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Design, Synthesis, and Biological Evaluation of Potential Prodrugs Related to the Experimental Anticancer Agent Indotecan (LMP400)

Peng-Cheng Lv[†], Mohamed S. A. Elsayed[†], Keli Agama[‡], Christophe Marchand[‡], Yves Pommier[‡], and Mark Cushman^{*,†}

Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, and the Purdue Center for Cancer Research, Purdue University, West Lafayette, IN 47907, Developmental Therapeutics Branch and Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892–4255

Abstract

Indenoisoquinoline topoisomerase I (Top1) inhibitors are a novel class of anticancer agents with two compounds in clinical trials. Recent metabolism studies of indotecan (LMP400) led to the discovery of the biologically active 2-hydroxylated analogue and 3-hydroxylated metabolite, thus providing strategically placed functional groups for the preparation of a variety of potential ester prodrugs of these two compounds. The current study details the design and synthesis of two series of indenoisoquinoline prodrugs and it also reveals how substituents on the O-2 and O-3 positions of the A ring, which are next to the cleaved DNA strand in the drug-DNA-Top1 ternary cleavage complex, affect Top1 inhibitory activity and cytotoxicity. Many of the indenoisoquinoline prodrugs were very potent antiproliferative agents with GI₅₀ values below 10 nanomolar in a variety of human cancer cell lines.

Graphical abstract

*Corresponding Author: Phone: 765-494-1465. Fax: 765-494-6970. cushman@purdue.edu.

[†]Purdue University

[‡]National Cancer Institute

The remaining authors have no competing and/or relevant financial interest(s) to disclose.

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ASSOCIATED CONTENT

Supporting Information. SMILES molecular strings and PDB files for the molecular models presented in Figures 3 and 4. This material is available free of charge via the internet at <http://pubs.acs.org>.

INTRODUCTION

Topoisomerases are ubiquitous enzymes that resolve the topological problems associated with DNA supercoiling during replication, transcription, and other nuclear processes.^{1–3} Human topoisomerase I (Top1) cleaves a single DNA strand by nucleophilic attack of the enzyme on a DNA phosphodiester to form a “cleavage complex” in which the 3' end of the broken DNA strand is covalently linked to the enzyme (Scheme 1). The broken (scissile) strand then undergoes “controlled rotation” around the unbroken strand to relax the DNA superhelical tension and remove supercoils. The catalytic cycle ends when the 5' end of the scissile strand carries out a nucleophilic attack on the phosphotyrosyl-DNA phosphodiester to religate the DNA and release the enzyme.^{4–7} Top1 inhibitors are classified as Top1 suppressors, which inhibit the DNA cleavage reaction, and Top1 poisons, which inhibit the DNA religation reaction (Scheme 1). Top1 is overexpressed in cancer cells and DNA damage responses are defective in some human tumors.^{1, 8, 9} Top1 poisons that stabilize the “cleavage complex” have therefore been developed as chemotherapeutic agents. The mechanism of cancer cell death produced by Top1 poisons involves collision of the DNA replication fork with the DNA cleavage site in the ternary DNA-drug-Top1 complex leading to double-strand breaks and cell death.¹

Representative Top1 poisons are shown in Figure 1. Camptothecin (**1**, Figure 1) is a natural product having Top1 as its only cellular target.¹⁰ Although topotecan (**2**) and irinotecan (**3**) are approved by the Food and Drug Administration (FDA) for the treatment of cancer,^{8, 11} camptothecin derivatives suffer from several major drawbacks, including poor aqueous solubility, dose-limiting toxicity, and bioavailability limitations resulting from lactone hydrolysis and binding of the ensuing hydroxyacid to plasma proteins.^{12–16} Moreover, both target mutations such as R364H¹⁷ and N722SX¹⁸ and induction of ABCG2^{19–21} and MXR²¹ ATP-binding cassette (ABC) drug efflux transporters compromise the anticancer activities of the camptothecins. These limitations have stimulated the search for noncamptothecin Top1 inhibitors as anticancer agents.

The Top1 poisoning activity of NSC314622 (**4**) was discovered after a COMPARE analysis of its cytotoxicity profile revealed a strong resemblance to that of other known Top1 poisons, including camptothecin (**1**) and the clinically useful anticancer drug topotecan (**2**).^{22, 23} Subsequently, MJ-III-65 (**5**) was found to be a potent Top1 poison after a hydroxyethylaminopropyl side chain was attached to the lactam nitrogen of **4**.²⁶ The indenoisoquinolines have several advantages over the camptothecins. First, different genes are likely to be targeted because the DNA cleavage site specificity of **4** is different from that of camptothecin (**1**), which may provide a different antitumor spectrum.²⁴ Second, the ternary Top1-DNA-drug cleavage complexes induced by the indenoisoquinolines are more stable than those formed in the presence camptothecin derivatives.²⁴ Third, in contrast to the camptothecin derivatives that have an unstable lactone ring, the indenoisoquinolines are chemically stable.²³ Fourth, the indenoisoquinolines are less affected by the R364H and N722S Top1 resistance mutations and drug efflux pumps than camptothecin.^{23, 25, 26} These advantages have stimulated interest in the synthesis of additional indenoisoquinolines. Two indenoisoquinoline Top1 poisons [indotecan (**6**, also known as LMP400 or NSC 724998) and indimitecan (**7**, also known as LMP776 or NSC 725776)] have entered Phase I clinical

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trials for treatment of cancer patients at the National Cancer Institute, and definite plans are being formulated to commence Phase II clinical trials.^{9, 27–29}

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Recent studies of the metabolism of indotecan (LMP400, **6**) and indimitecan (LMP776, **7**) led to the discovery of phenolic metabolites that had unexpectedly high Top1 inhibitory activity and cytotoxicity.³⁰ That investigation involved the synthesis of an array of phenolic synthetic standards that were also biologically active, which provided an unanticipated opportunity to synthesize prodrugs by esterification of the phenols. The present study focused on the utilization of the biologically active 2-hydroxylated indenoisoquinoline reference compound **8** (not a metabolite) and the biologically active metabolite **9** of LMP400.

