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Novel 1-8-Bridged Chiral Quinolones with Activity against Topoisomerase II: Stereospecificity of the Eukaryotic Enzyme

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A series of novel C-7 quinolyl-substituted enantiomers of ofloxacin were used to determine the stereospecificity of topoisomerase II for the C-11 methyl group in tricyclic quinolones. In all cases, the *S* isomer was the most active compound against the eukaryotic enzyme. It was ~2.2-fold more potent than the *R* isomer at inhibiting the overall catalytic activity of topoisomerase II (as monitored by DNA relaxation assays). A markedly greater difference in quinolone activity was observed in enzyme-mediated DNA cleavage reactions. While the *S* enantiomer stimulated nucleic acid breakage ~3.5-fold, the *R* compound did not enhance and, in fact, decreased initial DNA cleavage levels by ~50%. The activity of the racemic mixture more closely resembled that of the *R* enantiomer. In competition experiments, the DNA cleavage-enhancing effects of the *S* isomer were attenuated by the *R* compound. Taken together, these latter results indicate that the *R* enantiomer is an antagonist of *S* isomer-promoted topoisomerase II-mediated DNA cleavage. Finally, the cytotoxic potential of quinolyl-substituted ofloxacin analogs correlated with the ability to stimulate enzyme-mediated DNA cleavage. Thus, stereochemistry appears to be a governing factor for the potential development of tricyclic quinolones as topoisomerase II-targeted drugs with antineoplastic activity.

Quinolone-based drugs are a widely used class of oral antibiotics (11, 37, 42). The primary physiological target of these important pharmacological agents is DNA gyrase, a prokaryotic type II topoisomerase (11, 27, 36, 42). Quinolones cause bacterial death by converting DNA gyrase into a cellular poison (18). This is accomplished by stabilizing covalent enzyme-cleaved DNA complexes (6, 11, 35) which are naturally occurring intermediates in the catalytic cycle of all type II topoisomerases (26, 27).

While most medically relevant quinolones have a bicyclic parent ring structure, ofloxacin (see Fig. 1), one of the most potent members of this drug family currently in clinical use, is tricyclic in nature (3, 38). Unlike its bicyclic counterparts, the methyl-substituted 1-8 oxazine bridge of ofloxacin possesses a chiral center. This center, which is located at the C-11 position, is indicated in Fig. 1. Although the clinical formulation of ofloxacin is a racemic mixture, enantiomerically pure *R* and *S* stereoisomers have been characterized (5, 7, 10, 12, 15, 21). In all cases, the *S* isomer was substantially more active (≥10-fold) than its *R* antipode at inhibiting the DNA supercoiling reaction of gyrase or preventing the growth of bacterial cells. The activity of the racemic ofloxacin generally was one-half that of the pure *S* isomer (5, 7, 10, 12, 15, 21). Thus, it appears that the *R* enantiomer interacts poorly (at best) with DNA gyrase.

The effects of ofloxacin enantiomers on the catalytic activity of eukaryotic topoisomerase II also have been examined (12). As determined by the inhibition of enzyme-catalyzed DNA relaxation, an approximately twofold stereospecificity for the *S* configuration over the *R* configuration was observed. However, it should be noted that the potencies of both enantiomers toward topoisomerase II are low (12). Moreover, the effects of ofloxacin isomers on the critical DNA cleavage activity of the enzyme never were characterized.

Recently, a number of quinolones with greatly increased potencies (relative to ofloxacin) toward eukaryotic systems have been reported (2, 4, 16, 17, 28, 29, 39–41). The most active of these novel compounds possess aromatic substituents at the C-7 position (2, 16, 28, 29, 39, 40). Therefore, a stereoselective series of C-7 quinolyl-substituted ofloxacin analogs (CP-73,355 [racemate], CP-100,965 [*R* enantiomer], CP-100,964 [*S* enantiomer]) (16) (Fig. 1) were used to describe in further detail the stereospecificity of topoisomerase II. Results indicate that the eukaryotic enzyme displays an extreme prejudice in its DNA cleavage reaction for the *S* configuration at the C-11 position. In contrast to the *S* isomer (which stimulated topoisomerase II-mediated DNA cleavage ~3.5-fold), the *R* enantiomer showed no ability to enhance DNA cleavage and acted as an enzyme antagonist.

