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MECHANISM OF ACTION AND ANTITUMOR ACTIVITY OF (*S*)-10-(2,6-DIMETHYL-4-PYRIDINYL)-9-FLUORO-3-METHYL-7-OXO-2,3-DIHYDRO-7*H*-PYRIDOL[1,2,3-*de*]-[1,4]BENZOTHIAZINE-6-CARBOXYLIC ACID (WIN 58161)

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Abstract—(*S*)-10-(2,6-Dimethyl-4-pyridinyl)-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]benzothiazine-6-carboxylic acid (WIN 58161) is an enantiomerically pure quinolone with outstanding bacterial topoisomerase II (DNA gyrase, EC 5.99.1.3) inhibitory and antibacterial activity. Unlike most quinolones, WIN 58161 also exhibits significant inhibitory activity against mammalian topoisomerase II (EC 5.99.1.3). DNA gyrase and topoisomerase II inhibitory activities are enantioselective. Consequently, WIN 58161 and its enantiomer (WIN 58161-2) provide useful tools to probe the contribution of topoisomerase II inhibition to the mechanism of cytotoxicity of quinolones and the potential utility of quinolone–topoisomerase II inhibitors as antitumor agents. WIN 58161 inhibited both highly purified *Escherichia coli* DNA gyrase and HeLa cell topoisomerase II by the promotion of enzyme–DNA covalent complexes. WIN 58161 did not bind stably to DNA via intercalation and did not enhance the formation of topoisomerase I (EC 5.99.1.2)–DNA covalent complexes. At drug concentrations that are cytotoxic to P388 murine leukemia cells, WIN 58161 promoted intracellular DNA single-strand breaks (SSBs) that exhibited the hallmarks of being mediated by topoisomerase. DNA fragments were complexed with protein, and SSBs were readily resealed at 37° following drug removal. WIN 58161-2 was neither cytotoxic nor did it promote intracellular SSBs in P388. These observations suggest that the mechanism of cytotoxicity of WIN 58161 is predominantly, if not exclusively, a result of topoisomerase II inhibition. When studied in tumor-bearing mice, WIN 58161 exhibited a significant antitumor effect against each of five tumors tested, whereas neither toxicity nor antitumor activity was observed with WIN 58161-2. We conclude from these studies that WIN 58161 represents the prototype of a novel chemical class of topoisomerase II inhibitor with potential clinical utility in treating cancer.

Key words: topoisomerase II; DNA gyrase; quinolone; WIN 58161

Topoisomerases are a multi-gene family of enzymes [reviewed in Refs. 1–3] with proven utility as targets for both anticancer and antibacterial therapy [reviewed in Refs. 4–8]. Topoisomerases modulate DNA-linking relationships within or between DNA molecules and are essential participants in virtually all cellular activities that involve DNA processing. Examples of topoisomerase functions include the decatenation of interlinked DNA molecules that result from DNA replication and the relaxation of regions of high DNA superhelical tension that result from processes such as DNA replication and transcription.

DNA-linking relationships are modified by topoisomerases via a cycle of breakage and religation of DNA during the course of which a strand of DNA is passed through the break site. Topoisomerases are classified as either type I or type II, dependent upon whether they catalyze the passage of a single strand of DNA through a single-strand break or the passage of a double strand of DNA through a double-strand break. No clinically useful inhibitor of type I bacterial topoisomerases has been described, to date. However, inhibitors of eucaryotic topoisomerases of both type I and type II have been employed successfully in anticancer therapies. Similarly, inhibitors of bacterial topoisomerase II (DNA gyrase, EC 5.99.1.3) are effective antibacterial agents.

Inhibition of topoisomerase catalytic activity alone may, in some instances, be an effective means to achieve antibacterial and antineoplastic activity. Coumarin antibiotic activity is a consequence of the inhibition of the enzymatic activities of DNA gyrase through blocking the ATPase activity of the B subunit of DNA gyrase [9]. It has also been suggested that the antitumor mechanism of the investigational

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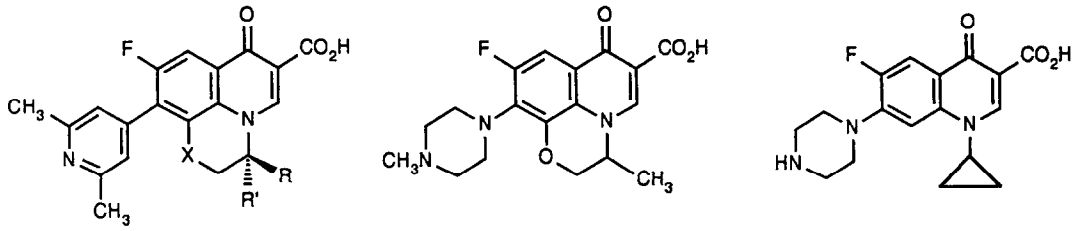

Fig. 1. Structures of quinolones used in the study.

antitumor agent merbarone involves inhibition of the catalytic activities of topoisomerase II (EC 5.99.1.3) [10]. However, the majority of topoisomerase-targeted therapeutics act via the stabilization of a transient reaction intermediate in which topoisomerase is covalently bound at the DNA break site via a phosphotyrosyl bond. This intermediate, referred to as the cleavable complex, is revealed as a protein-associated DNA strand break upon denaturation of topoisomerase. Inhibitors that act via this mechanism not only block catalytic function but also transform the topoisomerase enzyme into a cellular poison via the promotion of enzyme–DNA covalent complexes.

A number of distinct chemical classes of antineoplastics inhibit eucaryotic topoisomerases via stabilization of cleavable complex. Camptothecin and congeners are the most well studied topoisomerase I (EC 5.99.1.2) poisons. Eucaryotic topoisomerase II inhibitors that promote cleavable complex formation include both intercalating anthracyclines (i.e. doxorubicin, 5-iminodaunorubicin), anthracenediones (i.e. mitoxantrone), aminoacridines (i.e. *m*AMSA*) and ellipticines (i.e. datelliptinium), as well as the non-DNA intercalating epipodophyllotoxins (i.e. VP-16). In contrast, only nalidixic acid and the closely related class of 4-quinolone antibacterial agents stabilize bacterial topoisomerase II (DNA gyrase) cleavable complex.

