

Thanatop: A Novel 5-Nitrofuran that Is a Highly Active, Cell-Permeable Inhibitor of Topoisomerase II

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Abstract

A series of nitrofuran-based compounds were identified as inhibitors of estrogen signaling in a cell-based, high-throughput screen of a diverse library of small molecules. These highly related compounds were subsequently found to inhibit topoisomerase II *in vitro* at concentrations similar to that required for the inhibition of estrogen signaling in cells. The most potent nitrofuran discovered is ~10-fold more active than etoposide phosphate, a topoisomerase II inhibitor in clinical use. The nitrofurans also inhibit topoisomerase I activity, with ~20-fold less activity. Moreover, the nitrofurans, in contrast to etoposide, induce a profound cell cycle arrest in the G₀-G₁ phase of the cell cycle, do not induce double-stranded DNA breaks, are not substrates for multidrug resistance protein-1 export from the cell, and are amenable to synthetic development. In addition, the nitrofurans synergize with etoposide phosphate in cell killing. Clonogenic assays done on a panel of human tumors maintained *ex vivo* in nude mice show that the most active compound identified in the screen is selective against tumors compared with normal hematopoietic stem cells. However, this compound had only moderate activity in a mouse xenograft model. This novel class of topoisomerase II inhibitor may provide additional chemotherapeutic strategies for the development of cytotoxic agents with proven clinical utility. [Cancer Res 2007;67(9):4451–8]

Introduction

Transcription of estrogen-responsive genes is a complex, dynamic process that consists of repeated cycles of initiation by estrogen receptor α (ER α), recruitment and activation of DNA polymerase II, followed by limitation of transcription (1–4). The latter events are mediated by processes generally regarded as repressive; in particular, histone deacetylases (HDAC) and histone demethylases, in association with the SWI/SNF or the NuRD complex, remodel local chromatin structure to restrict transcriptional engagement. The complexity and cyclical nature of these events suggests that it may be possible to modulate estrogen signaling by using not only small molecules that directly interact with the ER but also with small molecules that target other processes involved in transcriptional cycling. We have provided proof of concept of this hypothesis with HDAC inhibitors, which have a profound antiestrogenic effect in the ER α -positive breast adenocarcinoma cell line MCF-7 (5). Additionally, topoisomerases I and II are engaged in transcriptional processes. Topoisomerase I is

a component of the transcription factor IID complex (6) and binds to nucleolin (7), SV40 T antigen (7), p53 (8), and heat shock protein 70 (9). Moreover, estrogen-dependent restructuring and transcriptional competence of the pS2 promoter has been shown to require the generation of a DNA double-stranded DNA (dsDNA) break that is promoted by topoisomerase II β and poly(ADP-ribose) polymerase (10). Collectively, these findings indicate that topoisomerases have a significant role in transcriptional processes.

Encouraged by proof of concept studies with histone deacetylase inhibitors that they could phenocopy the effect of antiestrogens and with the extent of the complexity of estrogen signaling (5), we did a cell-based, unbiased, high-throughput screen against a highly diverse compound library to discover novel small molecules that either activate or inhibit estrogen signaling. Six related nitrofuran derivatives were identified in the screen which inhibit estrogen signaling at micromolar levels and are overtly cytotoxic to dividing cells. These nitrofurans inhibit estrogen signaling and are potent inhibitors of topoisomerase II and, to a lesser extent, of topoisomerase I. These nitrofurans do not induce dsDNA breaks in cells, as detected by the phosphorylation of H2AX, an early marker of DNA damage. Prospectively, this indicates that the nitrofurans may be less mutagenic than etoposide. Moreover, unlike etoposide, which is derived from natural sources, the nitrofurans are chemically tractable, perhaps allowing their physicochemical properties to be modified to provide suitable clinical candidates.

Materials and Methods

Reagents and antibodies. Etoposide, etoposide phosphate, camptothecin, propidium iodide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. DMSO was purchased from Merck and vincristine sulphate from Calbiochem. Anti-phospho-H2AX (Ser¹³⁹) was purchased from Abcam. Anti-ER α (HC20) was purchased from Santa Cruz Biotechnologies. Topoisomerase I was purchased from GE Healthcare and topoisomerase II α was bought from USB. Topoisomerase II activity was also analyzed using material purchased from Topogen. The nitrofurans described in this article were prepared by Tripos as custom synthesis and supplied at a chemical purity of >90% by liquid chromatography-mass spectrometry UV at 254 nm with structures confirmed by elemental analysis and by ¹H-nuclear magnetic resonance.

Topoisomerase I and II assays. These assays were done according to the manufacturer's instructions. Briefly, 0.5 μ g supercoiled DNA, 1 unit topoisomerase I, and the compounds under investigation were incubated for 30 min at 30°C in 35 mmol/L Tris-HCl (pH 8.0), 72 mmol/L KCl, 5 mmol/L MgCl₂, 5 mmol/L DTT, 5 mmol/L spermidine, and 0.01% bovine serum albumin (BSA). The reactions were stopped by the addition of 6 \times loading dye solution (Fermentas) and then subjected to electrophoresis on 0.7% agarose gels, with or without ethidium bromide. Topoisomerase II assays were done as above except that 125 ng supercoiled DNA and 4 units topoisomerase II, together with the chemical compounds under investigation, were incubated for 30 min at 30°C in 10 mmol/L Tris-HCl (pH 7.9), 50 mmol/L NaCl, 50 mmol/L KCl, 5 mmol/L MgCl₂, 0.1 mmol/L EDTA, 15 μ g/ μ L BSA, and 1 mmol/L ATP.