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Prodrugs are bioreversible derivatives of drug molecules that undergo an enzymatic and/or chemical transformation in vivo to release the active parent drug, which can then exert the desired pharmacological effect.^{31–36} Irinotecan (**3**) provides a good example, since the carbamate is hydrolyzed after administration to the pharmacologically active phenol. The prodrug strategy can improve the physicochemical, biopharmaceutical, or pharmacokinetic properties of pharmacologically potent compounds, and thereby enhance the development and usefulness of a potential drug.^{37–42} Currently, 5–7% of the drugs approved worldwide can be classified as prodrugs.^{31, 34} Esters are the most common prodrugs used, and it is estimated that approximately 49% of all marketed prodrugs are activated by enzymatic hydrolysis.³³ Ester prodrugs are most often used to enhance the overall lipophilicity of the molecule and promote membrane permeability and oral absorption.³²

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Extensive structure-activity relationship studies have been published on the indenoisoquinolines, including modifications on the A ring,^{30, 43–54} B ring (side chain on the lactam nitrogen),^{48, 54–56} C ring,^{57, 58} and D ring.^{30, 42, 47, 48, 56, 57, 59, 60} However, previous investigations of indenoisoquinoline prodrugs are limited to a study of dihydroindenoisoquinoline prodrugs of indenoisoquinolines,⁶¹ and no work on ester prodrugs has previously been reported.

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The 4.64 μM MGM of indotecan (LMP400, **6**) is not consistent with its relatively high Top1 inhibitory activity (++++). One possible reason for this is limited aqueous solubility, and the relatively “flat” concentration vs. activity curves produced by indotecan in the NCI-60 cancer cell cytotoxicity assay are often characteristic of compounds with high potency and limited solubility. Indeed, both of the phenols **8** (MGM 0.412 μM) and **9** (MGM 0.076 μM) are expected to be more water soluble and they are also more cytotoxic than **6**. The present study was based on the hypothesis that prodrugs of **8** and **9** might have greater absorption from the GI tract than the phenols, and would be expected to be converted to the phenols in the blood plasma or in the cancer cells after absorption.^{62–67}

RESULTS AND DISCUSSION

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The synthesis of indenoisoquinoline **8** was performed according to the previously reported method with some modifications.⁵⁶ With the 2-hydroxylated indenoisoquinoline **8**³⁰ in hand, carbamate **10** was first prepared as shown in Scheme 2. Subsequently, four different esters

were prepared. The acetylated derivative **11** was obtained by reacting the phenol **8** with acetic anhydride in the presence of DMAP. Treatment of compound **8** with methyl 4-chloro-4-oxobutyrate in the presence of DMAP provided compound **12**. Reaction of compound **8** with benzoyl chloride in the presence of DMAP yielded compound **13**. The nicotinate ester **14** was prepared by EDC coupling compound **8** and nicotinic acid in the presence of DMAP.

At the same time, a series of *O*-3-modified indenoisoquinolines **15–19** were synthesized (Scheme 3) by similar methods as those used for compounds **10–14**. The sulfate compound **20**, which is a potential metabolite of indotecan, was prepared by treatment of compound **9** with SO₃-NMe₃ complex in refluxing anhydrous acetonitrile.

All of the new indenoisoquinoline derivatives were tested in Top1-mediated DNA cleavage assays. For this purpose, a ³²P 3'-end labeled 117-bp DNA fragment was incubated with Top1 at four concentrations of a tested compound. The DNA fragments were separated on 20% PAGE denaturing gels. The Top1 inhibitory activities were assigned on the basis of the visual inspection of the number and intensities of the gel bands corresponding to Top1-mediated DNA cleavage fragments and scored relative to the Top1 inhibitory activity of compounds **1** and **4**. Results are expressed in semiquantitative fashion: 0, no detectable activity; +, weak activity; ++, similar activity to compound **4**; +++, greater activity than **4**; + + +, equipotent with **1**. Ambiguous scores (e.g., between two values) are designated with parentheses (e.g., ++(+)) would be between ++ and +++). Representative PAGE results are shown in Figure 2.

As shown in Table 1, the 2-OH indenoisoquinoline **8** had good Top1 inhibitory activity at the ++(+) level. After conversion of the hydroxyl to a dimethylcarbamate, the observed Top1 inhibitory activity increased from ++(+) for compound **8** to +++ for compound **10**. Introduction of an acetyl group at the O-2 position yielded compound **11**, which was also a Top1 poison with activity at the ++ level. Subsequently, several hydrophobic substituents were attached to the O-2 position. Both compounds **12** and **13**, which have an aliphatic and aromatic substituent on the O-2 position, respectively, displayed reduced Top1 inhibitory activity at the + level. Compound **14**, which has a nicotinoyl moiety on the O-2 position, was found to be a promising Top1 poison with activity at the +++ level.

The Top1 inhibitory activities of *O*-3-modified indenoisoquinolines **16–20** were found to be either abolished or significantly reduced relative to the phenol **9**, which indicates that these derivatives are likely to be functioning as prodrugs since they maintain cytotoxicity despite having greatly diminished Top1 inhibitory activities. The two benzoates **13** and **18**, on the other hand, were significantly less cytotoxic than their respective parent compounds, and they are both weak Top1 poisons indicating that they may not be acting as prodrugs. In general, the *O*-2 modified indenoisoquinolines **10–14** are more potent Top1 poisons than their *O*-3 modified counterparts **15–19**.

The Top1-mediated DNA fragmentation patterns produced by camptothecin (**1**), indenoisoquinoline **5**, and compounds **10**, **11**, **13**, **14** and **20** are presented in Figure 2. The DNA sequence preferences for trapping the Top1-DNA cleavage complexes by these

indenoisoquinolines are similar to each other, but the pattern is different from camptothecin, indicating that the indenoisoquinolines target the genome differently than camptothecin.