MATERIALS AND METHODS

DNA topoisomerase II was isolated from the nuclei of 6- to 12-h-old *Drosophila melanogaster* embryos as described in the protocol of Shelton et al. (34). Negatively supercoiled bacterial plasmid pBR322 DNA was obtained from *Escherichia coli* DH1 by a Triton X-100 lysis which was followed by double banding in cesium chloride gradients containing ethidium bromide (31). The racemic (CP-73,355), *R* (CP-100,965), and *S* (CP-100,964) quinolones used in the present study were synthesized as HCl salts at Pfizer Central Research by a previously described procedure (8). Quinolones were dissolved at a concentration of 16.7 mM in 50 mM KOH and were diluted to a 5 mM stock with 10 mM Tris-HCl

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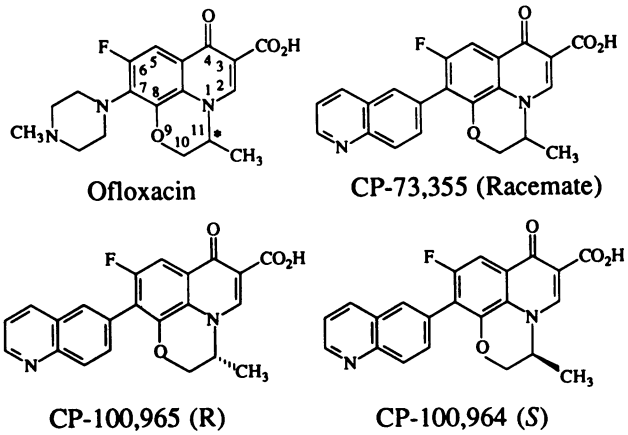

FIG. 1. Structures of stereospecific tricyclic quinolones.

(pH 6.5 or 7.0). Drug stocks were stored at 4°C and were never more than 1 day old when used for the experiments. Tris, ethidium bromide, and trypan blue dye were obtained from Sigma (St. Louis, Mo.), sodium dodecyl sulfate (SDS) was from E. Merck Biochemicals (West Point, Pa.), proteinase K was from United States Biochemicals (Cleveland, Ohio), ATP was from Pharmacia LKB Biotechnology (Piscataway, N.J.), penicillin was from Bristol-Myers Squibb Company (Princeton, N.J.), and streptomycin was from Pfizer Inc. (New York, N.Y.). All other chemicals were analytical reagent grade.

Inhibition of topoisomerase II-catalyzed DNA relaxation by quinolones. DNA relaxation assays were carried out by the protocol of Osheroff et al. (25). Reaction cocktails (final volume, 20 μl) contained 0.3 nM topoisomerase II, 5 nM negatively supercoiled pBR322 plasmid DNA, and 1 mM ATP in assay buffer (10 mM Tris-HCl [pH 7.9], 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2.5% [vol/vol] glycerol). Mixtures were incubated at 30°C for 15 min. Reactions were stopped by the addition of 2.5 μl of 0.77% SDS–77 mM EDTA. Two microliters of 10 mM Tris-HCl (pH 7.9), 0.05% bromophenol blue, 0.05% xylene cyanol, and 60% sucrose (wt/vol) was added, and samples were heated at 70°C for 2 min. Reaction products were resolved by electrophoresis in 1.0% agarose gels (EM Science, Gibbstown, N.J.) in 40 mM Tris-acetate (pH 8.0)–2 mM EDTA. Gels were stained for 30 min in an aqueous solution (1 μg/ml) of ethidium bromide, and DNA bands were visualized by transillumination with UV light (300 nm). Gels were photographed through Kodak 23A and 12 filters with Polaroid type 665 positive-negative film. The amount of DNA present was quantitated by scanning photographic negatives with an E-C Apparatus model EC910 scanning densitometer by using Hoefer GS-370 Data System software. The density of the bands was proportional to the amount of DNA present. The effects of quinolones on DNA relaxation were examined over a concentration range of 0 to 250 μM drug. An equivalent volume of drug diluent was added to control assays. Quinolone diluent had no effect on topoisomerase II-catalyzed DNA relaxation.