Cross-over inhibition between mammalian and bacterial topoisomerases is generally minimal. However, certain quinolone–DNA gyrase inhibitors do inhibit eucaryotic topoisomerase II [11–19]. (*S*)-10-(2,6-Dimethyl-4-pyridinyl)-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]-benzothiazene-6-carboxylic acid (WIN 58161; Fig. 1) is an enantiomerically pure quinolone with outstanding DNA gyrase inhibitory and antibacterial activity† [20]. As part of an ongoing study to evaluate the potential of quinolones to inhibit human topoisomerase II, WIN 58161 was found to also have significant inhibitory potency for mammalian topoisomerase II [19]. It was also shown that the (*S*)-methyl group of WIN 58161 is a requirement for activity in that the enantiomer (WIN 58161-2) is inactive. In the present paper, we have used WIN 58161 and WIN 58161-2 to probe the contribution of topoisomerase II inhibition to the cytotoxicity and antitumor activity of WIN 58161. We provide evidence of *in vivo* antitumor activity of a quinolone mediated by topoisomerase II inhibition, and we conclude that quinolones represent an additional chemical class of topoisomerase II inhibitor with potential as anticancer agents. A portion of this work has been published previously in abstract form.‡

MATERIALS AND METHODS

Reference drugs

VP-16 (VePesid®; etoposide; NSC 141540) and *m*AMSA (amsacrine; NSC 249992) were obtained from the Natural Products Branch, Division of Cancer Treatment, and the Drug Synthesis and Chemistry Branch, respectively, of the NCI. Camptothecin [NSC 100880; (*S*)-4-ethyl-4-hydroxy-1*H*-pyrano-[3′4′:6,7]indolizino[1,2-*b*]quinoline-3,14(4*H*,12*H*)-dione], ciprofloxacin [Cipro®; 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-

* Abbreviations: *m*AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; VP-16, 4′-demethylepipodophyllotoxin-9-[4,6-*O*-(*R*)-ethylidene-β-D-glucopyranoside]; VM-26, 4′-demethylepipodophyllotoxin-9-[4,6-*O*-2-thenylidene-β-D-glucopyranoside]; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; DMEM, Dulbecco's Modified Eagle's Medium; SSBs, DNA single-strand breaks; panc03, murine pancreatic adenocarcinoma No. 3; colo38, murine colon adenocarcinoma No. 38; mam16C, murine mammary adenocarcinoma No. 16C; qd, daily; and pGp-MDR, P-glycoprotein-mediated multidrug resistance.

† Reuman M, Daum SJ, Singh B, Wentland MP, Carabateas PM, Gruett MD, Coughlin SA, Sedlock DM, Rake JB and Lesher GY, Synthesis and antibacterial activity of some novel 1-substituted-7-pyridinyl-1,4-dihydro-4-oxoquinoline-3-carboxylic acids. *Twenty-ninth Interscience Conference on Antimicrobial Agents and Chemotherapy, Houston, TX*, 17–20 September 1989, Abstr. 1193.

‡ Coughlin SA, Danz DW, Robinson RG, Moskwa PS, Wentland MP, Lesher GY and Rake JB, Mechanism of action and antitumor activity of WIN 58161. *Proceedings of the Eighty-second Annual Meeting of the American Association for Cancer Research, Houston, TX*, 15–18 May 1991, Abstr. 2000.

(1-piperazinyl)-3-quinolinecarboxylic acid] and vinblastine (Velban®) were purchased from Sigma. All quinolones used in this study, other than ciprofloxacin, were synthesized by Sterling Winthrop Pharmaceuticals Research Division [19, 20].

Stock solutions of *m*AMSA, VP-16, and vinblastine were prepared in DMSO and stored at −20°. Solutions of *m*AMSA, VP-16, and vinblastine were diluted to 20% DMSO before use. Quinolones were solubilized in 0.1 N NaOH and diluted, thereafter, to 0.02 N NaOH. Solutions of quinolones were stored at room temperature or at −20°.

Cell lines

P388 murine leukemia cells were obtained from ATCC. HeLa Ohio (WIS) human cervical carcinoma cells were supplied by Dr. Roland Ruekert, University of Wisconsin at Madison. CEM human leukemic lymphoblast cells and the multidrug-resistant cell lines derived from CEM, VLB_{100} [21] and VM-1 [22] were supplied by Dr. William Beck, St. Jude Children's Research Hospital. HL-60 and HL-60/AMSA [23] were initially provided by Drs. M. Beran and B. Anderson of the Department of Hematology, The University of Texas, M.D. Anderson Cancer Center.

Reagents, enzymes and substrates

Escherichia coli DNA gyrase was supplied by Dr. Kenneth Marions, Memorial Sloan-Kettering Cancer Center. pBR322 plasmid DNA, calf thymus DNA, and Mono Q prepacked HR5/5 columns were purchased from Pharmacia-PL Biochemicals. Klenow fragment, *Eco*RI, calf thymus topoisomerase I and ultrapure Tris were purchased from Bethesda Research Laboratories. T_4 polynucleotide kinase and calf intestinal alkaline phosphatase were purchased from New England Biolabs. BSA (fraction V), purchased from Calbiochem, was heat inactivated at 1 mg/mL (60° for 3 hr) and concentrated by lyophilization before use. Ultrogel hydroxyapatite and phosphocellulose P11 were purchased from IBF and Whatman, respectively.

Preparation of [^{32}P]*-end-labeled pBR322 DNA*

*Eco*RI-linearized pBR322 DNA was labeled at the 3′-ends by the fill-in reaction with Klenow fragment and [α-^{32}P]ATP (final concentration 2.5 mCi/mL, ≥ 3000 Ci/mmol) or, following calf intestinal alkaline phosphatase treatment, at the 5′-ends with T4 polynucleotide kinase and [γ-^{32}P]ATP (final concentration 3 mCi/mL, ≥ 4500 Ci/mmol) [24]. The specific activity of end products was 5–8 × 10^6 cpm/μg.