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Table 1. Nitrofurans identified from the EMBL-DKFZ library are inhibitors of estrogen signaling

Object ID	Structure	IC ₅₀ ER signaling (μmol/L)	GI ₅₀ cytotox (μmol/L)
EMBL-153441		0.50	0.90
EMBL-153434		0.57	8.90
EMBL-102840		0.60	2.95
EMBL-153432		2.84	16.96
EMBL-153438		2.94	16.96
EMBL-153448		26.12	31.53
Furaltadone			
Nitrofurantoin			

NOTE: The EMBL identity number, structure, inhibition concentration required to inhibit estrogen signaling in MCF-7 cells by 50% (IC₅₀), and the concentration required to inhibit the growth of MCF-7 cells by 50% (GI₅₀) of the six 5-nitrofurans identified as inhibitors of estrogen signaling in a cell-based screen are shown. Also shown, for comparison, are the structures of furaltadone and nitrofurantoin, two nitrofurans used as anti-infective agents.

Cell lines, cell culture, and transfections. MCF-7, HEKPC7, and HEKMRP1 cells were grown in DMEM supplemented with 10% FCS. Hygromycin (100 μg/mL) was used to maintain expression within the MRP1 stable cell line. The HEKPC7 and HEKMRP1 cell lines (11) were a generous gift of Dr. Roger Deeley (Queen's University, Kingston, Ontario, Canada). Transfections were done using polyethylenimine (12).

Histone preparation and Western blot analysis. Cells (2×10^5) were seeded in six-well dishes and treated with test compounds for 16 h. Subsequently, 0.5 mL of 0.2 mol/L H₂SO₄ was applied onto the cells for 5 min. The cells were scraped, incubated on ice for 10 min, and then spun at 14,000 rpm for 15 min. The lysate was collected and proteins were precipitated by the addition of 0.25 volume of 100% trichloroacetic acid. After 10-min incubation on ice, precipitated proteins were collected by

centrifugation. The pellets were washed with 1 mL chloroform, air dried, and then resuspended in 100 μL water. Sample (50 μL) was then resolved by electrophoresis on 15% SDS-PAGE; proteins were then transferred on a polyvinylidene difluoride (PVDF) membrane (Millipore) for analysis by immunostaining.

For analysis by Western blot, after a 16-h treatment with compounds, cells were washed twice with PBS and directly lysed in sample buffer. Viscosity was reduced by brief sonication and proteins were resolved on a 10% SDS-PAGE gel. Proteins were transferred onto PVDF membrane, which was then subsequently blocked with casein, then incubated 2 h with primary antibody. Anti-phospho-H2AX (Ser¹³⁹) was diluted to 130 ng/mL and anti-ERα to 66 ng/mL. The membrane was then washed and incubated for 1 h with the corresponding horseradish peroxidase-conjugated

secondary antibody (DiaNova) and visualized by chemiluminescence with the enhanced chemiluminescence kit (Amersham).

Cell cycle analysis. Cytometric measurement of intracellular DNA content was done using propidium iodide staining (13); in essence, cells were trypsinized, permeabilized, stained, and subjected to flow cytometry with 10,000 events recorded. Cell fragments and aggregates of cells, identified by side scattering, were excluded from the analysis.

Chemosensitivity testing. The MTT assay was used to indirectly estimate cellular proliferation through mitochondrial reduction to the corresponding tetrazolium salt. In essence, 3,000 cells per well were seeded in a 96-well plate in medium supplemented with 10% FCS. They were exposed to test compounds 24 h later and then incubated for an additional 72 h before incubation with MTT for 3 h. DMSO concentrations were identical in all samples. Absorbance was determined at 550 nm. Two or more independent experiments were carried out, and within each experiment, estimations were done in quadruplicate.

Results

The European Molecular Biology Laboratory-German Cancer Research Center (EMBL-DKFZ) small-molecule library was screened against a cell-based assay in the ER α -positive cell line MCF-7, which was transfected with an ERE-tk-luc reporter plasmid that expresses luciferase under the control of an estrogen-responsive promoter (14). Approximately 55,000 compounds were screened in 96-well format at a concentration of 4 μ mol/L for an incubation period of 24 h. Around 1% of compounds inhibited luciferase production by greater than three times the SD of the assay. All compounds that potentially modulated estrogen signaling were subsequently validated in a counterscreen to

exclude compounds that directly inhibit luciferase activity. Of the confirmed inhibitors, six contained a nitrofurans group. Their structures, associated IC₅₀'s and GI (growth inhibition)₅₀'s against estrogen signaling and growth of MCF-7 cells, respectively, are shown in Table 1. We then concentrated on the most active member of this series, EMBL-153441, to evaluate the functional and mechanistic properties of this series of compounds. Importantly, the activity of EMBL-153441 on resynthesis gave similar values to the library compound in cell-based inhibition of estrogen signaling and in cytotoxicity assays.

The nitrofurans EMBL-102840 or EMBL-153441 and known inhibitors of either topoisomerase I or topoisomerase II activity abrogate ER α protein levels and compromise estrogen signaling in cell-based assays (Fig. 1C and D). Based on these findings, we systematically evaluated the nitrofurans identified from the screen in topoisomerase assays. As shown in Fig. 1B and C, EMBL-153441 inhibits topoisomerase II in both a decatenation assay, where induction of a dsDNA break is a prerequisite for activity, and in a plasmid relaxation assay. Indeed, the only nitrofurans found not to inhibit topoisomerase II activity is EMBL-153448, the least active member of the nitrofurans discovered in screen (data not shown). Moreover, concomitant addition of etoposide and EMBL-153441 results in greater inhibition than either compound alone, perhaps suggesting that each molecule targets a different site on topoisomerase II. Neither furaltadone nor nitrofurantoin, nitrofurans that are used as antibacterial agents (see Table 1 for structures), inhibits human topoisomerase II to appreciable levels (data not shown). In conclusion, the *in vitro* activity of EMBL-153441 on the