Stimulation of topoisomerase II-mediated DNA cleavage by quinolones. DNA cleavage reaction mixtures (final volume, 20 μl) contained 50 nM topoisomerase II and 5 nM negatively supercoiled pBR322 plasmid DNA in assay buffer. Samples were incubated at 30°C for 6 min. Topoisomerase II-DNA cleavage complexes were trapped by the addition of 2 μl of 10% SDS. EDTA (1.5 μl of a 250 mM solution) and proteinase K (2 μl of a 0.8-mg/ml solution) were added, and the reaction mixtures were incubated at 45°C for 30 min to digest topoisomerase II. DNA cleavage products were resolved by agarose gel electrophoresis and were quantitated by scanning densitometry as described above. The enhancement of DNA cleavage by quinolones was examined over a concentration range of 0 to 500 μM drug. In experiments that investigated the antagonistic nature of the *R* enantiomer, the *R* and *S* compounds were added simultaneously. Quinolone diluent was added to all control assays and had no effect on levels of DNA cleavage. Since experiments were carried out in the absence of ATP, assays monitored the topoisomerase II-mediated DNA cleavage that occurred prior to the DNA strand passage event of the enzyme (24, 26, 30).

Cytotoxicities of quinolones toward *D. melanogaster* Kc tissue culture cells. *D. melanogaster* Kc cells were cultured in T-flasks at 25°C in D22 medium that contained penicillin (100 U/ml) and streptomycin (150 μg/ml) (1). Two hours prior to the start of cytotoxicity studies, cultures were diluted to a concentration of ~4×10^6 cells per ml and the cells were transferred to spinner flasks. Initial cell viability averaged ~91% (as determined by trypan blue exclusion). Quinolone stocks or drug diluent (for control cultures) were sterile filtered before addition to cell suspensions. Cytotoxicity was determined at various final quinolone concentrations of up to 250 μM. Aliquots (100 μl) were removed from cultures at various times up to 50 h and were mixed with an equal volume of 0.1% trypan blue. Cells were counted with a hemacytometer. Only viable cells were included in growth curves.

RESULTS

Inhibition of topoisomerase II-catalyzed DNA relaxation by stereoselective C-7 quinolyl-substituted ofloxacin analogs. Clinically relevant quinolone antibiotics are poor inhibitors of eukaryotic type II topoisomerases (9, 11–14, 20, 22, 23, 25). For example, the concentration of ofloxacin required to inhibit catalytic enzyme activity by 50% (IC_{50}) has been reported to be in the millimolar range (12–14, 22). However, the substitution of an aromatic quinolyl group for the aliphatic methyl-piperazine ring at the C-7 position greatly enhances the activity of the quinolone. As seen in Fig. 2, the C-7 quinolyl series inhibited the overall catalytic activity of *D. melanogaster* topoisomerase II (as monitored by the ability to relax negatively supercoiled pBR322 plasmid DNA) in the micromolar range. The enzyme displayed an ~2.2-fold specificity for the *S* ($IC_{50} \approx 18$ μM) over the *R* ($IC_{50} \approx 40$ μM) isomer. The racemic quinolone was intermediate in potency. These results are consistent with those of the previous study of Hoshino et al. (12) on the inhibition of calf thymus topoisomerase II-catalyzed DNA relaxation by ofloxacin enantiomers.

Stereospecific enhancement of topoisomerase II-mediated DNA cleavage. The clinical potential of topoisomerase II-targeted antineoplastic agents results from the ability to enhance enzyme-mediated DNA cleavage (19, 33). Therefore, the effects of the quinolyl-substituted quinolone series on the critical double-stranded DNA cleavage reaction of topoisomerase II were assessed to further delineate the stereospecificity of the enzyme.

In comparison with their inhibition of DNA relaxation, the differential abilities of the *S* and *R* isomers to promote DNA breakage were striking. As seen in Fig. 3, the *S* isomer doubled levels of DNA cleavage at a concentration of ~95

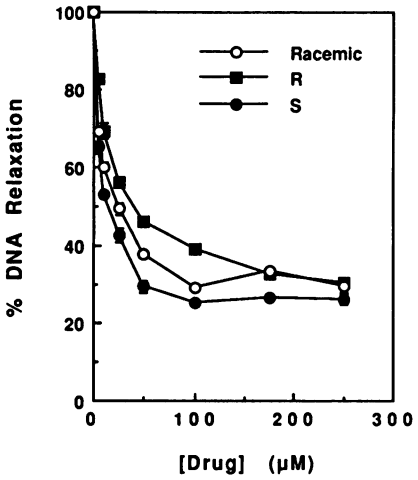

FIG. 2. Inhibition of topoisomerase II-catalyzed DNA relaxation by stereospecific tricyclic quinolones. The effects of the racemate, the *R*, and the *S* compounds are shown. Data represent averages of three independent assays. Standard deviations are represented by the vertical bars. Levels of enzyme activity were arbitrarily set to 100% in the absence of drug.

