °C in a humidified incubator, the plates were centrifuged briefly (1−2 min at 1000 rpm), and 100 $\mu$L of the growth medium was removed. Cell cultures were incubated with 50 $\mu$L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT, 1 mg/mL in Dubecco's PBS, for 4 h at 37 °C. The resulting purple formazan precipitate was solubilized with 200 $\mu$L of 0.4 N HCl in isopropyl alcohol. Absorbance was quantitated using a Bio-Rad model 3550 microplate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm. Absorbance values were transferred to a 486 personal

computer, and the $IC_{50}$ values were determined using the computer program EZ-ED50.[15]

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## References

(1) Chu, D. T. W.; Maleczka, R. E., Jr. Synthesis of 4-Oxo-4*H*-quino-[2,3,4-i,j][1, 4]benoxazine-5-carboxylic Acid Derivatives. *J. Heterocycl. Chem*. **1987**, *24*, 453−456.

(2) Chu, D. T. W.; Hallas, R.; Clement, J. J.; Alder, J.; McDonald, E.; Plattner, J. J. Synthesis and Antitumour Activities of Quinolone Antineoplastic Agents. *Drugs Exp. Clin. Res.* **1992**, *18*, 275−282.

(3) Chu, D. T. W.; Hallas, R.; Tanaka, S. K.; Alder, J.; Balli, D.; Plattner, J. J. Synthesis and Antitumour Activities of Tetracyclic Quinolone Antineoplastic Agents. *Drugs Exp. Clin. Res.* **1994**, *20*, 177−183.

(4) Clement, J. J.; Burres, N.; Jarvis, K.; Chu, D. T. W.; Swiniarski, J.; Alder, J. Biological Characterization of a Novel Antitumor Quinolone. *Cancer Res.* **1995**, *5*, 830−835.

(5) Shen, L. L.; Baranowski, J.; Pernet, A. G. Mechanism of Inhibition of DNA Gyrase by Quinolone Antibacterials. Specificity and Cooperativity of Drug Binding to DNA. *Biochemistry* **1989**, *28*, 3879−3885.

(6) Shen, L. L.; Kohlbrenner, W. E.; Weigl, D.; Baranowski, J. Mechanism of Quinolone Inhibition of DNA Gyrase. Appearance of Unique Norfloxacin Binding Sites in Enzyme-DNA Complexes. *J. Biol. Chem*. **1989**, *264*, 2973−2978.

(7) Shen, L. L.; Mitscher, L. A.; Sharma, P. N.; O′Donnell, T. J.; Chu, D. W. T.; Cooper, C. S.; Rosen, T.; Pernet, A. G. Mechanism of Inhibition of DNA Gyrase by Quinolone Antibacterials: A Cooperative Drug−DNA Binding Model. *Biochemistry* **1989**, *28*, 3886−3894.

(8) Willmott, C. J.; Maxwell, A. A Single Point Mutation in the DNA Gyrase A Protein Greatly Reduces Binding of Fluoroquinolones to the Gyrase-DNA Complex. *Antimicrob. Agents Chemother*. **1993**, *37*, 126−127.

(9) Permana, P. A.; Snapka, R. M.; Shen, L. L.; Chu, D. T. W.; Clement, J. J.; Plattner, J. J. Quinobenoxazines: A Class of Novel Antitumor Quinolones and Potent Mammalian DNA Topoisomerase II Catalytic Inhibitors. *Biochemistry* **1994**, *33*, 11333−11339.

(10) Kwok, Y.; Zeng, Q.; Hurley, L. H. Specific Interaction of Quinobenzoxazine A-62176 at Topoisomerase II Cleavage Sites: A 2:2 Quinobenzoxazine:$Mg^{2++}$ Self-Assembly Complex in the Presence of Topoisomerase II. *J. Biol. Chem*. **1998**, submitted.

(11) Fan, J.-Y.; Sun, D.; Yu, H.; Kerwin, S. M.; Hurley, L. H. Self-Assembly of a Quinobenzoxazine−$Mg^{2+}$ Complex on DNA: A New Paradigm for the Structure of a Drug−DNA Complex and Implications for the Structure of the Quinolone Bacterial Gyrase-DNA Complex. *J. Med. Chem*. **1995**, *38*, 408−424.

(12) Yu, H.; Hurley, L. H.; Kerwin, S. M. Evidence for the Formation of 2:2 Drug−$Mg^{2+}$ Dimers in Solution and for the Formation of Dimeric Drug Complexes on DNA from the DNA-Accelerated Photochemical Recation of Antineoplastic Quinobenzoxazines. *J. Am. Chem. Soc.* **1996**, *118*, 7040−7048.

(13) Marini, J. C.; Miller, K. G.; Englund, P. T. Decatenation of Kinetoplast DNA by Topoisomerases. *J. Biol. Chem*. **1980**, *255*, 4976−4979.

(14) Liu, L. F. DNA Topoisomerase Poisons as Antitumor Drugs. *Annu. Rev. Biochem*. **1989**, *58*, 351−375.

(15) Chen, S. F.; Behrens, D. L.; Behrens, C. H.; Czerniak, P. M.; Dexter, D. L.; Dusak, B. A.; Fredericks, J. R.; Gale, K. C.; Gross, J. L.; Jiang, J. B.; Kirshenbaum, M. R.; McRipley, R. J.; Papp, L. M.; Patten, A. D.; Perrella, F. W.; Seitz, S. P.; Stafford, M. P.; Sun, J. H.; Sun, T.; Von Hoff, D. D. XB596, a Promising Bis-naphthalimide Anticancer Agent. *Anti-Cancer Drugs* **1993**, *4*, 447−457.

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