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12-Substituted 2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridines as novel topoisomerase I-targeting antitumor agents

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ABSTRACT

2,3-Dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine and a few of its 12-substituted analogs are active as TOP1-targeting agents. Studies were performed to further evaluate the potential of this series of non-camptothecin TOP1-targeting agents. The influence of a hydroxymethyl, formyl, *N,N*-dimethylaminomethyl, 2-(*N,N*-dimethylamino)ethyl, 3-(*N,N*-dimethylamino)propyl), and 4-(*N,N*-dimethylamino)butyl substituent at the 12-position on TOP1-targeting activity and tumor cell growth was evaluated. In addition, the relative pharmacologic activities of the 12-carboxamide analog, as well as its *N*-methyl and *N,N*-dimethyl derivatives were assessed.

1. Introduction

Topoisomerases participate in processes such as DNA replication, repair, transcription, and recombination as well as chromosome condensation and segregation.[1,2] Topoisomerase I (TOP1) is the target of several antitumor agents that act by stabilizing the enzyme–DNA cleavage complex, which results in DNA damage and ultimately cell death.[3,4] Camptothecin (CPT) was the first compound identified as a TOP1-targeting agent.[5] Two clinical TOP1-targeting agents, topotecan (Hycamtin®) and irinotecan (CPT-11/Camptosar®) have since been developed. The improved water-solubility of topotecan and irinotecan relative to CPT was critical to their development into the clinic. These agents have incorporated, within their structure, the core structure of camptothecin, which includes a δ-lactone. This lactone moiety is susceptible to hydrolysis and the resulting carboxylic acid has a high affinity for human serum albumin.[6–8] In addition, it is known that both of these clinical agents are susceptible to transporter-mediated cellular efflux, which can limit intracellular accumulation and has been associated with multidrug resistance. Specifically over expression of MDR1 (P-glycoprotein) and breast cancer resistance protein (BCRP) have been associated with resistance to these camptothecins.[9–15] In view of these observations, non-camptothecin TOP1-targeting agents have been investigated for their potential to overcome these obstacles which could limit the effective drug concentration within certain tumor types.[16]

Several 5-(2-aminoethyl)dibenzo[*c,h*][1,6]naphthyridin-6-ones have been identified as exceptionally active topoisomerase I-targeting agents with potent antitumor activity.[16,17] One of the more extensively studied of these non-camptothecin TOP1-targeting agents is 5*H*-8,9-dimethoxy-5-(2-*N,N*-dimethylaminoethyl)-2,3-methylenedioxydibenzo[*c,h*][1,6]naphthyridin-6-one (ARC-111), **1** (Fig. 1).[18,19] The 11-aza analog of ARC-111, 11-2[2-*N,N*-dimethylaminoethyl]-2,3-dimethoxy-8,9-methylenedioxy-11*H*-isoquinolin[4,3-*c*]cinnolin-12-one **2** (Fig. 1) and related compounds have also been identified as potent TOP1-targeting agents.[16,20] Analogs within both of these series of compounds have proved to be active as antitumor agents in vivo when administered by gavage or parenterally to tumor-bearing mice.[21,22] The reversed lactam of ARC-111, **3** (Fig. 1) represents a third series of non-camptothecin TOP1-targeting agents consisting of various 6-substituted 6*H*-8,9-dimethoxy-6-(2-*N,N*-dimethylaminoethyl)-2,3-methylenedioxydibenzo[*c,h*][2,6]naphthyridin-5-ones. Several analogs within this series of compounds have shown exceptional TOP1-targeting activity and potent cytotoxic activity.[23,24] Earlier studies did indicate that appropriately substituted benzo[*i*]phenanthridine derivatives could also exhibit TOP1-targeting activity.[25,26] Only limited studies were performed on these benzo[*i*]phenathridines in light of their limited solubility and difficulties associated with their formulation for assessment of biological activity. Recently, it has been demonstrated that 12-carboxamide derivatives of benzo[*i*]phenan-