A molecular docking study was performed using the crystal structure (PDB ID: 1SC7) of a Top1-DNA-indenoisoquinoline ternary cleavage complex⁶⁸ in order to understand the Top1 inhibition results produced by the phenols **8** and **9** and by the prodrugs. The energy-minimized structure of the morpholine derivative **8**⁵⁴ (Figure 1) was docked into the crystal structure of a Top1-DNA cleavage site with GOLD using the centroid coordinates of the indenoisoquinoline ligand. The energy-minimized, top-ranked GOLD pose of compound **8** in ternary complex with DNA and Top1 is displayed in Figure 3. Compound **8** intercalates readily at the DNA cleavage site, between the +1 and -1 base pairs. Rings A and B stack with the scissile strand bases, while rings C and D stack with the noncleaved strand bases. The carbonyl group on the C ring forms a hydrogen bond to a nitrogen of the Arg364 side chain with an N-O distance of 2.5 Å, which is an essential contact for the Top1 inhibitory activity.³⁰ The previously reported crystal structure of an analogue of **4** having a 3'-carboxypropyl substituent on the lactam nitrogen in ternary complex with DNA and Top1 indicates that the A ring of the indenoisoquinoline is next to the cleaved DNA strand, where there is more room to accommodate substituents on the prodrug, and the present model of **8** in complex with DNA and Top1 is consistent with that X-ray crystal structure.⁶⁸ This explains the Top1 poisoning activity displayed by the prodrugs.

In order to gain a more detailed insight into the prodrug binding mode in the Top1-DNA-prodrug ternary complex and the resulting Top1 inhibitory activities, compound **14** was selected for a molecular docking study based on its relatively high Top1 inhibitory activity. As displayed in Figure 4, compound **14** is calculated to intercalate at the DNA cleavage site, between the +1 and minus;1 base pairs. Rings A and B stack with the scissile strand bases, while rings C and D stack with the noncleaved strand bases, which is consistent with the calculated binding mode of compound **8**. There is a hydrogen bond between the oxygen of the carbonyl group on the C ring in the minor DNA groove and a terminal nitrogen of the Arg364 side chain with an O N distance of 2.5 Å. The nicotinoyl substituent of the prodrug is readily accommodated in the complex as it protrudes through the cleaved DNA strand.

All of the indenoisoquinolines were tested for antiproliferative activity in the National Cancer Institute's 60 cell line screen (NCI-60).^{69, 70} The cells were incubated with the tested compounds at 100, 10, 1, 0.1, and 0.01 µM concentrations for 48 h before treatment with sulforhodamine B dye. Optical densities were recorded, and their ratios relative to that of the control were plotted as percentage growth against the log10 of the tested compound concentrations. The concentration that corresponds to 50% growth inhibition (GI₅₀) is calculated by interpolation between the points located above and below the 50% percentage growth. The mean-graph midpoint (MGM) is an estimated average of the GI₅₀ values derived from the NCI collection of 60 human cancer cell lines, where during the MGM calculation, anticancer agents with GI₅₀ values that are outside the testing range of 0.01 100 µM are arbitrarily assigned the values of 0.01 and 100 µM, respectively. The results are listed in Table 1. Most of the new compounds display significant potencies against various cell lines with GI₅₀ values in the nanomolar range, while compounds **10**, **13**, and **18** are in the micromolar range. Also, the GI₅₀ values of many of the substances vs. individual human

cancer cell lines are below the lowest concentration used (0.01 μM) in the standard NCI testing protocol. The nicotinoyl derivative **14** has good Top1 inhibitory activity at the +++ level as well as good antiproliferative activity with an MGM value of $0.158 \pm 0.065 \mu\text{M}$. In general there is a revealing lack of correlation between the rank order of observed cytotoxicities and inhibition of Top1. For example, the MGM cytotoxicity values of **9**, **15**–**17**, and **19** are all very close to each other, but the Top1 inhibitory activities range from +++ (+) to 0. This suggests that **15**–**17** and **19** are hydrolyzed intracellularly to the same biologically active species **9**. In the case of **8**, **11**, and **12**, the cytotoxicities of the assumed prodrugs **11** and **12** are actually greater than the parent **8** by a factor of almost one order of magnitude, which could possibly be explained by a more effective cellular penetration by **11** and **12** vs. **8**, followed by hydrolysis of **11** and **12** to **8**.

In order to investigate the possibility that hydrolysis of the prodrugs takes place extracellularly rather than intracellularly, compound **17** was added to cell-free cell culture medium and the mixture was incubated at 37 °C. Relatively slow conversion to the parent phenol **9** was observed, with 22% conversion after 99 hours. This result suggests that the prodrug is taken up by the cells and then hydrolyzed to the phenol during the 48-hour cell culture incubation period. This would also be consistent with the greater potency of **11** and **12** vs. **8**.

In addition to the stability studies in cell culture medium, the conversion of the acetate prodrug **16** to its parent drug **9** was evaluated in rat plasma. Figure 5 displays a plot of the percentage of the prodrug remaining in rat blood plasma at 37 °C as a function of time after an initial drug concentration of 5 μM . The selection of **16** for plasma stability analysis was made on the basis of its potent cytotoxicity in human cancer cell cultures (MGM 0.095 μM) together with its poor activity as a Top1 inhibitor. The hydrolysis of the prodrug **16** occurred very rapidly in rat plasma with a half-life of 0.90 min, suggesting that the parent drug **9** would be released quickly in the circulation after administration of **16**.

CONCLUSIONS

A series of indenoisoquinolines substituted in the 2- and 3-positions with ester side chains were designed and synthesized for possible deployment as prodrugs. A slight improvement in Top1 inhibitory activity was observed for compounds **10** and **14** vs. **8**. The rest of the prodrugs are less active vs. Top1 than their parent compounds. There is significant enhancement of the cytotoxicities of the acetate **11** and the succinate **12** vs. the parent phenol **8**, which would be consistent with more favorable cellular uptake followed by metabolism to the parent indenoisoquinolines. The fact that many of the derivatives have very potent cytotoxicities of similar magnitude despite being inactive or significantly less active Top1 poisons than the parent drugs supports the hypothesis that they are functioning as prodrugs of the parent compounds in human cancer cell culture.