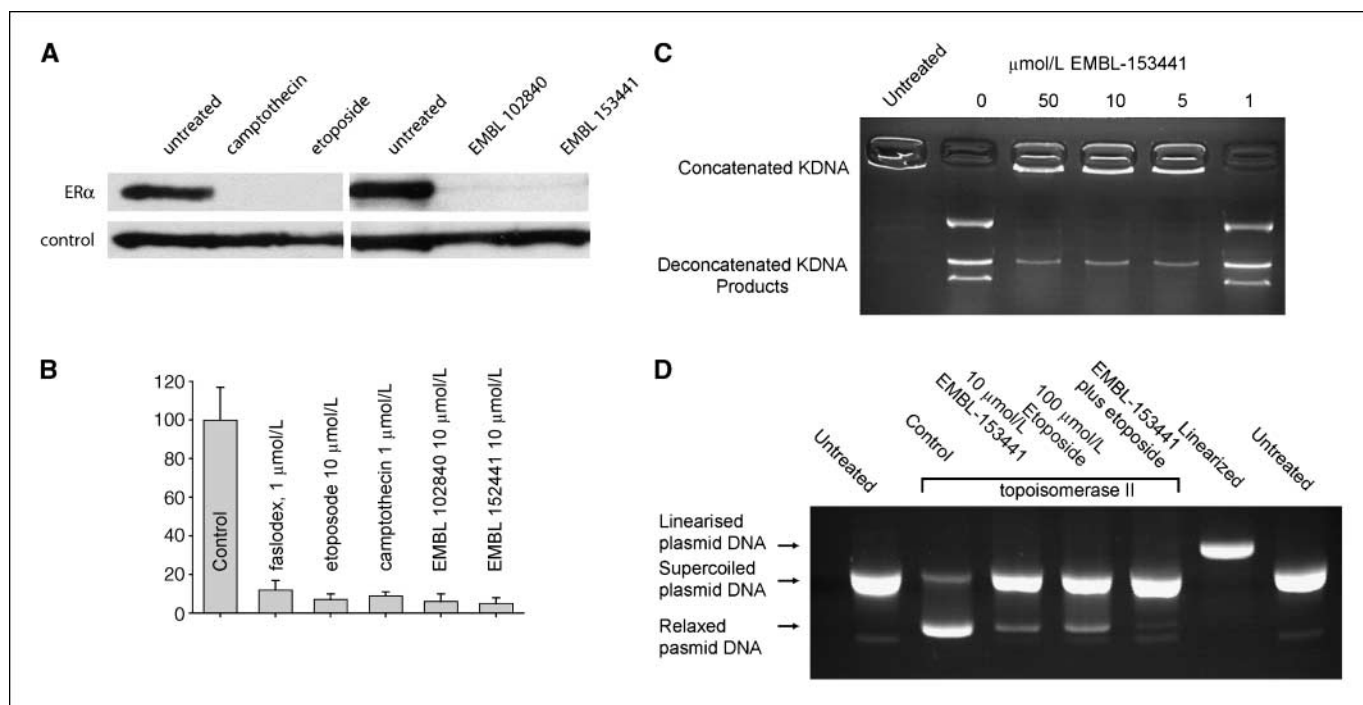


Figure 1. Inhibitors of topoisomerase I and II and the nitrofurans identified induced clearance of ER α protein from MCF-7 cells and inhibit topoisomerase II activity *in vitro*. Western blot analysis of ER α protein levels in MCF-7 cells shows that 16 h of treatment with an inhibitor of topoisomerase I activity (camptothecin; 1 μ mol/L) and topoisomerase II activity (etoposide phosphate; 20 μ mol/L) or with the nitrofurans EMBL-102840 and EMBL-153441 (10 μ mol/L) results in complete loss of ER α protein (A). Incubation of cells with the pure antiestrogen faslodex, topoisomerase inhibitors, or with EMBL-102840 or EMBL-153441 also compromises estrogen signaling in a cell-based reporter assay where luciferase expression is under control of an estrogen-responsive promoter (B). Topoisomerase II activity, through introducing dsDNA breaks, has the ability to resolve concatenated kinetoplast DNA. This process is inhibited by micromolar concentrations of EMBL-153441 (C). This effect is also seen in a plasmid relaxation assay (D), where ~10-fold more etoposide is required to produce inhibition of topoisomerase II than EMBL-153441. Moreover, some evidence of cooperativity between etoposide and EMBL-153441 on coaddition is apparent (D).