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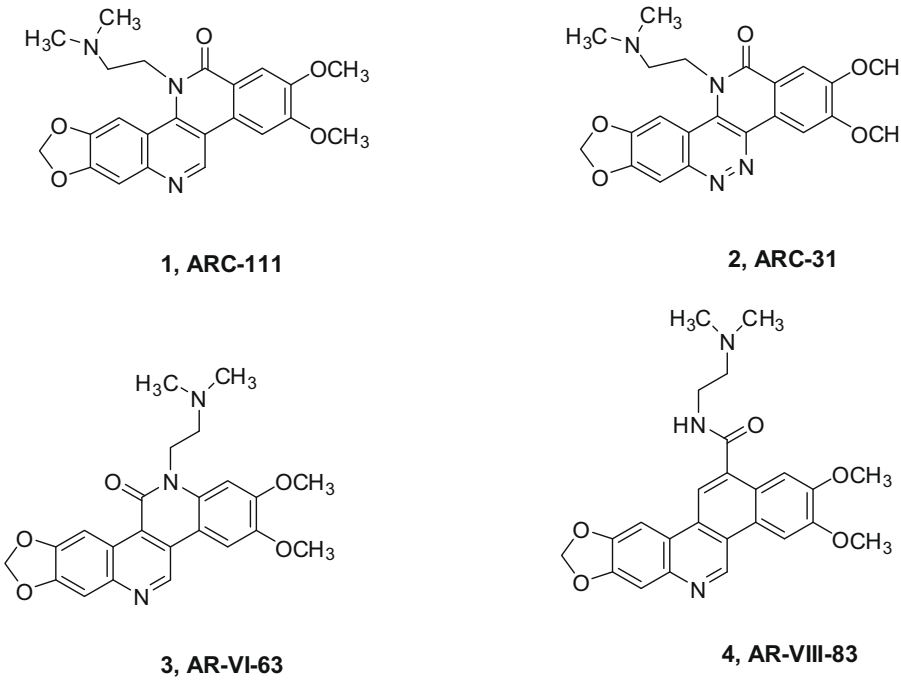


Figure 1. Structure of ARC-111 and other structurally similar non-campothtecin topoisomerase I-targeting agents.

thridine also have the potential to be further developed as an additional series of non-camptothecin TOP1-targeting agents.[23] The potential of these compounds as TOP1-targeting agents was made apparent by the biological activities associated with **4**.[23,27] The presence of the aminoalkyl group attached to the 12-carboxamide provided functionality in the case of **4** significantly improved the hydrophilic property of this compound. These results rekindled our interest in exploring whether similarly substituted benzo[*i*]phenanthridines, particularly those with polar substituents at the 12-position, would exhibit similar biological activity. Specifically targeted for synthesis and biological evaluation were a series of 12-substituted 2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridines.

2. Results and discussion

2.1. Synthesis

The synthetic approach for the preparation of the 12-(*N*,*N*-dimethylamino)methyl analogue of 2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine is outlined in Scheme 1. The synthesis of **5** was accomplished as previously described by photocyclization of 3-(6,7-methylenedioxyquinolin-4-yl)-2-(2-iodo-4,5-dimethoxyphenyl)acrylic acid ethyl ester.[23,27] Compound **5** was reduced to corresponding benzyl alcohol **6** using LAH. Oxidation of **6** with MnO_2 afforded **7** cleanly. Treatment of **7** with dimethylamine followed by reduction with triacetyloxyborohydride at 0 °C provided **8** in good yield.

Methods used for the preparation of the 12-(2-aminoethyl)- and 12-[(2-*N*,*N*-dimethylamino)ethyl]- benzo[*i*]phenanthridine derivatives are outlined in Scheme 2. Condensation of **7** with nitromethane gave **9** in 75% yield. Compound **9** was first reduced to **10** using $NaBH_4$, which was then further reduced to the primary amine **11** using Zn/AcOH. N,N-Dimethylation of **11** was accomplished using formalin in the presence of $NaBH_3CN$ to provide **12** in 61% yield.