EXPERIMENTAL SECTION

General

Solvents and reagents were purchased from commercial vendors and were used without any further purification. Melting points were determined using capillary tubes with a Mel-Temp apparatus and are uncorrected. Infrared spectra were obtained using KBr pellets. IR spectra were recorded using a Perkin-Elmer 1600 series or Spectrum One FTIR spectrometer. ¹H NMR spectra were recorded at 300 MHz using a Bruker ARX300 spectrometer with a QNP probe. ESIMS analyses were performed at the Purdue Campus-Wide Mass Spectrometry Center on a Finnegan-MATT LCQ Classic mass spectrometer. Analytical thin layer chromatography was done on Baker-flex silica gel IB2-F plates, and compounds were visualized with short wavelength UV light and ninhydrin staining. Silica gel flash chromatography was accomplished using 230–400 mesh silica gel. HPLC analyses were completed on a Waters 1525 binary HPLC pump/Waters 2487 dual λ absorbance detector system using a 5 μ M C₁₈ reverse phase column. Compound purities were estimated by reversed phase C₁₈ HPLC, with UV detector at 254 nm, and the major peak area of each tested compound was 95% of the combined total peak area. All yields refer to isolated compounds. The rat plasma stability analysis of **16** was performed at Cyprotex US, LLC 313 Pleasant St. Watertown, MA 02472.

3-Methoxy-6-(3-morpholinopropyl)-5,12-dioxo-6,12-dihydro-5H-[1,3]dioxolo[4',5':5,6]indeno[1,2-c]isoquinolin-2-yl Dimethylcarbamate (10)

A solution of compound **8** (0.100 g, 0.216 mmol) in chloroform (10 mL) was treated with dimethylcarbamoyl chloride (0.050 g, 0.52 mmol) in the presence of DMAP (0.200 g). The mixture was stirred at 23 °C for 30 h. The mixture was diluted to a volume of 150 mL with CHCl₃, washed with H₂O (2 x 50 mL) and saturated aq NaCl (50 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by flash column chromatography (SiO₂, ~40 g), eluting with 1% MeOH in CHCl₃, to yield the product as a solid (0.060 g, 52%): mp 268–270 °C (dec). IR (film) 2938, 1718, 1650, 1508, 1168, 1031, 863 cm⁻¹; ¹H NMR (CDCl₃) δ 8.28 (s, 1 H), 7.73 (s, 1 H), 7.38 (s, 1 H), 7.26 (s, 1 H), 7.04 (s, 1 H), 6.06 (s, 2 H), 4.57–4.52 (t, *J* = 7.5 Hz, 2 H), 3.93 (s, 3 H), 3.69 (s, 4 H), 3.16 (s, 3 H), 3.03 (s, 3 H), 2.53 (s, 6 H), 2.01 (s, 2 H); ESIMS *m/z* (rel intensity) 536 (MH⁺, 100); HRESIMS *m/z* (rel intensity) 536.2041 (MH⁺), calcd for C₂₈H₃₀N₃O₈ 536.2033. HPLC purity: 98.83% (C-18 reverse phase, MeOH-H₂O, 90:10).

3-Methoxy-6-(3-morpholinopropyl)-5,12-dioxo-6,12-dihydro-5H-[1,3]dioxolo[4',5':5,6]indeno[1,2-c]isoquinolin-2-yl Acetate (11)

A solution of compound **8** (0.093 g, 0.20 mmol) in chloroform (10 mL) was treated with Ac₂O (0.050 g, 0.45 mmol) in the presence of DMAP (0.200 g). The mixture was stirred at 23 °C for 5 h. The mixture was diluted to a volume of 100 mL with CHCl₃, washed with H₂O (2 x 50 mL) and saturated aq NaCl (50 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by flash column chromatography (SiO₂, ~30 g), eluting with 1.25% MeOH in CHCl₃, to yield the title compound as a solid (0.052 g, 50%): mp 272–274 °C. IR (film) 2948, 1770, 1661, 1514, 1379, 1029, 866 cm⁻¹; ¹H NMR (CDCl₃) δ 8.26 (s, 1 H), 7.75 (s, 1 H), 7.40 (s, 1 H), 7.06 (s, 1 H), 6.07 (s, 2 H), 4.46–4.51

(t, $J = 7.8$ Hz, 2 H), 3.93 (s, 3 H), 3.75 (s, 4 H), 2.52 (s, 6 H), 2.32 (s, 3 H), 1.99 (s, 2 H); ESIMS m/z (rel intensity) 507 (MH^+ , 100); HRESIMS m/z (rel intensity) 507.1761 (MH^+), calcd for $C_{27}H_{27}N_2O_8$ 507.1767. HPLC purity: 96.34% (C-18 reverse phase, MeOH- H_2O , 85:15).

3-Methoxy-6-(3-morpholinopropyl)-5,12-dioxo-6,12-dihydro-5*H*-[1,3]dioxolo[4',5':5,6]indeno[1,2-*c*]isoquinolin-2-yl Methyl Succinate (12)

A solution of compound **8** (0.134 g, 0.288 mmol) in chloroform (10 mL) was treated with methyl 4-chloro-4-oxobutyrates (0.050 g, 0.33 mmol) in the presence of DMAP (0.200 g). The mixture was stirred at 23 °C for 4 h. The mixture was diluted to a volume of 150 mL with $CHCl_3$, washed with H_2O (2 x 30 mL) and saturated aq NaCl (80 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by flash column chromatography (SiO_2 , ~40 g), eluting with 1% MeOH in $CHCl_3$, to yield the product as a solid (0.068 g, 41%): mp 219–220 °C. IR (film) 2931, 1773, 1658, 1309, 1125, 1031, 786 cm^{-1} ; 1H NMR ($CDCl_3$) δ 8.19 (s, 1 H), 7.70 (s, 1 H), 7.32 (s, 1 H), 6.96 (s, 1 H), 6.02 (s, 2 H), 4.39–4.44 (t, $J = 7.8$ Hz, 2 H), 3.90 (s, 3 H), 3.74 (s, 7 H), 2.95–2.99 (t, $J = 7.2$ Hz, 2 H), 2.75–2.79 (t, $J = 6.9$ Hz, 2 H), 2.52 (s, 6 H), 1.93–1.98 (m, 2 H); ESIMS m/z (rel intensity) 579 (MH^+ , 100); HRESIMS m/z (rel intensity) 579.1975 (MH^+), calcd for $C_{30}H_{31}N_2O_{10}$ 579.1979. HPLC purity: 99.05% (C-18 reverse phase, MeOH- H_2O , 90:10).