μM and maximally stimulated nucleic acid breakage ~3.5-fold. In marked contrast, the *R* enantiomer showed no ability to enhance DNA cleavage and actually decreased nucleic acid breakage to ~50% below basal (i.e., no drug) levels.

Unlike the conclusion that can be drawn from studies on prokaryotic systems (i.e., that the *R* isomer of ofloxacin shows little ability to interact with DNA gyrase) (5, 7, 10, 12, 15, 21), this latter result implies that the present *R* compound interacts with *D. melanogaster* topoisomerase II but functions as an antagonist of enzyme-mediated DNA cleavage. This hypothesis was tested by two additional experiments.

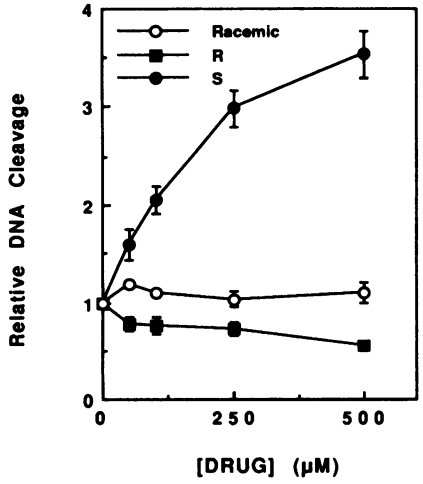

FIG. 3. Effects of stereospecific tricyclic quinolones on topoisomerase II-mediated DNA cleavage. Results for the racemate, the *R*, and the *S* compounds are shown. Double-stranded DNA cleavage was monitored by the conversion of negatively supercoiled plasmid to linear molecules. Data represent averages of three independent assays. Standard deviations are represented by the vertical bars. Levels of enzyme-mediated DNA cleavage generated in the absence of drug were arbitrarily set to 1.

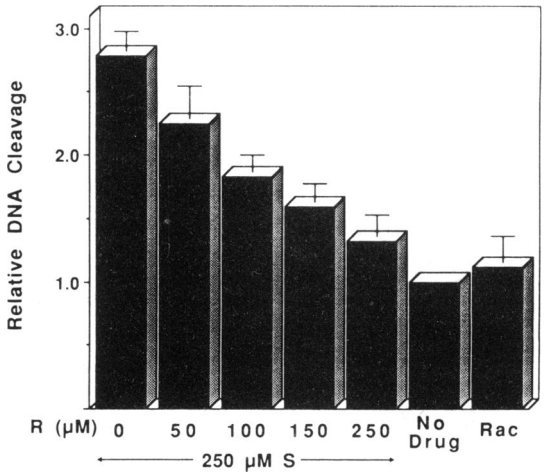

FIG. 4. Antagonism of topoisomerase II-mediated DNA cleavage by the *R* enantiomer. Bars represent levels of DNA cleavage generated at a constant concentration of 250 μM *S* isomer over a range of 0 to 250 μM *R* compound. Levels of DNA cleavage generated in the presence of no drug or 500 μM racemate (Rac) are shown for comparison. Data represent averages of two to three independent experiments. Standard errors or deviations (as appropriate) are shown.

The first experiment determined the effects of the racemic quinolone on enzyme-mediated DNA cleavage. If the *R* enantiomer is an inert stereoisomer, the racemate should be ~50% as active as the *S* enantiomer at stimulating DNA cleavage. Conversely, if the *R* isomer is a strongly interacting antagonist of topoisomerase II-mediated DNA cleavage, the activity of the racemate should approximate that of the *R* enantiomer. As shown in Fig. 3, the racemic mixture affected enzyme-mediated DNA cleavage in a manner that closely resembled that of the *R* isomer. The maximal stimulation of nucleic acid breakage observed was only 1.2-fold, and a slight inhibition of DNA cleavage was seen at increased drug concentrations.