Topoisomerase II purification

Topoisomerase II was purified extensively from a late log-phase suspension culture of HeLa cells (5–7 × 10^5 cells/mL). The purification procedure employed is essentially that described by Drake *et al.* [25] with the exception that phosphocellulose (Whatman P11) was substituted for Mono S as the final stage of purification. The topoisomerase II pool from Mono Q was diluted by dropwise addition of 1/2 vol. of P buffer (15 mM potassium phosphate, pH 7.0, 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 10 mM sodium bisulfite, 1.0 mM PMSF) to a final concentration of NaCl of approximately 0.2 M and loaded (0.1 mL/min) onto a 0.5 × 6.0 cm P11 column previously equilibrated with P buffer containing 0.2 M NaCl. The column was washed with 9 mL of equilibration buffer and eluted with a 20-mL linear gradient of 0.2 to 1.0 M NaCl in P buffer. This column resolved topoisomerase II, which eluted at approximately 0.6 M NaCl, from the residual contaminating topoisomerase I, which eluted at 0.8 M NaCl. Preparations of topoisomerase II were diluted with glycerol to 50% (v/v) and stored at −20° for over a year without detectable loss of cleavage activity. The total yield of topoisomerase II obtained from approximately 5×10^{10} HeLa cells with this purification method was 10^6 units, where 1 unit equals that amount of topoisomerase II that forms precipitable complex with 1 ng of 3′-end-labeled pBR322 DNA under standard topoisomerase II cleavage assay conditions in the presence of 10 μg/mL *m*AMSA.

Cleavable complex assay

Drug-promoted cleavable complex formation was assayed by quantitating the amount of [^{32}P]-end-labeled pBR322 DNA covalently complexed to topoisomerase II or DNA gyrase as a function of drug concentration. Cleavage assays were assembled at 4°. Assays were initiated by the addition of 5 μL of test compound or solvent to 25 μL of assay mixture to yield the following final concentrations in 30-μL reactions: topoisomerase II [50 mM Tris–Cl, pH 7.9, 44 mM NaCl, 10 mM $MgCl_2$, 0.6 mM DTT, 0.5 mM EDTA, 30 μg/mL heat-inactivated BSA, 0.5 mM ATP, 5.5% (w/v) glycerol, 4 ng 3′-[^{32}P]-end-labeled pBR322 DNA and 10 units topoisomerase II]; and DNA gyrase [50 mM Tris–Cl, pH 7.5, 24 mM KCl, 4 mM $MgCl_2$, 5 mM DTT, 0.14 mM EDTA, 1.8 mM spermidine, 9 μg/mL tRNA, 6.5% (w/v) glycerol, 0.36 mg/mL heat-inactivated BSA, 4 ng 3′-[^{32}P]-end-labeled pBR322 DNA and 18 ng DNA gyrase]. Cleavage reactions were incubated for 20 min at 37° (topoisomerase II) or for 45 min at 30° (DNA gyrase). Cleavage reactions were terminated by rapid denaturation of enzyme by the addition of 3 μL of 10% SDS. Enzyme–DNA cleavable complexes were either precipitated by the method of Trask *et al.* [26] or treated with 0.1 mg/mL proteinase K (Sigma) (30 min, 37°) for analysis of DNA strand breaks by autoradiography following agarose gel electrophoresis. Precipitates were collected and washed with a Brandell cell harvester on GF/B glass fiber filter membranes (Whatman). Filters were dried, and precipitated cpm were determined by liquid scintillation counting with 5 mL Readisafe (Beckman) liquid scintillation fluid.

DNA intercalation assay

Experimental compounds were tested for DNA intercalation by topoisomerase I relaxation of closed circular plasmid DNA to equilibrium in the presence of test compound [27].

Quantitation of intracellular DNA SSB

Intracellular DNA SSB were measured by alkaline elution [28] in P388 murine leukemia cells.

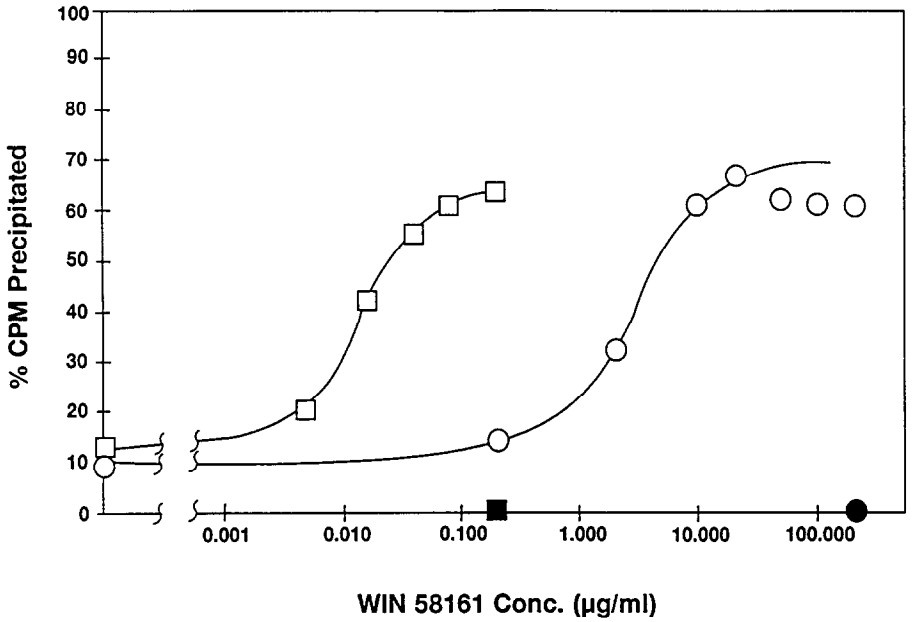

Fig. 2. Induction of *E. coli* DNA gyrase- and HeLa cell topoisomerase II-mediated cleavable complex formation by WIN 58161. Cleavable complex formation was measured by SDS/K^+ precipitation, as described in Materials and Methods, in the presence (open symbols) or absence (closed symbols) of (□) DNA gyrase or (○) topoisomerase II.

Cytotoxicity

Cytotoxicity was evaluated by determining clonogenic survival of cells following a 1-hr exposure to test compound. Clonogenic survival of P388, CEM, VM-1, and VLB_{100} was determined by soft agar colony formation in 0.3% Bacto agar (Difco) in DMEM containing 30% fetal bovine serum. The plating efficiency for P388 was 40–70% following 7 days at 37° in the presence of 5% CO_2. The plating efficiencies of CEM, VM-1, and VLB_{100} cells were 15–30, 30–40, and 5–10%, respectively, following 10–14 days at 37° in the presence of 5% CO_2.

In vivo *antitumor evaluation*

Evaluations of *in vivo* antitumor activity were conducted either at Wayne State University or at Southern Research Institute. All research involving animals described in this publication was performed in accord with Sterling Winthrop Pharmaceuticals Research Division's Policy on Animal Use and all national and federal legislation. All Wayne State and Southern Research Institute animal facilities and programs are accredited by the American Association for Accreditation of Laboratory Animal Care.