3-Methoxy-6-(3-morpholinopropyl)-5,12-dioxo-6,12-dihydro-5*H*-[1,3]dioxolo[4',5':5,6]indeno[1,2-*c*]isoquinolin-2-yl Benzoate (13)

A solution of compound **8** (0.94 g, 0.20 mmol) in chloroform (10 mL) was treated with benzoyl chloride (0.050 g, 0.36 mmol) in the presence of DMAP (0.200 g). The mixture was stirred at 23 °C for 27 h. The mixture was diluted to a volume of 100 mL with $CHCl_3$, washed with H_2O (2 x 25 mL) and saturated aq NaCl (50 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by flash column chromatography (SiO_2 , ~40 g), eluting with 2% MeOH in $CHCl_3$, to yield the product as a solid (0.065 g, 57%): mp 261–263 °C (dec). IR (film) 2342, 1744, 1650, 1508, 1308, 1032, 788 cm^{-1} ; 1H NMR ($CDCl_3$) δ 8.39 (s, 1 H), 8.15–8.22 (m, 2 H), 7.80 (s, 1 H), 7.64–7.67 (m, 1 H), 7.52–7.56 (m, 3 H), 7.07 (s, 1 H), 6.08 (s, 2 H), 4.57–4.52 (t, $J = 7.2$ Hz, 2 H), 3.91 (s, 3 H), 3.67 (s, 4 H), 2.59 (s, 6 H), 2.07 (s, 2 H); ESIMS m/z (rel intensity) 569 (MH^+ , 100); HRESIMS m/z (rel intensity) 569.1930 (MH^+), calcd for $C_{32}H_{29}N_2O_8$ 569.1924. HPLC purity: 96.56% (C-18 reverse phase, MeOH- H_2O , 80:20).

3-Methoxy-6-(3-morpholinopropyl)-5,12-dioxo-6,12-dihydro-5*H*-[1,3]dioxolo[4',5':5,6]indeno[1,2-*c*]isoquinolin-2-yl Nicotinate (14)

A solution of compound **8** (0.120 g, 0.26 mmol) in chloroform (10 mL) was treated with nicotinic acid (0.200 g, 1.62 mmol) in the presence of EDC·HCl (0.160 g, 0.83 mmol) and DMAP (270 mg). The mixture was stirred at 23 °C for 3 h. The mixture was diluted to a volume of 120 mL with $CHCl_3$, washed with H_2O (2 x 60 mL) and saturated aq NaCl (60 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by flash column chromatography (SiO_2 , ~40 g), eluting with 1.25% MeOH in $CHCl_3$, to yield the title compound as a solid (0.066 g, 44%): mp 277–279 °C (dec). IR

(film) 2956, 1750, 1508, 1381, 1272, 1116, 865 cm^{-1} ; ^1H NMR (CDCl_3) δ 9.42 (s, 1 H), 8.89 (s, 1 H), 8.47–8.49 (d, J = 8.1 Hz, 1 H), 8.41 (s, 1 H), 7.81 (s, 1 H), 7.47–7.52 (m, 1 H), 7.41 (s, 1 H), 7.08 (s, 1 H), 6.09 (s, 2 H), 4.51–4.56 (t, J = 6.9 Hz, 2 H), 3.92 (s, 3 H), 3.67 (s, 4 H), 2.58 (s, 6 H), 2.07 (s, 2 H); ESIMS m/z (rel intensity) 570 (MH^+ , 100); HRESIMS m/z (rel intensity) 570.1872 (MH^+), calcd for $\text{C}_{31}\text{H}_{28}\text{N}_3\text{O}_8$ 570.1876. HPLC purity: 95.84% (C-18 reverse phase, MeOH, 100).

2-Methoxy-6-(3-morpholinopropyl)-5,12-dioxo-6,12-dihydro-5H-[1,3]dioxolo[4',5':5,6]indeno[1,2-c]isoquinolin-3-yl Dimethylcarbamate (15)

A solution of compound **9** (0.100 g, 0.216 mmol) in chloroform (15 mL) was treated with dimethylcarbamoyl chloride (0.031 g, 0.32 mmol) in the presence of DMAP (0.015 g). The mixture was stirred at 23 °C for 30 h. The mixture was diluted to a volume of 100 mL with CHCl_3 , washed with H_2O (2 x 50 mL) and saturated aq NaCl (50 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by flash column chromatography (SiO_2 , ~40 g), eluting with 2.5% MeOH in CHCl_3 , to yield the product as a solid (0.081 g, 70%): mp 290–292 °C. IR (film) 2960, 2350, 1870, 1732, 1508, 1163, 1032, 863 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.01 (s, 1 H), 7.93 (s, 1 H), 7.26 (s, 1 H), 6.82 (s, 1 H), 6.09 (s, 2 H), 4.50 (m, 2 H), 3.98 (s, 3 H), 3.82 (m, 6 H), 3.14 (s, 3 H), 3.02 (s, 3 H), 2.61 (m, 4 H), 2.05 (m, 2 H); ESIMS m/z (rel intensity) 536 (MH^+ , 100); HRESIMS m/z (rel intensity) 536.2037 (MH^+), calcd for $\text{C}_{28}\text{H}_{30}\text{N}_3\text{O}_8$ 536.2033. HPLC purity: 95.63% (C-18 reverse phase, MeOH- H_2O , 90:10).