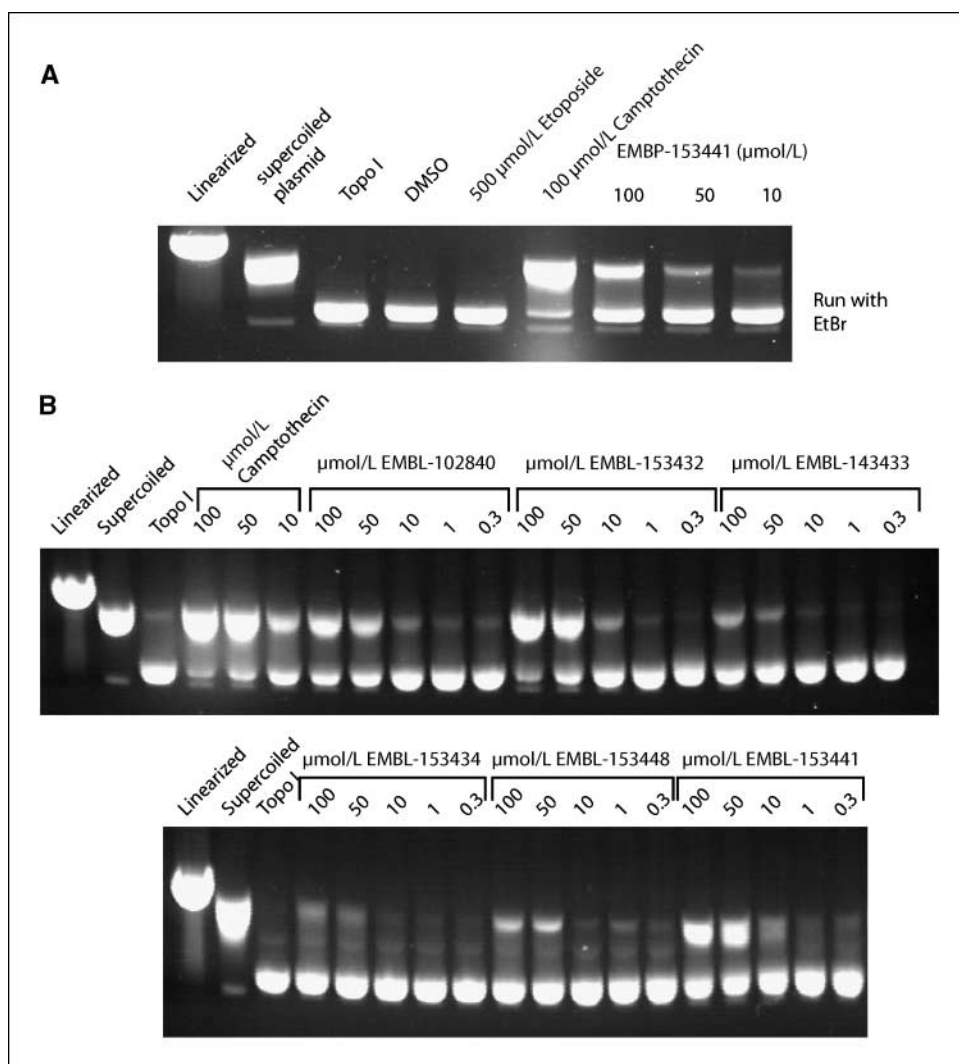


Figure 2. Nitrofurans also inhibit topoisomerase I activity *in vitro*. Topoisomerase I activity was evaluated in a plasmid relaxation assay, using camptothecin as a known inhibitor. *A*, whereas the topoisomerase II inhibitor etoposide does not inhibit topoisomerase I, the nitrofuran EMBL-153441 does inhibit, albeit with >10-fold less activity than the inhibition of topoisomerase II. *B*, the activity of all six nitrofurans identified in the screen in inhibiting topoisomerase I activity. The ranking of inhibition of each compound in inhibition of estrogen signaling and topoisomerase I and topoisomerase II activity is identical in all three assays.

inhibition of topoisomerase II is generally concordant with its cell-based activity in inhibiting estrogen signaling or in cellular cytotoxicity; in particular, EMBL-153441 is ~10-fold more active than etoposide.

We also evaluated the ability of EMBL-153441 to inhibit topoisomerase I activity, using a plasmid relaxation assay and camptothecin as a positive control. As shown in Fig. 2, EMBL-153441 does inhibit topoisomerase I activity, albeit with ~10-fold less activity than the *in vitro* inhibition of topoisomerase II. Additionally, four of the other five nitrofurans discovered in the screen also inhibit topoisomerase I activity, generally in the 10 to 50 µmol/L range (Fig. 2).

We then characterized the effect that EMBL-153441 had on the distribution of cells within the cell cycle and found that EMBL-153441 induces a G₁-G₀ cell cycle arrest, in contrast to etoposide, which induces arrest in the G₂-M phase of the cell cycle (Fig. 3A). DNA repair machinery will attempt to repair etoposide-induced dsDNA damage, through an initial recruitment of histone H2AX followed by its subsequent phosphorylation on Ser¹³⁹ (15). We used this effect as a surrogate marker to ascertain if EMBL-153441, like etoposide, induces DNA damage. As shown in Fig. 3B, EMBL-153441, in contrast to etoposide, does not induce phosphorylation of histone H2AX. Collectively, arrest in G₀-G₁

and lack of observable DNA damage suggest that EMBL-153441 has a different mechanism of action on topoisomerase II than that of etoposide. By implication, this suggests that etoposide and EMBL-153441 may synergize with each other, if each target different sites on topoisomerase II. We first determined dose concentration curves for both compounds and used these to simulate the result of an additive effect on the inhibition of cell growth (Fig. 3C, *left*). The observed result indicates that the extent of inhibition induced by concomitant addition of etoposide phosphate and EMBL-153441 is greater than that anticipated for a purely additive effective, with a modest synergy occurring, with each compound increasing the activity of the other by ~3-fold (Fig. 3C, *right*).