Tumors. P388 murine leukemia and the following transplantable solid tumors of mice were used for *in vivo* testing: B16 melanoma panc03, colo38, and mam16C. All tumors are in the Developmental Therapeutics Program frozen tumor repository, maintained by the Biological Testing Branch, Frederick, MD. Each has a detailed description, code identification, and a list of references at the National Tumor Repository. Tumors were maintained in the mouse strain of origin and were transplanted in the appropriate F1 hybrid (or the strain of origin) for therapy trials.

Chemotherapy. For panc03, colo38, and mam16C, mice were implanted bilaterally s.c. on day 0 with 30–60 mg tumor fragments, and chemotherapy was started 3 days after tumor implantation. For P388 and B16 studies, the tumor cells were implanted i.p. with either 10^6 cells or 0.5 mL of a 10% brei, respectively, on day 0 and chemotherapy was started on day 1. Titered controls were included to facilitate the calculation of tumor cell kill.

Endpoints for assessing antitumor activity. Quantitative endpoints used to assess antitumor activity included percent increased life span (%ILS), tumor cell kill ($\log_{10}$ cell kill) and tumor growth inhibition (T/C). Endpoints were calculated as follows:

$$\%ILS = (D_t - D_c)/D_c \times 100$$

where D_t is the median day of death for treated and D_c is the median day of death for control groups.

$$\text{Log}_{10}\text{ cell kill (total)} = T - C/(3.32)\,(Td)$$

where T − C is the tumor differences in either the median day to achieve a tumor mass of 1000–1500 mg (panc03, colo38, mam16C) or the median day of death (P388, B16) between the treated (T) and the control (C) groups and the Td is the tumor doubling time (in days), the latter estimated from the best fit straight line from a log–linear growth plot of the control group tumors in exponential growth. The conversion of the T − C values to $\log_{10}$ cell kill is possible because the Td for tumors regrowing post-

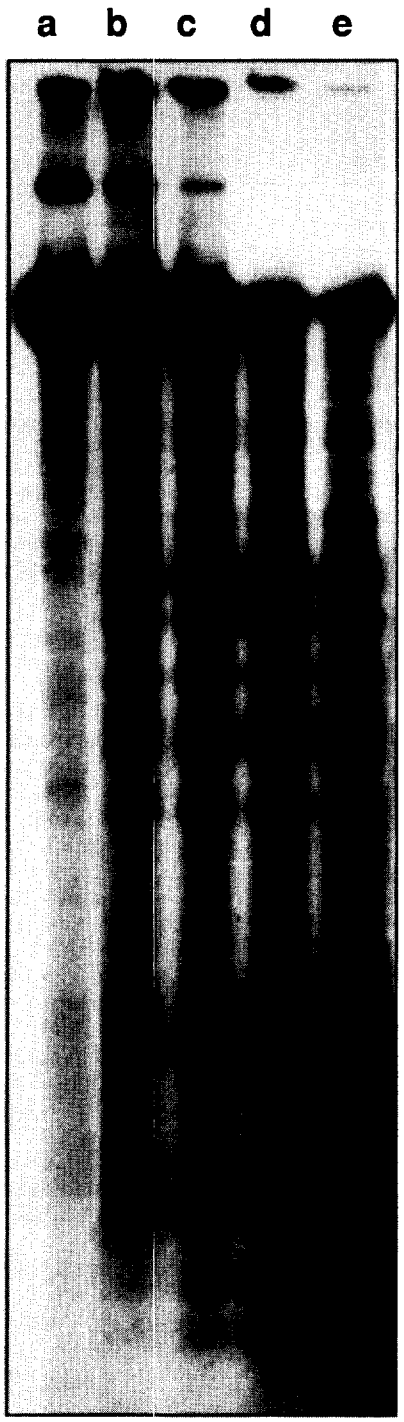

Fig. 3. Induction of site-specific DNA cleavage by HeLa cell topoisomerase by WIN 58161. Topoisomerase II cleavable complex assay products were proteolytically digested, resolved by agarose gel electrophoresis, and detected by autoradiography as described in Materials and Methods. Cleavage reactions contained: lanes (a) 0, (b) 4, (c) 8, (d) 16, and (e) 32 μg/mL WIN 58161.

treatment approximated the Td values of the tumors in the untreated control mice.

$$\% \text{ T/C} = (\text{T/C}) \times 100$$

where T and C are median tumor weights of the treatment and control groups, respectively, at the time that the median tumor weight of the control group tumors reached approximately 750–1500 mg in size. Tumor weights were estimated from two-dimensional measurements: tumor weight (mg) = $(a \times b^2)/2$, where a and b are the tumor length and width (mm), respectively.

RESULTS AND DISCUSSION

WIN 58161 was found to promote the formation of protein–DNA covalent complexes in the presence of either highly purified *E. coli* DNA gyrase or HeLa cell topoisomerase II and 3′-[^{32}P]-end-labeled pBR322 DNA (Fig. 2). Protein–DNA complexes were not detected in reactions containing BSA in the absence of either topoisomerase II or DNA gyrase. This observation suggests that WIN 58161 does not directly cross-link protein to DNA, but rather promotes the formation of both topoisomerase II and DNA gyrase cleavable complexes.

To demonstrate this more conclusively, topoisomerase II cleavage reactions were digested with proteinase K and subjected to agarose gel electrophoresis. The result of this analysis revealed WIN 58161-dependent, site-specific, DNA strand cleavage in the presence of topoisomerase II (Fig. 3). Furthermore, in the absence of topoisomerase II, WIN 58161 did not cause single- or double-strand DNA breaks as examined by agarose gel electrophoresis of drug-treated supercoiled plasmid DNA (data not shown). The property of enhancing topoisomerase-mediated DNA strand cleavage by drug is a definitive characteristic of antineoplastic topoisomerase poisons. The DNA cleavage pattern generated by topoisomerase II in the presence of WIN 58161 was distinct from that promoted by either *m*AMSA or VP-16, consistent with reports that different chemical classes of topoisomerase II poisons promote unique DNA fragmentation patterns (data not shown).