2-Methoxy-6-(3-morpholinopropyl)-5,12-dioxo-6,12-dihydro-5H-[1,3]dioxolo[4',5':5,6]indeno[1,2-c]isoquinolin-3-yl Acetate (16)

A solution of compound **9** (0.047 g, 0.10 mmol) in chloroform (20 mL) was treated with Ac_2O (0.016 g, 0.15 mmol) in the presence of DMAP (0.025 g, 0.20 mmol). The mixture was stirred at 23 °C for 3 h. The mixture was diluted to a volume of 100 mL with CHCl_3 , washed with H_2O (2 x 50 mL) and saturated aq NaCl (50 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by flash column chromatography (SiO_2 , ~30 g), eluting with 1.25% MeOH in CHCl_3 , to yield the title compound as a solid (0.035 g, 68%): mp >350 °C. IR (film) 2346, 1773, 1656, 1508, 1308, 1115, 670 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.10 (s, 1 H), 7.90 (s, 1 H), 7.43 (s, 1 H), 7.08 (s, 1 H), 6.09 (s, 2 H), 4.49 (s, 2 H), 3.98 (s, 3 H), 3.80 (s, 4 H), 2.58 (s, 6 H), 2.34 (s, 3 H), 2.05 (m, 2 H); ESIMS m/z (rel intensity) 507 (MH^+ , 100); HRESIMS m/z (rel intensity) 507.1772 (MH^+), calcd for $\text{C}_{27}\text{H}_{27}\text{N}_2\text{O}_8$ 507.1767. HPLC purity: 95.26% (C-18 reverse phase, MeOH- H_2O , 90:10).

2-Methoxy-6-(3-morpholinopropyl)-5,12-dioxo-6,12-dihydro-5H-[1,3]dioxolo[4',5':5,6]indeno[1,2-c]isoquinolin-3-yl Methyl Succinate (17)

A solution of compound **9** (0.060 g, 0.13 mmol) in chloroform (10 mL) was treated with methyl 4-chloro-4-oxobutylate (0.050 g, 0.33 mmol) in the presence of DMAP (0.100 g). The mixture was stirred at 23 °C for 4 h. The mixture was diluted to a volume of 100 mL with CHCl_3 , washed with H_2O (2 x 25 mL) and saturated aq NaCl (50 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by flash

column chromatography (SiO₂, ~40 g), eluting with 1.25 % MeOH in CHCl₃, to yield the product as a solid (0.039 g, 53%): mp >350 °C. IR (film) 2348, 1717, 1650, 1508, 1116, 670 cm⁻¹; ¹H NMR (CDCl₃) δ 8.05 (s, 1 H), 7.89 (s, 1 H), 7.42 (s, 1 H), 7.04 (s, 1 H), 6.07 (s, 2 H), 4.43–4.48 (t, *J* = 7.5 Hz, 2 H), 3.96 (s, 3 H), 3.76 (s, 4 H), 3.65 (s, 3 H), 2.93–2.97 (t, *J* = 6.9 Hz, 2 H), 2.74–2.78 (t, *J* = 6.6 Hz, 2 H), 2.53 (s, 6 H), 1.99 (s, 2 H); ESIMS *m/z* (rel intensity) 579 (MH⁺, 100); HRESIMS *m/z* (rel intensity) 579.1974 (MH⁺), calcd for C₃₀H₃₁N₂O₁₀ 579.1979. HPLC purity: 97.00% (C-18 reverse phase, MeOH-H₂O, 80:20).

2-Methoxy-6-(3-morpholinopropyl)-5,12-dioxo-6,12-dihydro-5*H*-[1,3]dioxolo[4',5':5,6]indeno[1,2-*c*]isoquinolin-3-yl Benzoate (18)

A solution of compound **9** (0.85 g, 0.183 mmol) in chloroform (15 mL) was treated with benzoyl chloride (0.038 g, 0.275 mmol) in the presence of DMAP (0.044 g, 0.366 mmol). The mixture was stirred at 23 °C for 24 h. The mixture was diluted to a volume of 50 mL with CHCl₃, washed with H₂O (2 x 25 mL) and saturated aq NaCl (50 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by flash column chromatography (SiO₂, ~40 g), eluting with 2.5% MeOH in CHCl₃, to yield the product as a solid (0.062 g, 61%): mp 316–318 °C. IR (film) 2957, 2348, 1651, 1559, 1309, 1116, 864 cm⁻¹; ¹H NMR (CDCl₃) δ 8.20–8.27 (m, 2 H), 8.14 (s, 1 H), 8.03 (s, 1 H), 7.63–7.70 (m, 1 H), 7.50–7.56 (m, 2 H), 7.44 (s, 1 H), 7.08 (s, 1 H), 6.09 (s, 2 H), 4.50 (s, 2 H), 3.99 (s, 3 H), 3.80 (s, 4 H), 2.58 (s, 6 H), 2.05 (s, 2 H); ESIMS *m/z* (rel intensity) 569 (MH⁺, 100); HRESIMS *m/z* (rel intensity) 569.1921 (MH⁺), calcd for C₃₂H₂₉N₂O₈ 569.1924. HPLC purity: 96.75% (C-18 reverse phase, MeOH-H₂O, 85:15).

2-Methoxy-6-(3-morpholinopropyl)-5,12-dioxo-6,12-dihydro-5*H*-[1,3]dioxolo[4',5':5,6]indeno[1,2-*c*]isoquinolin-3-yl Nicotinate (19)

A solution of compound **9** (0.153 g, 0.33 mmol) in chloroform (30 mL) was treated with nicotinic acid (0.061 g, 0.50 mmol) in the presence of DCC (0.075 g, 0.36 mmol) and a catalytic amount of DMAP. The mixture was stirred at 23 °C for 6 h. The mixture was diluted to a volume of 100 mL with CHCl₃, washed with H₂O (2 x 60 mL) and saturated aq NaCl (60 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by flash column chromatography (SiO₂, ~40 g), eluting with 2.5% MeOH in CHCl₃, to yield the title compound as a solid (0.097 g, 53%): mp 274–276 °C (dec). IR (film) 2961, 1749, 1657, 1504, 1261, 1033, 864 cm⁻¹; ¹H NMR (CDCl₃) δ 9.41 (s, 1 H), 8.87 (s, 1 H), 8.45–8.48 (d, *J* = 7.8 Hz, 1 H), 8.16 (s, 1 H), 8.04 (s, 1 H), 7.46 (s, 2 H), 7.10 (s, 1 H), 6.11 (s, 2 H), 4.52 (m, 2 H), 3.97 (s, 3 H), 3.80 (s, 4 H), 2.60 (s, 6 H), 2.07 (s, 2 H); ESIMS *m/z* (rel intensity) 570 (MH⁺, 100); HRESIMS *m/z* (rel intensity) 570.1884 (MH⁺), calcd for C₃₁H₂₇N₃O₈ 570.1876. HPLC purity: 95.53% (C-18 reverse phase, MeOH-H₂O, 85:15).