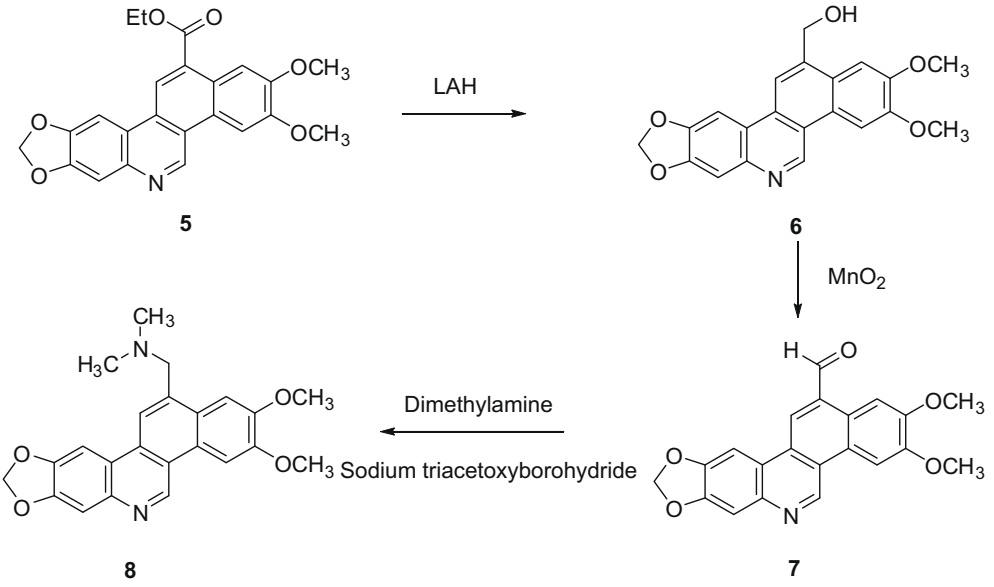
The ethyl ester **5** was converted to its carboxylic acid derivative **13**, which served as the intermediate for the preparation of the 12-carboxamides **14–16.** This carboxylic acid was converted to its acid chloride, which was used without further purification and treated with either ammonia or the appropriate alkylamine to provide **14–16** (see Scheme 3).

The synthetic methods used for the preparation of the 12-[3-(*N*,*N*-dimethylamino)propyl] and 12-[4-(*N*,*N*-dimethylamino)butyl] benzo[*i*]phenanthridine derivatives are outlined in Scheme 4. Wittig reaction with **7** and 2-(dimethylaminomethyl)triphenylphosphonium bromide in THF in the presence of LiHMDS provided the 12-(3-dimethylamino)prop-1-enyl derivative **17**, which was converted to **18** in the presence of 5% Pd/C and hydrogen. In a similar manner **7** was treated with 3-(dimethylamino)propyltriphenylphosponium bromide in the presence of LiHMDS to give the 12-(4-dimethylamino)but-1-enyl derivative **19**, which was hydrogenated to give **20**. Treatment of **7** with freshly prepared dimethylaminoethyl magnesium chloride provided the 12-(4-dimethylamino-1-hydroxy)butyl derivative **21**.