Antineoplastic topoisomerase II poisons are represented by a diverse number of chemical classes. Of these, the epipodophyllotoxins are the only class reported to not stably intercalate into DNA. Quinolone antibacterial agents also do not bind directly to DNA. The ability of WIN 58161 to intercalate into DNA was examined by the relaxation of closed circular plasmid DNA to equilibrium by excess topoisomerase I in the presence of WIN 58161. WIN 58161 showed no evidence of DNA intercalation (Fig. 4) by this analysis at concentrations ranging from 0.16 to 640 μM. The absence of stable DNA intercalation by WIN 58161 was confirmed in an ethidium bromide displacement assay (data not shown). Thus, WIN 58161 represents the prototype of a novel chemical class of non-intercalating topoisomerase II inhibitor.

In some topoisomerase I–DNA relaxation experiments, high concentrations of WIN 58161 caused a shift in the position of the ladder topomers to the

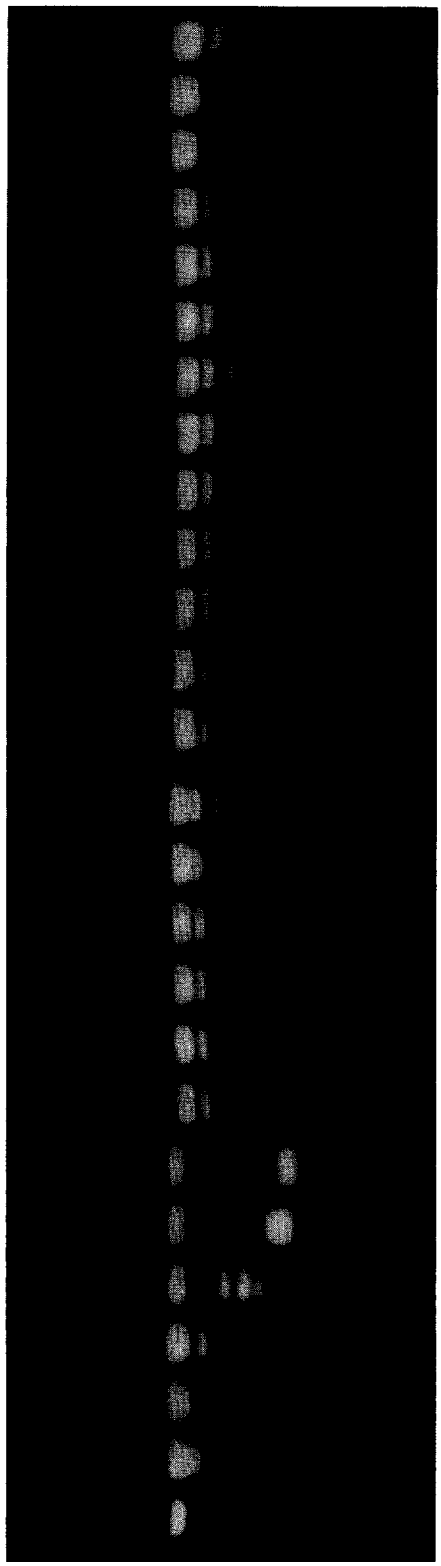

Fig. 4.

Table 1. Topoisomerase II and DNA gyrase inhibitory activity of WIN 58161 and several structurally related quinolone antibacterial agents

Test compound	EC_{50}* (μM)	
	Topoisomerase II	DNA gyrase
WIN 58161	9.4	0.024
WIN 58161-2	>260	0.75
1	31	0.026
2	>540	0.22
Ofloxacin	>280	0.11
Ciprofloxacin	>300	0.03
Norfloxacin	NT	0.19
*m*AMSA	0.76	NT

* Inhibition of topoisomerase II and DNA gyrase was assayed by SDS/K^+ precipitation of cleavable complexes of enzyme and 3′-[^{32}P]-end-labeled pBR322 DNA. The EC_{50} is the concentration of drug with activity equal to 50% of the maximal DNA–enzyme complexes obtained with reference inhibitors. Reference inhibitors used for topoisomerase II and DNA gyrase were *m*AMSA and norfloxacin, respectively. NT = not tested.

position of nicked DNA. To examine the possibility that WIN 58161 has a low level of topoisomerase I inhibitory activity, WIN 58161 was tested further. The agent failed to promote protein–DNA covalent complexes in the presence of calf thymus topoisomerase I and 5′-[^{32}P]-end-labeled pBR322 DNA, and at high concentration it inhibited topoisomerase I covalent complex formation (data not shown). Therefore, it is likely that WIN 58161 stabilizes only cleavable complexes of type II topoisomerases.

The topoisomerase II inhibitory activity of WIN 58161 was much greater than that observed for reference antibacterial quinolones (Table 1). Extensive structure–activity relationship studies for WIN 58161 and related quinolone topoisomerase II inhibitors revealed that the 2,6-dimethyl-4-pyridinyl group was highly beneficial to activity [18, 19]. In general, prototypic 7-substituents (e.g. 1-piperazinyl) of highly potent gyrase-interactive quinolones (e.g. ofloxacin, ciprofloxacin) were not contributory to mammalian enzyme activity.

Regarding the effect on topoisomerase II inhibitory potency upon modifying the 1,8-bridge of WIN 58161, the following observations were noted: (a) replacing the sulfur with oxygen to give **1** slightly decreased potency 3-fold, (b) there is a requirement for the (*S*)-methyl group in that no activity was seen

Fig. 4. DNA intercalative properties of WIN 58161. The DNA intercalative binding ability of WIN 58161 was examined by topoisomerase I-relaxation of closed circular plasmid DNA to equilibrium in the presence of test compound [27]. The intercalating (*m*AMSA) and non-intercalating (VP-16) reference topoisomerase II inhibitors are included for comparison. DNA intercalation assays contained (lane a) no drug; (lanes b–g) *m*AMSA at (b) 10, (c) 20, (d) 40, (e) 80, (f) 160, and (g) 320 μg/mL; (lanes h–m) VP-16 at (h) 20, (i) 40, (j) 80, (k) 160, (l) 320, and (m) 640 μg/mL; and (lanes n–z) WIN 58161 at (n) 0.16, (o) 0.31, (p) 0.63, (q) 1.3, (r) 2.5, (s) 5.0, (t) 10, (u) 20, (v) 40, (w) 80, (x) 160, (y) 320, and (z) 640 μg/mL.