The second experiment directly assessed the potential for antagonism by the *R* enantiomer. If the *R* compound indeed functions as an antagonist, it should block the stimulation of topoisomerase II-mediated DNA cleavage promoted by the *S* isomer. Therefore, the levels of quinolone-induced DNA cleavage (at a constant concentration of *S* isomer) were determined at increasing concentrations of the *R* enantiomer (Fig. 4). The approximately threefold increase in nucleic acid breakage promoted by 250 μM *S* isomer was attenuated in the presence of the *R* enantiomer. Upon the addition of 250 μM *R* compound, the effects of the *S* isomer were diminished by more than 80%. Taken together, these latter findings provide strong evidence that the *R* isomer is an antagonist of *S* isomer-promoted topoisomerase II-mediated DNA cleavage.

Stereospecific cytotoxicity of C-7 quinolyl-substituted ofloxacin analogs. A recent study (4) indicated that topoisomerase II is the primary cytotoxic target for quinolones in *Saccharomyces cerevisiae* and that cell death is triggered by drug stimulation of enzyme-mediated DNA cleavage rather than by inhibition of overall catalytic activity. To determine whether quinolones promote cell death by a similar mechanism in cells from higher eukaryotes, the cytotoxicities of the present stereospecific ofloxacin analogs toward *D. melanogaster* Kc cells were characterized. This tissue culture

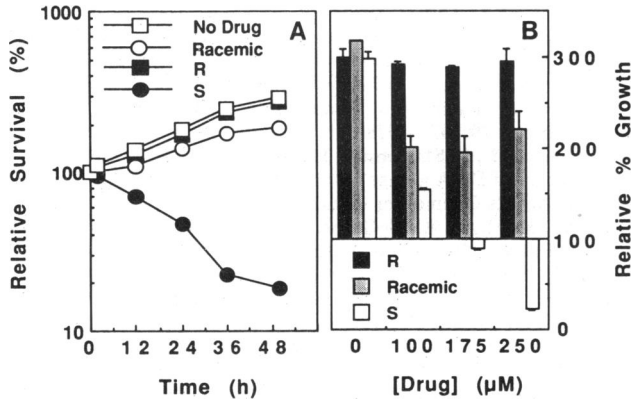

FIG. 5. Cytotoxicities of stereospecific tricyclic quinolones toward *D. melanogaster* Kc cells. (A) Time course for cytotoxicity determined in the continuous presence of no drug or 250 μM racemate, *R*, or *S* compound. Data are representative of two independent experiments. (B) Drug concentration titration determined following a 50-h exposure of cells to the racemate, *R*, or *S* compound. Data represent averages of two independent experiments. Standard errors are shown. Relative cell survival was arbitrarily set to 100% at time zero.

line is made up of undifferentiated embryonic cells (1). If quinolones kill Kc cells by the mechanism described previously in *S. cerevisiae* (4), the *S* isomer (which stimulates topoisomerase II-mediated DNA cleavage) should be toxic to Kc cells, while the *R* enantiomer and the racemic form (both of which inhibit enzyme activity but do not promote significant DNA cleavage) should allow growth of cultures.

Results of cytotoxicity studies are shown in Fig. 5. A drug concentration of 250 μM was used to generate the time course shown in Fig. 5A. This quinolone concentration is more than six times greater than the IC_{50} for inhibition of topoisomerase II-catalyzed DNA relaxation by the *R* isomer (the least potent quinolone examined). Consistent with the studies on *S. cerevisiae*, the *S* enantiomer was toxic to Kc cells and killed >80% of the initial culture following a 50-h exposure. Moreover, cells treated with the *S* compound displayed morphological changes and uncharacteristic aggregation patterns. Conversely, no cell death was observed in cultures that contained either the *R* isomer or the racemate. Both of these latter quinolones allowed high rates of cell growth that were ~95 and ~65% that of the control (no drug) culture, respectively.

To further assess the cytotoxicity of the ofloxacin analogs, the effects of these quinolones on the growth of Kc cells were determined over a range of drug concentrations (Fig. 5B). A 50-h time point was used to generate the data shown. The *S* isomer affected cell growth at all concentrations tested. The minimal quinolone concentration required to block cell growth (i.e., the transition from cytostatic to cytotoxic drug effects) was ~160 μM. At this concentration, the *S* enantiomer enhanced the DNA cleavage mediated by the *D. melanogaster* type II enzyme ~2.5-fold (Fig. 3). Once again, no cytotoxicity was observed at any concentration of the *R* isomer or the racemate that was used.