Resistance to etoposide, with concomitant cross-resistance to a range of cytotoxic agents, including the anthracyclines, epipodophyllotoxins and *Vinca* alkaloids, is a frequent clinical outcome to treatment (16). Two classes of proteins, P-glycoprotein and multidrug resistance protein (MRP), members of the ATP binding cassette transporter superfamily, are predominantly responsible for these effects. We used a matched pair of cell lines (11), to evaluate if EMBL-153441 is a substrate for MRP-1 action. As shown in Fig. 4, although expression of MRP-1 induces an ~10-fold increase in resistance to etoposide, it does not induce significant resistance

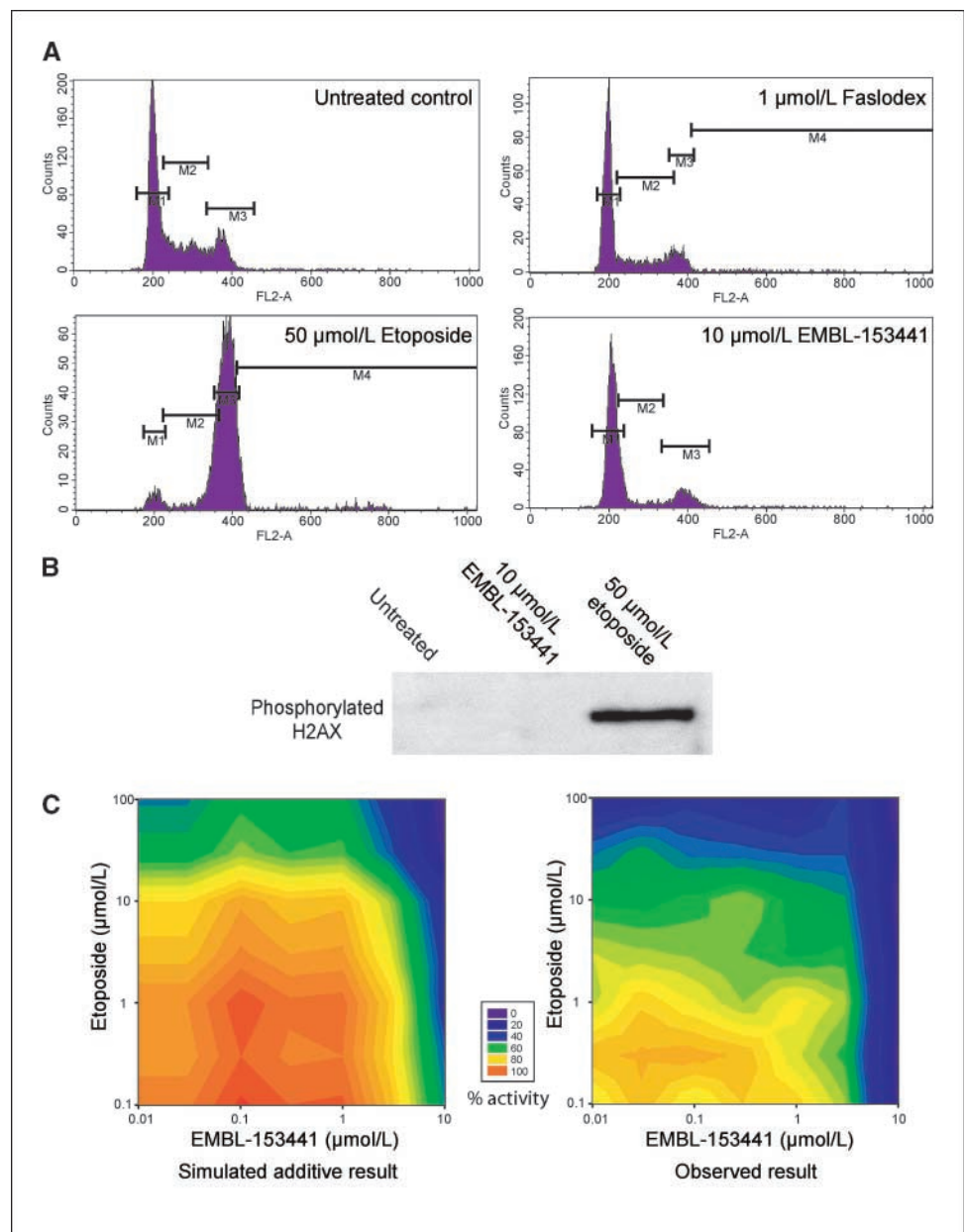
to EMBL-153441, indicating that an increase in MRP-1 expression does not compromise cell killing induced by EMBL-143441.

We then addressed the capability of EMBL-153441 to inhibit the growth of human tumors maintained in nude mice, both in an *ex vivo* colony-forming assay and *in vivo*. Xenografts, as described previously (17), were selected to represent a range of human tumor types. EMBL-153441 and etoposide phosphate were profiled for dose responsiveness against nine human tumor xenografts as follows: colon cancer, CXF 280; non-small cell lung cancers, LXFE 1422 and LXFL 529; small cell lung cancer, LXFS 650; mammary cancers, MAXF 1322, MAXF 401, and MAXF 857; ovarian cancer, OVXF 1353; and renal cancer, RXF 944LX. Also tested was the response of human hematopoietic stem cells, obtained from umbilical cord blood, to both compounds. The average concentration required to inhibit colony formation in this panel of cell types by 50%, 70%, and 90% are shown in Table 2. EMBL-153441 is ~3-

fold more active than etoposide in inhibiting the growth of this panel of cells. Moreover, the difference between the average efficacy of EMBL-153441 and etoposide phosphate increases to ~4-fold (4.0 versus 14.6 $\mu\text{mol/L}$ GI_{70} s), when hematopoietic cells are excluded from the data set.