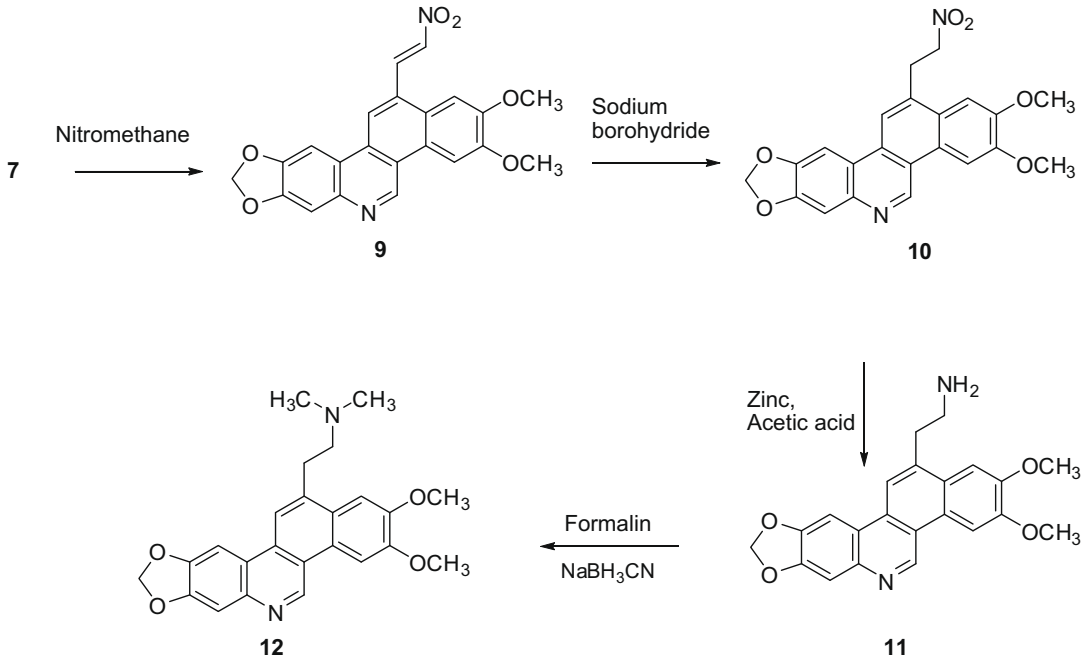
Compound **22** was prepared as illustrated in Scheme 5 by treatment of **7** with the appropriate ethylenediamine followed by reduction with sodium cyanoborohydride.

2.2. TOP1-targeting activity and cytotoxicity

The relative TOP1-targeting activities and the results of cellular assays of these 12-substituted 2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridines in both RPMI8402 and P388, as well as their respective camptothecin resistant variants, CPT-K5 and P388/CPT-45 are provided in Table 1. The poor solubility of the carboxylic acid derivative **13** prevented an assessment of its biological activity and the limited solubility of the carboxamide **14** likely compromised efforts to obtain an accurate determination of its relative activity in these in vitro assays. Four of the benzo[*i*]phenanthridine derivatives targeted in this study for synthesis and biological evaluation, **6**, **11**, **12**, and **20**, had TOP1-targeting activity comparable to CPT.



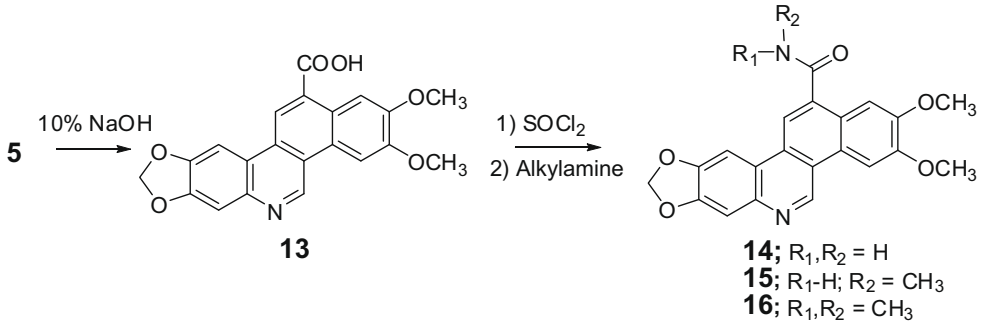
Scheme 1. Preparation of the 12-hydroxymethyl-, 12-formyl-, and 12-(*N*,*N*-dimethylamino)methyl- analogues of 2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine.