Three lines of evidence indicate that the cytotoxic nature of the *S* enantiomer results from its ability to enhance topoisomerase II-mediated DNA breakage rather than to inhibit overall catalytic activity. First, the inhibition of DNA relaxation at 250 μM *S*, *R*, or racemate was similar (residual enzyme activity was 26, 31, or 30%, respectively) (Fig. 2), but only the *S* isomer was cytotoxic in nature. Second, even at 50 μM *S* (the concentration that produced a similar inhibition of DNA relaxation as was observed with 250 μM *R*), the *S* isomer inhibited cell growth ~45% (data not shown). In contrast, 250 μM *R* decreased the rate of cell growth (compared with the no drug control) by only 6%. Third, the inhibition of DNA relaxation by the *S* enantiomer did not change appreciably between 50 and 250 μM quinolone. However, over the same concentration range, the cytotoxicity of the compound increased dramatically and levels of drug-promoted DNA cleavage rose approximately threefold (Fig. 3).

DISCUSSION

The stereospecificity of *D. melanogaster* topoisomerase II for the C-11 position of tricyclic quinolones was determined with a novel series of ofloxacin analogs. These analogs replaced the methyl-piperazine group at the C-7 position of ofloxacin with a quinolone ring (12–14, 16, 22). This substitution greatly enhances drug activity against the eukaryotic enzyme (16). In all assays used, topoisomerase II displayed a preference for the *S* over the *R* configuration of the C-11 methyl group.

Studies on prokaryotic systems demonstrate that DNA gyrase can discern the stereoconfiguration of ring substituents in chiral quinolones (5, 7, 10, 12, 15, 21, 30, 32). With regard to ofloxacin, the bacterial enzyme displays a high (≥10-fold) specificity for the *S* over the *R* configuration at the C-11 position (5, 7, 10, 12, 15, 21). The fact that the racemate is approximately one-half as active against bacterial systems as is the *S* compound (5, 7, 10, 12, 15, 21) indicates that the *R* enantiomer is a poor effector of the prokaryotic type II enzyme. In marked contrast, the *R* isomer of the quinolyl-substituted ofloxacin analogs used in the present study was a potent antagonist of DNA cleavage mediated by the eukaryotic enzyme. As a consequence, the present racemate showed little activity against either *D. melanogaster* topoisomerase II or tissue culture cells. This finding implies that characterization of racemic quinolone mixtures may lead to inappropriate conclusions concerning the activities of the individual stereoisomers.

Although the *S* compound inhibited enzyme-catalyzed DNA relaxation with a potency that was ~2.2-fold greater than that of the *R* enantiomer, its effect on topoisomerase II-mediated DNA cleavage was dramatically different from that of the latter stereoisomer. It is not clear at present why only the *S* isomer enhances nucleic acid breakage. However, it is tempting to speculate that it is due to alterations in the positioning of the quinolone enantiomers at the enzyme-DNA interface. Thus, the position of the *S* isomer inhibits the DNA strand passage activity of topoisomerase II and at the same time increases the longevity of the enzyme-DNA cleavage complex, while the position of the *R* isomer (even though it likely overlaps that of the *S* compound) produces only the former effect. The eventual resolution of this mechanistic point will probably require a crystallographic structure of the topoisomerase II-DNA-quinolone complex. However, it is notable that the above hypothesis is consistent with the antagonistic nature of the *R* enantiomer.

A number of quinolone-based compounds are potent effectors of topoisomerase II and show activity against eukaryotic cells (2, 4, 16, 17, 28, 29, 39–41). Thus, this drug class represents a potential source of novel agents for the treatment of human cancers. The present study demonstrates that, like DNA gyrase, the eukaryotic type II enzyme

can distinguish quinolone stereoconfiguration and argues for the use of enantiomerically pure compounds in all future discoveries of chiral quinolones as antineoplastic agents. Furthermore, the finding that the cytotoxicities of the *R* and *S* compounds mimick the extreme stereoselectivity of drug-promoted nucleic acid breakage strongly suggests that the ability to enhance topoisomerase II-mediated DNA cleavage is the critical indicator for the potential development of quinolones as anticancer drugs.

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