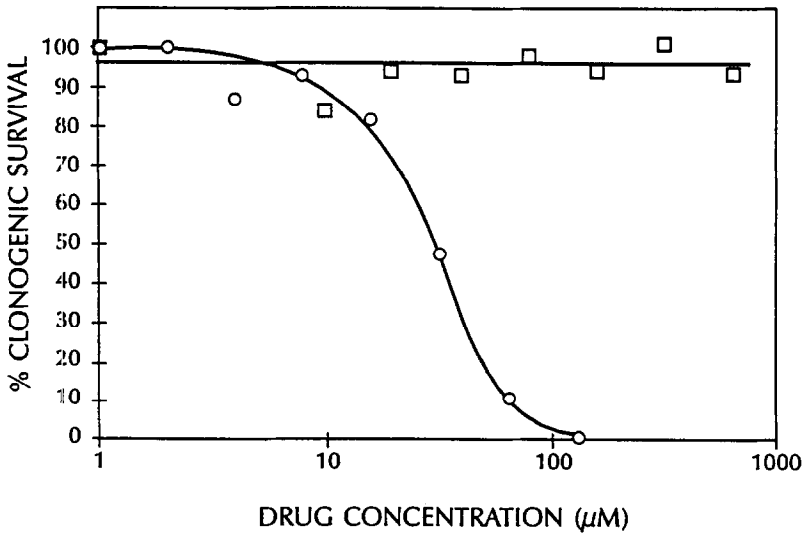

Fig. 5. Cytotoxic evaluation of WIN 58161 and WIN 58161-2. Cell survival was determined by colony formation in soft agar, as described in Materials and Methods, following a 1-hr exposure of P388 murine leukemia cells to either (○) WIN 58161 or (□) WIN 58161-2.

with the enantiomers (WIN 58161-2 and **2**) of WIN 58161 and **1** as well as analogues of **1** where the methyl group was replaced by ethyl or hydrogen, and (c) the 1,8-bridge of WIN 58161 and **1** is not a prerequisite for activity; the corresponding 1-cyclopropyl-8-fluoro analogue [18] has comparable activity to WIN 58161.

To explore the potential utility of WIN 58161 as an antitumor agent, the mechanism of cytotoxicity of WIN 58161 for P388 murine leukemia cells was investigated. The cytotoxic potencies of WIN 58161 and WIN 58161-2 were examined in log phase cultures of P388 murine leukemia cells. Following a 1-hr exposure to drug, cells were removed from drug by centrifugation, resuspended in fresh medium without drug, serially diluted, and plated in soft agar to quantitate clonogenic survival. Concentrations of WIN 58161 greater than 10 μM were cytotoxic to P388 cells (IC_{50} = 23 μM), whereas WIN 58161-2 had no effect on the survival of P388 cells at concentrations up to and including 640 μM (Fig. 5). Thus, the relative cytotoxicities of WIN 58161 and WIN 58161-2 were similar to the relative inhibitory potencies exhibited by these two compounds against topoisomerase II. This result is consistent with the conclusion that cell death, following exposure to WIN 58161, is a consequence of topoisomerase II inhibition. Moreover, the complete lack of cytotoxicity observed for WIN 58161-2 suggests that topoisomerase II inhibition may be the only mechanism of cytotoxicity for WIN 58161 at the concentrations tested.

To examine the relationship of topoisomerase II inhibition to the cytotoxicity of WIN 58161 more directly, we sought to relate intracellular topoisomerase II inhibition to the cytotoxicity of WIN 58161. Antineoplastic topoisomerase II poisons all produce intracellular DNA SSBs. To investigate the relationship of intracellular SSB formation to the mechanism of cytotoxicity of WIN 58161, intracellular SSBs were measured by alkaline elution following a 1-hr exposure of log phase P388 cells to either WIN 58161 or WIN 58161-2. WIN 58161 was found to generate intracellular SSBs in a concentration-dependent manner, whereas no SSBs were observed following exposure of cells to WIN 58161-2 (Fig. 6). Having thus obtained evidence suggesting that the cytotoxicity of WIN 58161 was a consequence of intracellular SSBs, our next line of investigation was to determine whether the SSBs resulting from exposure of P388 cells to WIN 58161 were topoisomerase mediated.

Topoisomerase-mediated intracellular SSBs can be distinguished from other types of SSBs such as those resulting from ionizing radiation, alkylating agents and DNA repair processes. SSBs generated by topoisomerase inhibitors have topoisomerase covalently attached at the break site. A comparison of the alkaline elution rate from P388 cell lysates analyzed under standard deproteinizing conditions and lysates that were not treated with proteinase K revealed that the rate of elution of DNA from P388 cells following treatment with WIN 58161 was greatly reduced in the absence of proteinase K treatment (Fig. 7). It can be concluded, therefore, that WIN 58161, like antineoplastic topoisomerase II poisons, causes both SSBs and protein–DNA cross-links in P388 cells exposed to WIN 58161. An additional characteristic by which topoisomerase-mediated intracellular SSBs can be distinguished from those that do not involve topoisomerase is the rapidity with which SSBs are resealed following the removal of drug. To investigate the reversibility of SSBs promoted by WIN 58161, intracellular SSBs were quantitated by alkaline elution in P388 cells exposed to WIN 58161 for 1 hr at 37° followed either by

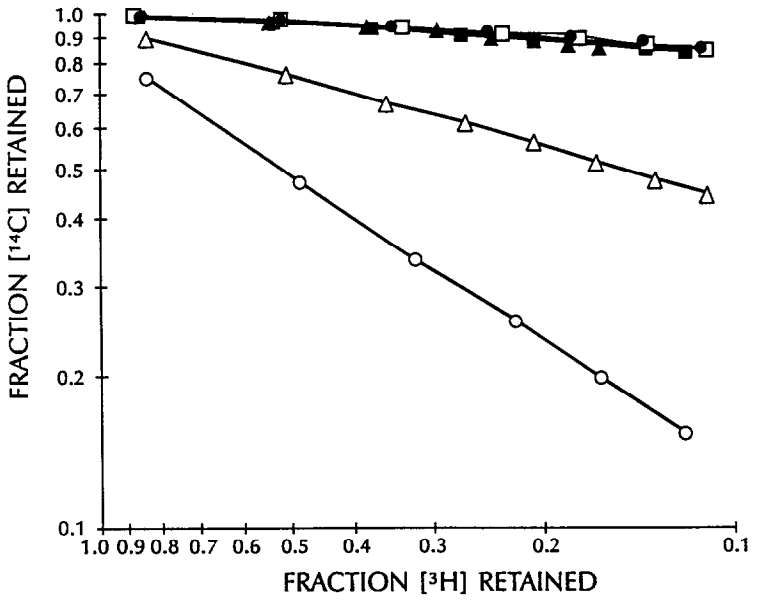

Fig. 6. Intracellular SSB formation following exposure to WIN 58161 and WIN 58161-2. SSBs were measured by alkaline elution under deproteinizing conditions in P388 cells, as described in Materials and Methods. Prior to analysis, cells were incubated for 1 hr (□), in the absence of drug; or in the presence of (△), 10 μM WIN 58161; (○) 20 μM WIN 58161; (▲) 10 μM WIN 58161-2; or (●) 50 μM WIN 58161-2.