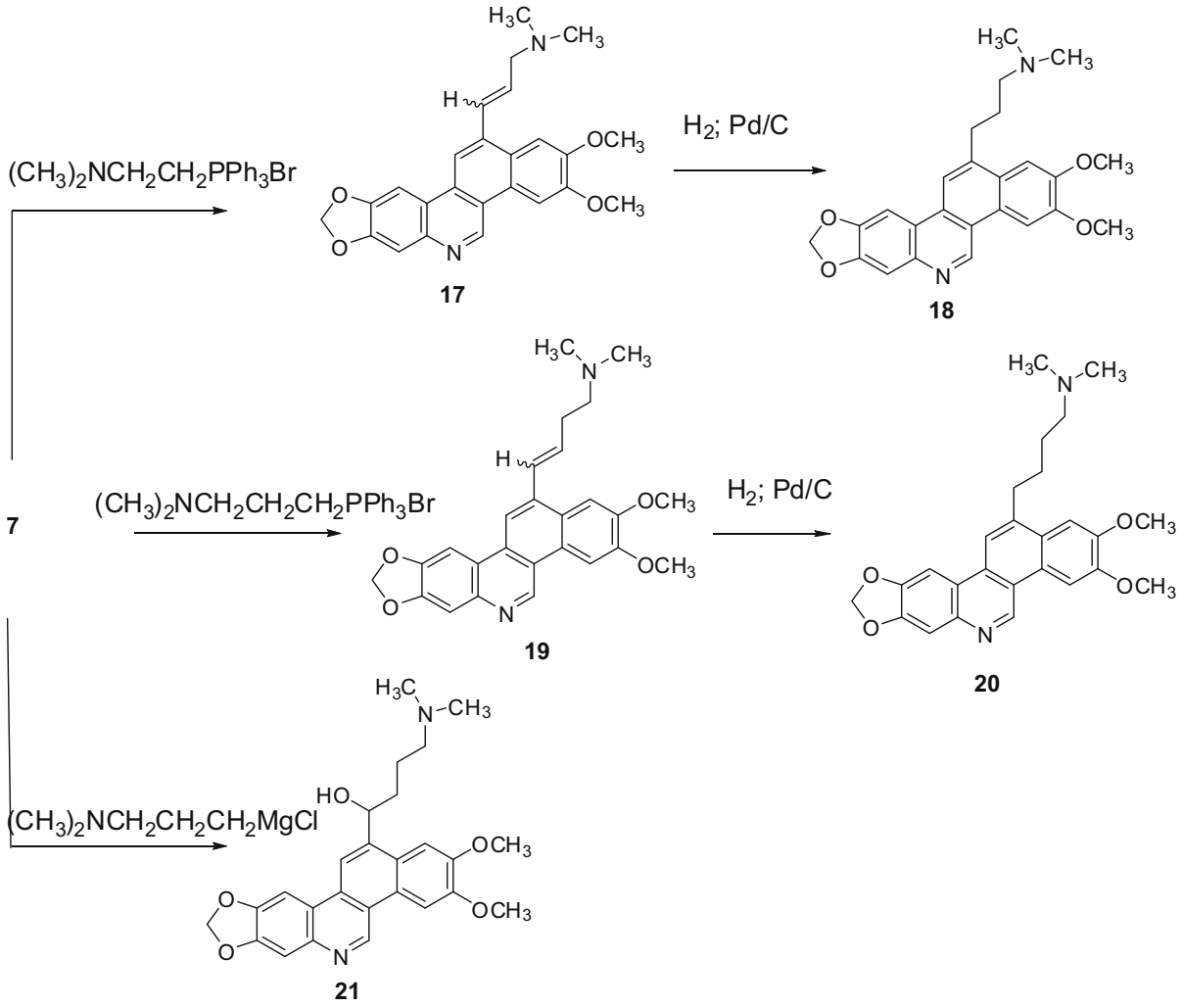
Scheme 2. Preparation of 12-(2-nitroethenyl)-, 12-(2-nitroethyl)-, 12-(2-aminoethyl)- and 12-(*N*,*N*-dimethylaminoethyl)- analogues of 2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine.