The individual response of each cell type is presented on a logarithmic scale relative to the mean inhibition (GI_{50}) of the test panel (Fig. 5A). In this analysis, more sensitive cell types have bars to the left of the mean value, with bars to the right indicative of cell types more resistant to that of average. Mean graph analysis can be used to generate a fingerprint of the antiproliferative profile of a compound. Although the profiles for both compounds are generally concordant, specific differences do occur. Notably, hematopoietic stem cells are, in relative terms, ~30-fold more sensitive to etoposide phosphate than to EMBL-153441. We selected the most sensitive cell type, LXFS 650, derived from a small cell lung

Figure 3. EMBL-153441 induces an accumulation of cells in the $\text{G}_0\text{-G}_1$ phase of the cell cycle, does not induce double-stranded breaks in DNA, and shows moderate synergy with etoposide phosphate in inhibiting cell growth. MCF-7 cells were treated for 16 h with either DMSO solvent control, 1 $\mu\text{mol/L}$ faslodex (a potent inhibitor of estrogen signaling that binds to the $\text{ER}\alpha$), 50 $\mu\text{mol/L}$ etoposide phosphate, or 10 $\mu\text{mol/L}$ EMBL-153441. Cells were taken into suspension, fixed, permeabilized, and treated with RNase, and their DNA was stained with diamidinopropidium iodide. The stained suspension was then subjected to analysis by flow cytometry, with 10,000 events recorded for each condition. As expected, faslodex induces an increase in the proportion of cells in the $\text{G}_0\text{-G}_1$ phase of the cell cycle, with etoposide phosphate inducing an accumulation of cells in $\text{G}_2\text{-M}$. EMBL-153441 induces a profound $\text{G}_0\text{-G}_1$ arrest, with a considerable reduction in cells in S phase (A). We then compared, by Western blot analysis, the induction of phosphorylated H2AX, a marker of dsDNA breaks, on treatment of MCF-7 cells for 16 h with DMSO solvent control, etoposide phosphate (50 $\mu\text{mol/L}$), or EMBL-153441 (10 $\mu\text{mol/L}$). Only etoposide induced H2AX phosphorylation, indicating that at doses that inhibit estrogen signaling and cell growth, EMBL-153441 does not induce DNA damage (B). We then evaluated if synergy occurs between etoposide phosphate and EMBL-153441. Independent dose response curves were determined for etoposide phosphate and for EMBL-153441. These were used to simulate a purely additive effect (left) of adding both drugs to MCF-7 cells. This is compared with the actual results (right) obtained in a checkerboard experiment that estimated the inhibition of cell growth obtained with combinations of etoposide phosphate and EMBL-153441 (C). There is a clear, although modest, synergy between etoposide phosphate and EMBL-153441, where each enhances the inhibitory effect of the other by ~3-fold.



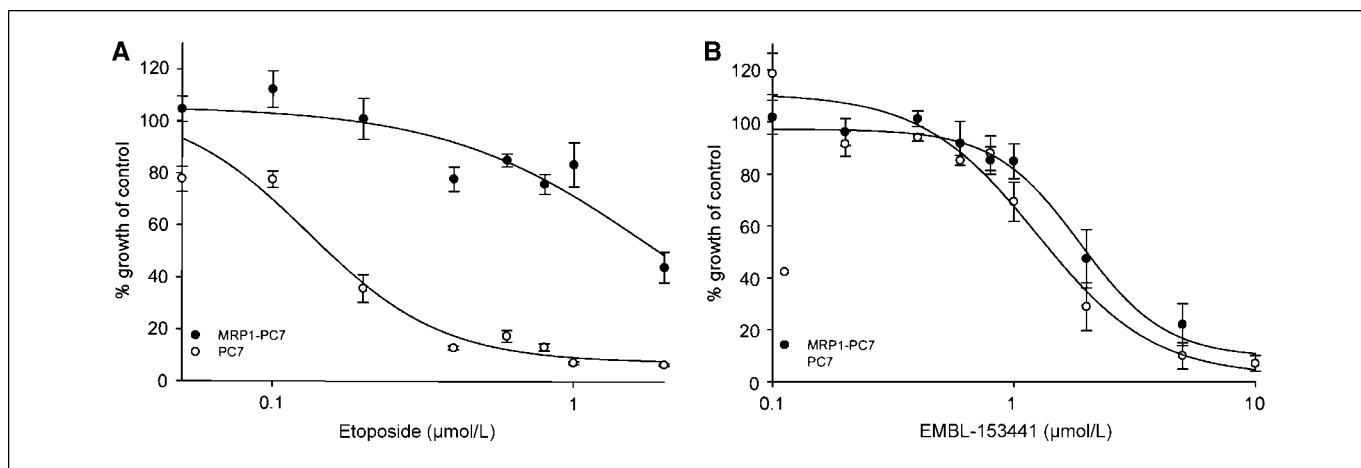


Figure 4. EMBL-153441 is not subject to cellular resistance by MRP-1. A matched pair of human embryonic kidney (HEK) cells, parental and a stable cell line expressing MRP-1, were used to determine if EMBL-153441 is subject to cellular export by MRP-1. Although MRP-1 overexpression decreases the sensitivity of HEK cells to etoposide (A), as determined by mitochondrial reduction of MTT, it does not alter sensitivity to EMBL-153441 (B), indicating that this later compound is not a substrate for MRP-1 export.

carcinoma tumor, to study in a nude mouse/human tumor xenograft model. Before commencing these studies, a series of doses of EMBL-153441 was administered by the i.p. route to mice to ascertain a maximum tolerated dose (MTD), defined as the dose that resulted in a 10% loss of body weight. The MTD was found to be 100 mg/kg. As shown in Fig. 4B, despite having an *ex vivo* IC₉₀ of ~120 nmol/L against the LXFS 650 tumor in the colony-forming assay, only moderate antitumor activity was obtained with EMBL-153441 administered daily by i.p. at a dose of 100 mg/kg. This result, in conjunction with the high MTD of EMBL-153441, suggests that optimization of the physicochemical variables, especially to improve aqueous solubility, is necessary to obtain improved activity.