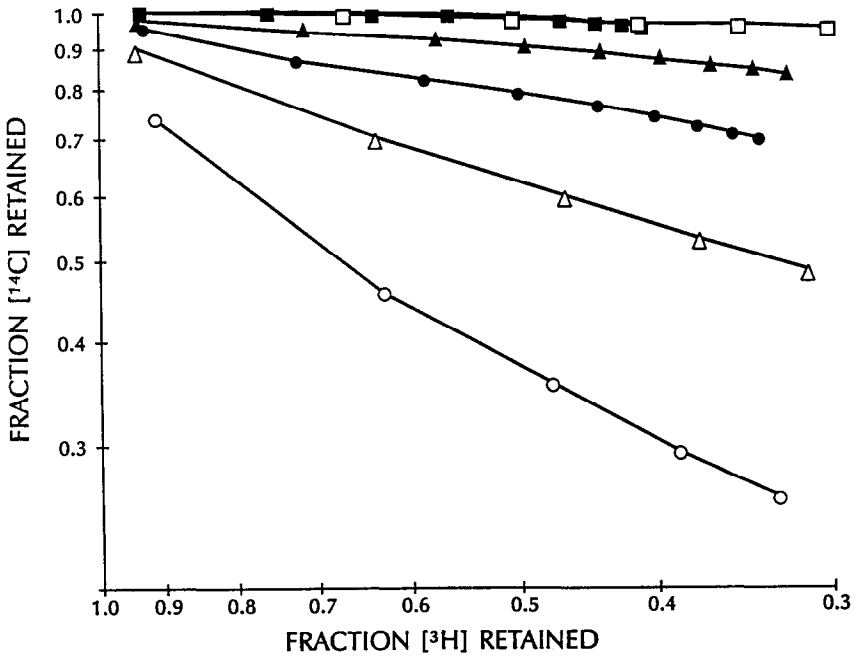

Fig. 7. Concealment of WIN 58161-promoted intracellular SSBs by protein. SSBs were measured by alkaline elution under either deproteinizing conditions (open symbols), or in the absence of proteinase treatment (closed symbols), as described in Materials and Methods. Prior to analysis cells were incubated for 1 hr (squares) in the absence of drug, or in the presence of (triangles) 10 μM WIN 58161 or (circles) 20 μM WIN 58161.

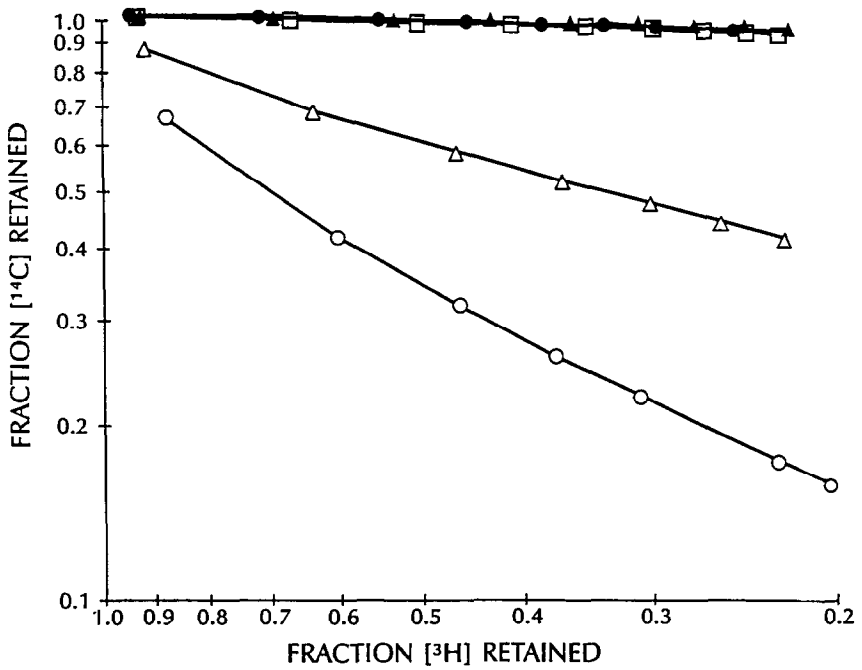

Fig. 8. Reversibility of WIN 58161-promoted intracellular SSBs. SSBs were measured by alkaline elution under deproteinizing conditions as described in Materials and Methods. P388 cells were treated for 1 hr in the presence or absence of drug after which cells were exchanged into drug-free medium. SSBs were measured in cells pretreated in this manner either following (open symbols) 1 hr on ice or (closed symbols) 1 hr at 37°. Drug treatment consisted of (squares) no drug, (triangles) 10 μM WIN 58161, or (circles) 20 μM WIN 58161.

20 min on ice or 20 min at 37° after the exchange of cells into fresh drug-free medium. As is typically observed with antineoplastic topoisomerase II poisons, no WIN 58161-promoted SSBs were detectable 20 min following drug removal (Fig. 8). These results, while strongly implicating topoisomerase II in the mechanism of SSB formation by WIN 58161, are also consistent with a dual mechanism of action whereby WIN 58161 generates SSBs and protein–DNA cross-links by separate processes. This alternative conclusion, however, is unlikely as WIN 58161 failed to generate either DNA strand breaks or protein–DNA cross-links when incubated with DNA in the absence of topoisomerase II. Therefore, we conclude from these studies that the cytotoxicity of WIN 58161 is a direct consequence of the compound's inhibition of topoisomerase II.