2-Methoxy-6-(3-morpholinopropyl)-5,12-dioxo-6,12-dihydro-5*H*-[1,3]dioxolo[4',5':5,6]indeno[1,2-*c*]isoquinolin-3-yl Hydrogen Sulfate (20)

A mixture of SO₃-NMe₃ (0.280 g, 2.02 mmol) and Et₃N (0.77 mL, 4.032 mmol) was added to a well-stirred mixture of compound **9** (0.156 g, 0.336 mmol) in anhydrous MeCN (8 mL) at 23 °C under argon. The reaction mixture was heated at reflux under argon for 40 h. The

reaction mixture was cooled to 23 °C, decanted, and concentrated under reduced pressure. The crude product was applied twice to a column of silica gel (eluent 1–20% MeOH-CHCl₃). The fractions containing the target compound were concentrated, and the target compound **20** (0.094 g, 52%) was obtained by preparative silica gel TLC (2 mm, CH₃OH-CHCl₃, 1:5): mp 283–285 °C (dec). ¹H NMR (DMSO, 300 MHz) 9.42 (s, 1 H), 8.24 (s, 1 H), 7.91 (s, 1 H), 7.55 (s, 1 H), 7.13 (s, 1 H), 6.21 (s, 2 H), 4.46 (m, 2 H), 3.88 (s, 3 H), 3.55 (s, 4 H), 3.07–3.11 (m, 2 H), 2.37 (s, 4 H), 1.90 (m, 2 H); ESIMS *m/z* (rel intensity) negative ion 543.1 [(M – H)[–], 100]; HRESIMS *m/z* (rel intensity) 543.1077 [(M – H)[–]], calcd for C₂₅H₂₃N₂O₁₀S 543.1073. HPLC purity: 96.67% (C-18 reverse phase, MeOH-H₂O, 90:10).

Topoisomerase I-Mediated DNA Cleavage Reactions

Human recombinant Top1 was purified from baculovirus as previously described.⁷² DNA cleavage reactions were prepared as previously reported with the exception of the DNA substrate.⁵⁶ Briefly, a 117-bp DNA oligonucleotide (Integrated DNA Technologies) encompassing the previously identified Top1 cleavage sites in the 161-bp fragment from pBluescript SK(–) phagemid DNA was employed. This 117-bp oligonucleotide contains a single 5'-cytosine overhang, which was 3'-end-labeled by fill-in reaction with [α-³²P]dGTP in React 2 buffer (50 mM Tris-HCl, pH 8.0, 100 mM MgCl₂, 50 mM NaCl) with 0.5 unit of DNA polymerase I (Klenow fragment, New England BioLabs). Unincorporated [³²P]dGTP was removed using mini Quick Spin DNA columns (Roche, Indianapolis, IN), and the eluate containing the 3'-end-labeled DNA substrate was collected. Approximately 2 nM radiolabeled DNA substrate was incubated with recombinant Top1 in 20 μL of reaction buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, and 15 μg/mL BSA] at 25 °C for 20 min in the presence of various concentrations of compounds. The reactions were terminated by adding SDS (0.5% final concentration) followed by the addition of two volumes of loading dye (80% formamide, 10 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue). Aliquots of each reaction mixture were subjected to 20% denaturing PAGE. Gels were dried and visualized by using a phosphorimager and ImageQuant software (Molecular Dynamics). For simplicity, cleavage sites were numbered as previously described in the 161-bp fragment.

Stability of Compound 17 in Cell Culture Medium

RPMI 1640 medium was purchased from Sigma-Aldrich (St. Louis, MO). Compound **17** (10 μM) was incubated at 37 °C with RPMI 1640 medium. After 99 h, an aliquot was taken from the incubation mixture and filtered prior to analysis using liquid chromatography mass spectrometry (Waters, Germany). The flow rate was 0.75 mL/min and the eluent was recorded with a DAD at 280 nm.

Rat Plasma Stability Studies

In duplicate analyses, compound **16** and reference compounds (enalapril as high clearance control and warfarin as the low clearance one) were incubated at 5 μM concentration with rat plasma (Sigma-Aldrich P2516 – Plasma) at 37 °C. An aliquot was removed from each experimental reaction after 0, 1, 3, 5 and 15 minutes and mixed with three volumes of ice-cold Stop Solution (methanol, containing propranolol as an internal standard). Stopped

reactions were incubated at least ten minutes at -20 °C. The samples were centrifuged to remove precipitated protein, and the supernatants were analyzed by LC-MS/MS (Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software) using positive ionization mode to quantitate the remaining parent. Propranolol was utilized as an analytical internal standard (IS). Data were converted to % remaining by dividing the concentration at each time point by the time zero concentration value.

Molecular Modeling

The Top1 crystal structure for docking was prepared, and the docking protocol was validated as previously described.⁵⁶ The ternary complex ligand centroid coordinates for docking were defined using the ligand in the Top1-DNA-MJ238 crystal structure (PDB code 1SC7) as the center of the binding pocket (x = 21.3419, y = 3.9888, z = 28.2163). The ligand was then deleted. Indenoisoquinolines to be modeled were constructed in SYBYL. Atom types were assigned using SYBYL atom typing. Hydrogens were added, and the ligands were minimized by the conjugate gradient method using the MMFF94s force field with MMFF94 charges, a distance-dependent dielectric function, and a 0.01 kcal mol⁻¹ Å⁻¹ energy gradient convergence criterion. Each ligand was docked into the mutant crystal structure using GOLD 3.2 with default parameters, and the coordinates were defined by the crystal structure as described above. The top four poses for each ligand were examined. The highest-ranked poses for these ligands were merged into the crystal structure, and the entire complex was subsequently subjected to minimization using a standard Powell method, the MMFF94s force field and MMFF94 charges, a distance-dependent dielectric function, and a 0.05 kcal mol⁻¹ Å⁻¹ energy gradient convergence criterion. During the energy minimization, the ligand and a 7 Å sphere surrounding the ligands were allowed to move while the structures outside this sphere were frozen in an aggregate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Representative Top1 poisons.