Discussion

We report the discovery of a novel class of topoisomerase II inhibitor, linear nitrofurans, which inhibit topoisomerase II activity *in vitro* with IC₅₀ values in the low micromolar range. These compounds are generally cytotoxic to cells and seem to inhibit topoisomerase II through a mechanism distinct from etoposide. We have named this class of compound thanatop, from the Greek word *thanatos* (death) and *top* (from topoisomerase).

Etoposide, a semisynthetic derivative of podophyllotoxin isolated from the American mandrake (*Podophyllum peltatum*), has been in clinical use for more than two decades. Etoposide inhibits topoisomerase II α and topoisomerase II β late in the catalytic cycle, when the enzyme acts to religate the dsDNA break that permits changes in the topological status of DNA. Consequently, topoisomerase II inhibited by etoposide functions to allow DNA replication to occur but provokes a mitotic crash in late G₂. Etoposide may primarily kill cells by stabilizing a covalent enzyme-cleaved DNA complex, known as the cleavage complex. The accumulation of cleavage complexes in treated cells leads to the generation of permanent DNA strand breaks, which trigger recombination/repair pathways, mutagenesis, and chromosomal translocations. When such breaks overwhelm the cell, they initiate apoptotic pathways. In consequence, etoposide converts topoisomerase II from an essential enzyme to a potent cellular toxin that fragments the genome. Chromosomal rearrangements involving the breakpoint cluster region of the *mixed lineage leukemia*

(*MLL*) gene on chromosome 11q23 are a hallmark of therapy-related acute myeloid leukemias that are induced by etoposide (18). Etoposide-induced acute myelogenous leukemia, usually involving the breakpoint cluster region of the *MLL* gene on chromosome 11q23, is a severe consequence of therapy that occurs in 2% to 12% of patients (19). Other major adverse effects of treatment with etoposide are myelosuppression and cardiotoxicity.

Nitrofurans have a long and somewhat checkered history as antibiotics in human therapy. Although they undoubtedly have immense therapeutic value as broad spectrum antimicrobial agents (20), with resistance to nitrofurans being an infrequent occurrence, nitrofurans are now regarded as being sufficiently mutagenic to preclude their use in human treatment for infections or in the treatment of animals in the human food supply (21). The generally accepted mechanism of action of nitrofurans as antibiotics is that they are reduced by bacterial nitroreductases to highly reactive metabolites that then covalently react with and poison essential components of the bacterial cellular machinery (22, 23). We therefore wished to determine if the nitrofurans identified as inhibitors of estrogen signaling did so specifically; three nitrofurans (i.e., EMBL-153448, furaltadone, and nitrofurantoin) had relatively poor or no inhibition of estrogen signaling

Table 2. The average effect that EMBL-153441 and etoposide phosphate have on the panel of nine tumors maintained *ex vivo* in nude mice and on hematopoietic stem cells to inhibit growth by 50%, 70%, and by 90%

Compound	Inhibition of colony formation ($\mu\text{mol/L}$)		
	Mean IC ₅₀	Mean IC ₇₀	Mean IC ₉₀
EMBL-153441	0.43	1.13	4.04
Etoposide phosphate	1.15	3.37	7.43

NOTE: EMBL-153441 is ~3-fold more active than etoposide phosphate.

(Table 1) and also did not inhibit human topoisomerase I or II activity at 100 $\mu\text{mol/L}$ (data not shown), indicating that the nitrofuran group alone is insufficient to inhibit topoisomerase activity. In addition, activity was seen *in vitro* using purified topoisomerases under conditions where reduction of the nitrofuran is unlikely. From these observations, we conclude that the active nitrofurans specifically inhibit topoisomerase II, although we cannot currently preclude that they act on additional targets. Although the nitrofuran moiety is a known mutagen, it may be that, as the nitrofurans do not directly induce dsDNA breaks, they may be less prone to provoke treatment-induced leukemias than etoposide. Given the severity of most cancers and their high unmet clinical need, nitrofurans may remain suitable therapeutic agents for cancer if sufficient activity and pharmacologic properties can be attained.

Nitrofurans have been described previously as antiproliferative agents (24, 25), although the mechanism of action was not demonstrated. Both articles report cytotoxic effects on cells that are of the same order as those reported in this article. Inhibition of growth in an *in vivo* mouse i.p. tumor/i.p. challenge model was also observed, with cell growth transiently inhibited following a single administration of nitrofuran. Additionally, 5-nitrofuran derivatives of fatty acid hydrazides induce a G_0 - G_1 arrest in the cell cycle of human myeloid leukemic cell lines (25). One plausible explanation for an accumulation of cells in G_1 , reflecting the difference in the mechanistic action of the nitrofurans and etoposide, is that nitrofurans prevent DNA replication through debilitating topoisomerase II activity early in the catalytic cycle. Cells may then proceed through the cell cycle until halted by a block in DNA replication. This possibility is consistent with the profound suppression of cells in S phase when treated with EMBL-153441 (Fig. 3A).

EMBL-153441 inhibited the panel of human cell lines evaluated with ~ 3 -fold greater activity at the IC_{70} value than etoposide

phosphate. Strikingly, and in contrast to etoposide phosphate, human hematopoietic stem cells were, compared with the most sensitive cell types, relatively nonsensitive to EMBL-153441. In absolute terms, etoposide phosphate was ~ 30 -fold more toxic to human hematopoietic stem cells than EMBL-153441. *In vivo* profiling of EMBL-153441 in a nude mouse xenograft model, with the tumor placed subdermal between the scapulae, was challenged by once daily dosing at 100 mg/kg and showed a moderate reduction in tumor volume over a 14-day period. Given the sensitivity of the tumor in the colony-forming assay (GI_{90} of 120 nmol/L), this result indicates that the administered compound had poor access to the tumor. In particular, EMBL-153444 has very limited solubility ($\sim 25 \mu\text{g/mL}$ in PBS; data not shown) and had to be administered i.p. using DMSO as solvent carrier. Further optimization of this compound series will concentrate on modifying the physicochemical properties to enhance potency, but in particular, their drug-like characteristics, to ensure an increase in the systemic distribution, availability, and persistence of compound. This may be entirely feasible, as it seems that considerable variation at the end of the molecule that opposes the nitrofuran group can be tolerated without loss of potency (data not shown).