It is noteworthy that WIN 58161 represents a novel chemical class of non-intercalating topoisomerase II inhibitor, previously represented only by the epipodophyllotoxins. A novel chemical class of antineoplastic topoisomerase II inhibitor might be of particular value if the class did not share cross-resistance with topoisomerase II inhibitors that are currently used in the treatment of cancer. The two predominant mechanisms of resistance reported for topoisomerase II inhibitors result in multi-drug resistance (MDR). In pGp-MDR, resistance is due to the overexpression of P-glycoprotein, a membrane protein that regulates drug efflux. VLB_{100} is a pGp-MDR cell line derived from the CEM human leukemic lymphoblast cell line, originally derived as resistant to vinblastine [21]. In addition to resistance to *Vinca* alkaloids, VLB_{100} is cross-resistant to multiple chemical classes of topoisomerase II inhibitor including VP-16, *m*AMSA, and doxorubicin. A second mechanism of anticancer drug resistance that confers resistance to topoisomerase II inhibitors is a consequence of an alteration in either the topoisomerase II enzyme or the level of the enzyme in cells. VM-1 is a VM-26-resistant cell line derived from CEM in which drug resistance to topoisomerase II inhibitors is a consequence of an alteration in topoisomerase II [22]. Neither VLB_{100}

Table 2. Lack of cross-resistance of the VLB_{100} and VM-1 CEM cell lines to WIN 58161

	IC_{50}* (μM)		
Drug	CEM	VLB_{100}	VM-1
WIN 58161	51	42	40
VP-16	5.1	31	29
*m*AMSA	0.68	5.3	5.2
Vinblastine	3.4	185	3.1

* Cytotoxicity was measured by clonogenic survival in soft agar as described in Materials and Methods. The IC_{50} values are the averages of two or more determinations.

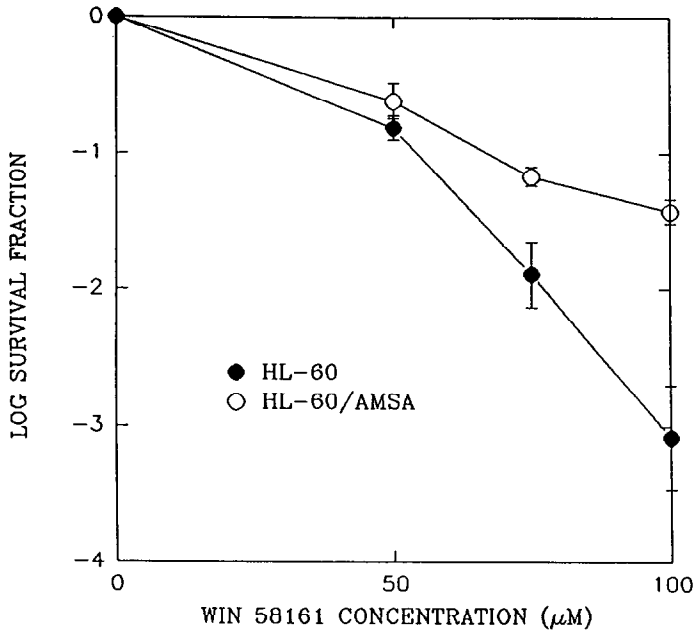

Fig. 9. Cytotoxicity of WIN 58161 to HL-60 and HL-60/AMSA cells. Cells were exposed to various concentrations of the drug for 1 hr at 37°. Points are means of three independent experiments ± 1 SD.

Table 3. *In vivo* antitumor activity of WIN 58161

Tumor	Drug route/ schedule	Maximum tolerated total dose* (mg/kg)	%T/C	%ILS
Panc03	s.c., qd 3–9	781	17	
Colo38	s.c., qd 3–9	504	0	
Mam16C	s.c., qd 1–4	263	15	
B16	i.p., qd 1,5,9	1500		66
P388	i.p., qd 1,5,9	1014		90

* Maximum tolerated total dose is the maximum non-lethal dose.

Table 4. *In vivo* antitumor evaluation of WIN 58161 enantiomers against early stage colo38

Test compound	Drug route/ schedule	Total dose (mg/kg)	%T/C*	T−C (days)	Log_{10} cell kill
WIN 58161	s.c., qd 3–14	1920	0	47	5.1
WIN 58161-2	s.c., qd 3–11	2638	>100		

* Determined on day 18. The average size of control tumors was 887 mg. There were no tumor-free survivors in either the control or treated mice (5 mice/group). There were no drug-induced deaths with either agent. WIN 58161 produced a −8% body weight loss at the nadir (day 15). WIN 58161-2-treated mice had a +4.8% weight gain.

nor VM-1 was resistant to WIN 58161 (Table 2). WIN 58161 was also evaluated for cytotoxicity against the Chinese hamster ovary cell line CHO and VPMR-5 [29], a VP-16-resistant cell line derived from CHO, which expresses an altered form of topoisomerase II (supplied by Dr. Warren Ross, University of Florida at Gainesville). VPMR-5 was also not cross-resistant to WIN 58161 (data not shown). In addition, investigators at the M.D. Anderson Cancer Center examined the cross-resistance of HL-60/AMSA to WIN 58161. HL-60/AMSA is a human leukemia cell line with a high

level of resistance for intercalating topoisomerase II inhibitors [30, 31]. HL-60/AMSA was only minimally resistant to WIN 58161 (Fig. 9), substantiating the hypothesis that the mechanism of resistance in HL-60/AMSA imparts resistance exclusively for intercalating topoisomerase II inhibitors. These results indicate that the most frequently observed mechanisms of laboratory-derived resistance to topoisomerase II inhibitors do not alter the cytotoxicity of WIN 58161 and suggest that WIN 58161 may have utility against clinically resistant disease.

WIN 58161 exhibited a significant antitumor effect in each of five *in vivo* antitumor models in which it was evaluated (Table 3). These models represent a diverse range of tumor types including leukemia and solid tumors. It can be concluded that the *in vivo* antitumor activity obtained with WIN 58161 is a consequence of topoisomerase II inhibition. WIN 58161-2, the non-topoisomerase II-inhibitory enantiomer of WIN 58161, failed to reduce tumor growth in a side-by-side comparison with WIN 58161 for *in vivo* antitumor activity against early stage colon adenocarcinoma 38. In this evaluation, WIN 58161 was highly active, yielding a 0% T/C and a 5.1 $\log_{10}$ cell kill, whereas WIN 58161-2 was completely inactive (Table 4). We conclude from these studies that WIN 58161 represents the prototype of a novel chemical class of topoisomerase II inhibitor with potential clinical utility in treating cancer.

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