Figure 2.

Top1-mediated DNA cleavage induced by indenoisoquinolines **20**, **14**, **10**, **13** and **11**: lane 1, DNA alone; lane 2, Top1 + DNA; lane 3, **1**, 1 μM ; lane 4, **5**, 1 μM ; lanes 5-8, **20**, **14**, **10**, **13**, and **11** at 0.1, 1, 10, and 100 μM , respectively, from left to right. Numbers and arrows on the left indicate cleavage site positions.

Figure 3.

The hypothetical binding mode of compound **8** in ternary complex with DNA and Top1. The diagram is programmed for wall-eyed (relaxed) viewing. Compound **8** is shown as green sticks, the protein residues are displayed as blue sticks, and the base pairs are displayed as lines. The structure was generated starting from the Top1-DNA-MJ238 crystal structure (PDB 1SC7).

Figure 4.

The hypothetical binding model of compound **14** in ternary complex with DNA and Top1. The diagram is programmed for wall-eyed (relaxed) viewing. Compound **14** is shown as green sticks, the protein residues are displayed as blue sticks, and the base pairs are displayed as lines. The structure was generated starting from the Top1-DNA-MJ238 crystal structure (PDB 1SC7).

Figure 5. Stability analysis of the acetate prodrug **16** in rat plasma at 37 °C. The initial drug concentration was 5 μ M.

Scheme 1.

Scheme 2^a

^aReagents and conditions: (a) for **10**: dimethylcarbamoyl chloride, DMAP, CHCl₃, 23 °C, 30 h (52% yield); for **11**: Ac₂O, DMAP, CHCl₃, 23 °C, 5 h (50% yield); for **12**: CH₃OCOCH₂CH₂COCl, DMAP, CHCl₃, 23 °C, 4 h (41% yield); for **13**: benzoyl chloride, DMAP, CHCl₃, 23 °C, 27 h (57% yield); for **14**: nicotinic acid, EDC·HCl, DMAP, CHCl₃, 23 °C, 3 h (44% yield).

Scheme 3^a

^aReagents and conditions: (a) for **15**: dimethylcarbamoyl chloride, DMAP, CHCl₃, 23 °C, 30 h (70% yield); for **16**: Ac₂O, DMAP, CHCl₃, 23 °C, 3 h (68% yield); for **17**: CH₃OCOCH₂CH₂COCl, DMAP, CHCl₃, 23 °C, 4 h (53% yield); for **18**: benzoyl chloride, DMAP, CHCl₃, 23 °C, 24 h (61% yield); for **19**: nicotinic acid, DCC, DMAP, CHCl₃, 23 °C, 6 h (53% yield); for **20**: SO₃·NMe₃, CH₃CN, Et₃N, reflux, 40 h (52% yield).

Table 1

Antiproliferative Potencies and Topoisomerase I Inhibitory Activities of A-ring Modified Indenoisoquinolines.

Compd	Cytotoxicity (GI ₅₀ in μM) ^d								MGMP ^b	Top 1Cleavage ^e
	lung HOP-62	Colon HCT-116	CNS SF-539	melanoma UACC-62	Ovarian OVCAR-3	renal SN12C	prostate DU-145	breast MCF-7		
6	1.78	1.15	0.040	0.030	74.1	0.813	0.155	0.374	4.64 ± 1.25	+++
8	0.257	0.279	0.335	0.282	0.871	0.195	0.257	0.144	0.412 ± 0.005 ^d	++(+)
10	1.698	6.07	1.86	4.47	12.0	2.40	1.55	0.022	3.46 ± 0.999	+++
11	0.018	0.029	<0.01	0.018	0.138	<0.01	<0.01	<0.01	0.043 ± 0.019	++
12	<0.01	0.014	<0.01	<0.01	0.066	<0.01	<0.01	<0.01	0.049 ± 0.008	+
13	0.079	0.85	0.037	0.096	4.57	0.054	0.051	0.033	1.5 ± 0.17	+
14	0.012	0.030	<0.01	0.022	0.295	<0.01	0.013	<0.01	0.158 ± 0.065	+++
9	0.01	0.02	<0.01	<0.01	0.03	<0.01	<0.01	<0.01	0.076 ± 0.028	+++(+)
15	0.032	0.026	0.021	0.017	0.105	0.025	0.032	<0.01	0.070 ± 0.028	++(+)
16	<0.01	<0.01	<0.01	<0.01	0.269	<0.01	<0.01	<0.01	0.095 ± 0.045	0
17	0.016	0.020	<0.01	0.018	0.037	<0.01	0.014	<0.01	0.036 ± 0.018	0
18	2.14	5.89	0.135	0.059	30.9	0.195	0.724	0.015	3.19 ± 0.622	+
19	<0.01	<0.01	<0.01	<0.01	0.015	<0.01	<0.01	<0.01	0.035 ± 0.000	+
20	0.389	0.389	0.263	0.263	1.12	0.355	0.417	0.209	0.912	++

^aThe cytotoxicity GI₅₀ values are the concentrations corresponding to 50% growth inhibition.^bMean graph midpoint for growth inhibition of all human cancer cell lines successfully tested, ranging from 10⁻⁸ to 10⁻⁴ molar.^cCompound-induced DNA cleavage due to Top1 inhibition is graded by the following rubric relative to 1 μ M camptothecin: 0, no inhibitory activity; +, between 20 and 50% activity; ++, between 50 and 75% activity; +++, between 75% and 95% activity; +++++, equipotent.^dFor MGM GI₅₀ values in which a standard error appears, the GI₅₀ values for individual cell lines are the average of two determinations; values without standard error are from one determination.