Primary lung tumors (small cell and non-small cell lung adenocarcinomas) are often characterized by preliminary chemosensitivity, with initial patient response rates $>80\%$ achieved with combination chemotherapy; however, long-term survival is very poor, with 5-year survival rates between 5% to 10% (26). The consistent and rapid emergence of drug resistance in lung cancer compromises treatment, which can be overcome by dose escalation, to 5- to 10-fold higher doses. At such chemotherapeutic intensity, a deleterious effect on hematopoiesis becomes limiting and requires supportive intervention, such as autologous bone marrow transplantation. Additionally, suitable combinations of non-cross-resistant agents may preclude resistance. Such principles of elevated dosing

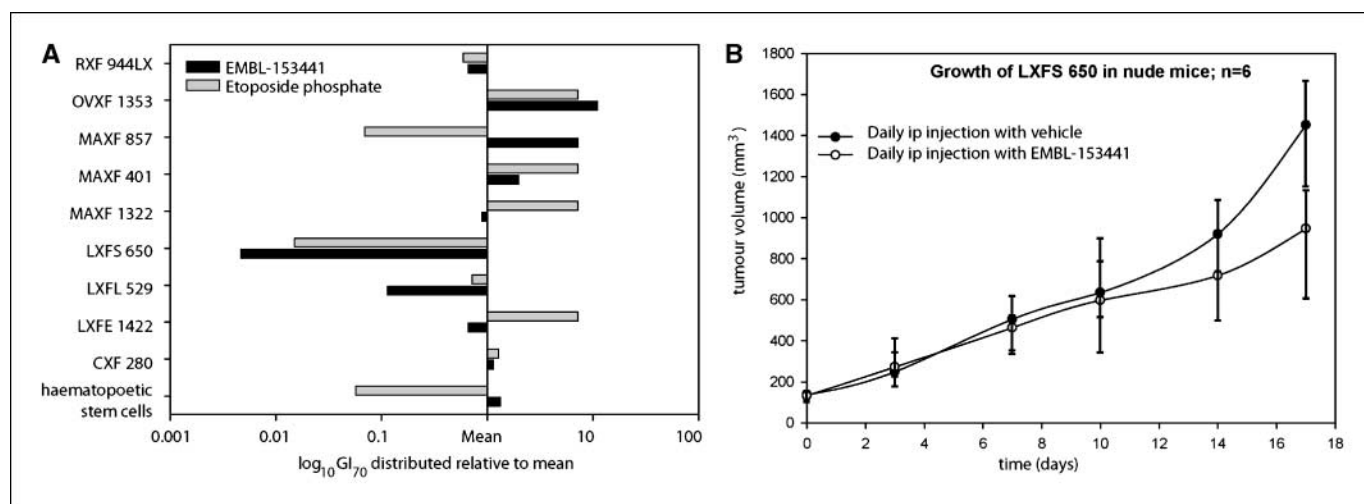


Figure 5. EMBL-153441 inhibits the growth of human tumors in colony-forming assays and in a nude mouse xenograft model. The effect of EMBL-153441, compared with etoposide phosphate, on an *ex vivo* panel of human tumors was determined. A panel of nine human tumors was maintained as xenografts in nude mice, which were excised, disaggregated, and used to seed plates where the medium was immobilized with 0.75% agar. Colonies, arising from the tumor stem cell population, were allowed to grow to a diameter of $>50 \mu\text{m}$, after which they were stained and counted. Normal human hematopoietic stem cells, obtained from cord blood, were used to indicate the myelosuppressive effect of test compounds. Mean graph analysis of the individual cell types under evaluation show that EMBL-153441 and etoposide phosphate have a generally similar response profile, with primary lung tumors most sensitive to both compounds (A). Human hematopoietic stem cells are ~ 30 -fold more sensitive to etoposide phosphate than to EMBL-153441. We then evaluated the most sensitive tumor, LXFS 650, in a xenograft/nude mouse model. Fragments of tumor were implanted s.c. between the scapulae and allowed to grow to a volume of $\sim 100 \text{mm}^3$, after which daily i.p. administration of EMBL-153441 at 100 mg/kg in DMSO, or solvent control, was initiated. A modest reduction in tumor growth was seen with the nitrofuran EMBL-153441; Points, tumor volume; bars, 95% values of variation within each data set (B).

and combination chemotherapy, established in laboratory models, have resulted in the design of effective regimes for the treatment of several tumor types (26).

In light of these considerations, particularly as EMBL-153441 is not subject to cellular resistance through MRP-1, the nitrofurans class of topoisomerase inhibitor has potential in the rational combination chemotherapeutic regimes. In particular, use with etoposide merits further consideration. Etoposide and the nitrofurans inhibit topoisomerase II through different mechanisms. This is reflected in the synergy that exists between both compounds in cell killing. Although the effect is modest, at ~3-fold, it is greater than an additive effect and would be significant if translated into the clinic.

The thanatops are a new class of antiproliferative agent that are synthetically tractable, which target topoisomerase II, and, within

the limited range of compounds identified, also target topoisomerase I, albeit to a lesser extent. The synthetic amenability of the nitrofurans suggests that further improvements in terms of potency, drug-like characteristics, and selectivity toward topoisomerase I or II could be achieved to develop potential clinical candidates.

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