All of these compounds, however, were at least an order of magnitude less potent when evaluated for cytotoxic activity in RPMI8402 or P388 cells. Camptothecin resistant variants have been established for both of these cell lines. CPT-K5 is the variant of RPMI8402 cells, wherein a mutant form of TOP1 has been attributed to its camptothecin resistance.[28] P388/CPT45 is the variant of P388. The lack of expression of TOP1 has been associated with the resistance to camptothecin of P388/CPT45 cells relative to its parent cell line.[29] Cross-resistance to these cell lines by a cytotoxic agent can be indicative of TOP1 as a principal target associated with its cytotoxicity in the MTT assay. In marked contrast to **1** (ARC-111) as well as **3** and **4**, several of the benzo[*i*]phenanthridines synthesized and evaluated in this study did not exhibit cross-resistance to these camptothecin-resistant cell lines. Only the 12-(2-aminoethyl) and the 12-[2-(*N*,*N*-dimethylamino)ethyl] derivatives, **11** and **12**, together with the 12-carboxamides **14–16** exhibited significant cross-resistance to both CPT-K5, as well as P388/CPT45 cells. It is of interest to note that all three 12-carboxamides clearly demonstrated significant cross-resistance, which is consistent to that previously observed with other 12-carboxamide derivatives.[27]

While **14–16** are not among the more potent TOP1-targeting agents, the comparative assay data clearly indicate that TOP1-targeting activity is associated with their activity in the MTT assay.

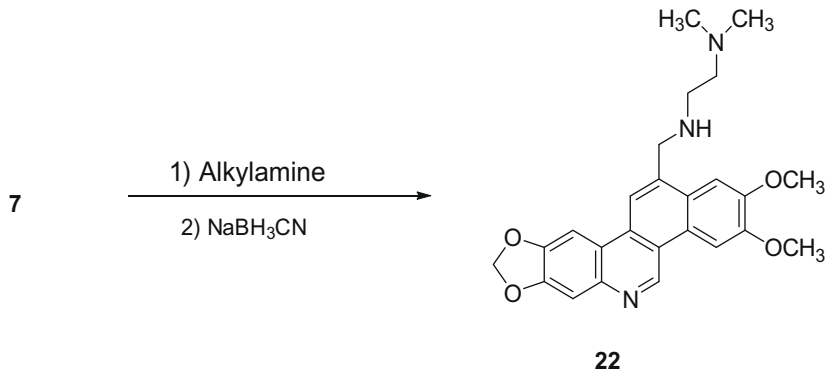


Scheme 3. Preparation of the carboxamide derivatives **14**–**16**.



Scheme 4. Preparation of 3- and 4-carbon linked *N*,*N*-dimethylalkylamines substituted at the 12-position of 2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine.

Based upon the extent of DNA cleavage with purified enzyme in the presence of these test compounds, several compounds, such as **6** and **20**, had similar potency to camptothecin as TOP1-targeting agents. The absence of significant cross-resistance with these cell lines, however, suggests that mechanisms other than TOP1-targeting activity are primarily responsible for MTT assay data for compounds 6–10 and **17–22**. The MTT assay cannot distinguish between a cytotoxic response and a growth inhibitory response. These substituted benzo[*i*]phenanthridines are planar molecules, unlike 5-substituted dibenzo[*c*,*h*][1,6]naphthyridin-6-one derivatives, which have substituents within the bay-region of these polycyclic molecules. Molecules that can intercalate within DNA may inhibit cell growth. With increased planarity, it is more likely that these derivatives could exhibit an enhanced potential for intercalation into DNA. It is possible, therefore, that their dominant effect for some of these analogs could be to inhibit cell growth. Cytotoxic activity mediated by stabilization of the TOP1–DNA cleavable complex, therefore, could be masked by their ability to inhibit cell growth through DNA intercalation. The most potent of the benzo[*i*]phenanthridines evaluated in this study using the MTT assay is **7**, which had relatively



Scheme 5. Preparation of **22**.

Table 1
Relative TOP1-targeting activity and cytotoxicity of **1**, **3** and various 12-substituted 2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridines

Compound	TOP1-mediated cleavage[a]	Cytotoxicity IC_{50} (μM)			
		RPMI8420	CPT-K5	P388	P388/CPT45
1	0.3	0.002	0.90	0.001	0.23
3	0.6	0.0007	0.21	0.002	0.015
4	0.1	0.003	1.0	0.003	0.32
6	0.25	0.03	0.03	0.03	0.03
7	4.7	0.003	0.003	0.002	0.002
8	1.1	0.033	0.033	0.005	0.015
9	9.2	0.033	0.035	0.025	0.025
10	7.7	0.38	0.30	0.28	0.29
11	0.3	0.025	0.25	0.016	0.30
12	0.5	0.03	0.45	0.03	0.30
14	0.8	4.0	>10	1.6	>10
15	1.2	0.04	>10	0.03	0.30
16	1.1	0.6	>10	0.48	>10
17	7.7	0.045	0.083	0.03	0.037
18	10	0.18	0.14	0.038	0.10
19	15	0.30	0.22	0.03	0.06
20	0.2	1.0	2.3	1.4	2.1
21	9.2	0.30	2.1	0.41	0.51
22	1.9	0.024	0.03	0.02	0.024
CPT	0.2	0.004	>10	0.004	>10

[a] Topoisomerase I cleavage values are reported as REC, relative effective concentration, these are concentrations relative to topotecan, whose value is arbitrarily assumed to be 1, that are able to produce 10% cleavage of the plasmid DNA in the presence of human topoisomerase I. CPT is five times more than topotecan in this assay and has a REC value of 0.2.

weak TOP1-targeting activity when compared to other 12-substituted 2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridines.

The cytotoxicity data for KB3-1 cells and for the variants KBV-1 and KBH5.0 are listed in Table 2. KBV-1 cells overexpress the efflux transporter MDR1,[30] and KBH5.0 cells overexpress the efflux transporter BCRP.[18] Topotecan and SN38 are substrates for the efflux transporters MDR1 and BCRP. Decreased cytotoxicity against KBV-1 cells relative to the parent cell line KB3-1 is indicative of substances that are substrates for the efflux transporter MDR1. Similarly, resistance to the cytotoxic effects of a substance observed in KBH5.0 cells relative to its parent cell line KB3-1 is indicative of a compound being a substrate for the BCRP efflux transporter. As was observed for **4**, several of these benzo[*i*]phenanthridine derivatives including **9**, **11**, **18**, **20** and **21** are substrates for MDR1 with relative resistance ranging from 6 to 44 based upon comparative IC_{50} values. The limited solubility of **14** precluded an accurate determination of its relative cytotoxicity in these in vitro assays. These data also indicated that none of the compounds evaluated were comparable to **4** as substrates for the BCRP efflux transporter.

These data indicate that select 12-substituted 2,3-dimethyoxy-8,9-methylenedioxybenzo[*i*] phenanthridine derivatives can have potent TOP1-targeting activity. Based upon TOP1-targeting activity and the results of our cellular assays, the 12-carboxamide **15** and the 12-(2-aminoethyl) derivatives **11** and **12** are among the more promising derivatives. Further studies are required in laboratory animals to evaluate the efficacy of these various 12-substituted benzo[*i*]phenathridines as antitumor agents.

2.3. Experimental

Melting points were determined with a Meltemp capillary melting point apparatus. Column chromatography refers to flash chromatography conducted on SiliTech 32–63 μm, (ICN Biomedicals, Eschwege, Ger.) using the solvent systems indicated. Infrared spectral data were obtained using a Thermo-Nicolet Avatar 360 Fourier transform spectrometer and are reported in cm^{-1}. Proton (^1H NMR) and carbon (^{13}C NMR) nuclear magnetic resonance were recorded on a Varian Gemini-200 Fourier transform spectrometer. NMR spectra (200 MHz ^1H and 50 MHz ^{13}C) were recorded in the deuterated solvent indicated with chemical shifts reported in δ units downfield from tetramethylsilane (TMS). Coupling constants are reported in Hertz (Hz). Mass spectra were obtained from Washington University Resource for Biomedical and Bio-organic Mass Spec-

Table 2
Cytotoxicity of **1**, **3** and various 12-substituted 2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridines

Compound	Cytotoxicity IC_{50} (μM)		
	KB3-1 (wt)	KBV-1 ($^{+}$MDR1)	KBH5.0 ($^{+}$BCRP)
1	0.005	0.002	0.006
3	0.003	0.004	0.004
4	0.005	0.22	0.06
6	0.045	0.075	0.08
7	0.004	0.016	0.019
8	0.03	0.17	0.06
9	0.03	1.78	0.04
10	0.30	0.44	0.49
11	0.05	0.5	0.20
12	0.025	0.08	0.07
14	1.5	>10	>10
15	0.031	0.10	0.08
16	0.60	0.70	0.38
17	0.16	0.37	0.29
18	0.04	0.5	0.18
19	0.32	0.4	0.32
20	0.4	4.0	0.95
21	0.17	1.9	0.55
22	0.025	0.027	0.038
TPT	0.04	0.44	0.44
SN38	0.004	0.046	0.30

trometry within the Department of Chemistry at Washington University, St. Louis, MO. All starting materials and reagents were purchased from Aldrich. Solvents were purchased from Fisher Scientific, and were A.C.S. grade or HPLC grade. Methylene chloride was freshly distilled from calcium hydride. All other solvents were used as provided without further purification.

2.3.1. 12-Hydroxymethyl-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (6)

A solution of **5** (0.55 mmol) in THF drop wise was added at 0 °C to a stirred solution of LAH (1.1 mmol) in THF (20 mL). The resulting reaction suspension was stirred for 2 h at 0 °C, and then carefully quenched by addition of water (0.05 mL), 15% NaOH (0.05 mL) and water (0.15 mL) sequentially. The resulting reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The residue was chromatographed using 10:1 $CHCl_3$/MeOH to provide a yellow powder in 55% yield; mp 267–269 °C; IR (KBr) 3448; ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 4.05 (s, 3H), 4.12 (s, 3H), 5.19 (s, 2H), 6.12 (s, 2H), 7.53 (s, 1H), 7.45 (s, 1H), 7.84 (s, 1H), 8.01 (s, 1H), 8.25 (s, 1H), 9.64 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 55.3, 55.7, 61.9, 99.2, 101.8, 103.0, 104.4, 106.3, 116.0, 119.6, 119.8, 124.7, 124.7, 129.4, 140.2, 141.7, 145.7, 147.8, 148.8, 148.9, 149.8; HRMS calcd for $C_{21}H_{17}NO_5H$: 364.1185; found 364.1179.

2.3.2. 12-Formyl-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]-phenanthridine (7)

A solution of **6** (1.0 mmol), MnO_2 (10 mmol) in DMF (30 mL) was stirred for 2 h at room temperature. The resulting reaction mixture was filtered through a Celite bed and filtrate was concentrated under reduced pressure. The residue was chromatographed using 20:1 CH_2Cl_2/MeOH to provide a brown powder in 74% yield; mp 260–263 °C; IR (KBr) 1685; ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 4.12 (s, 3H), 4.17 (s, 3H), 6.20 (s, 2H), 7.58 (s, 1H), 7.97 (s, 1H), 8.17 (s, 1H), 8.74 (s, 1H), 8.92 (s, 1H), 9.93 (s, 1H), 10.50 (s, 1H); HRMS calcd for $C_{21}H_{15}NO_5H$: 362.1028; found 362.1023.

2.3.3. 12-Dimethylaminomethyl-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (8)

To a solution of **7** (0.03 mmol) in anhydrous CH_2Cl_2 and DMF (5:1, 30 mL) was added dimethylamine (2 M in THF, 0.15 mmol) at room temperature. The resulting reaction solution was cooled to 0 °C and sodium triacetoxyborohydride (0.15 mmol) was added. After stirring at 0 °C for 5 min, AcOH (10 μL) was added. The reaction mixture was warmed up to room temperature overnight, and then quenched with water (0.1 mL). The resulting mixture was partitioned between sat $NaHCO_3$ and CH_2Cl_2. The organic layer was concentrated and the residue was chromatographed using 10:1 CH_2Cl_2/MeOH to provide a yellow powder in 60% yield; mp 210–216 °C; ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 2.39 (s, 6H), 3.94 (s, 2H), 4.09 (s, 3H), 4.15 (s, 3H), 6.16 (s, 2H), 7.56 (s, 1H), 7.83 (s, 1H), 7.95 (s, 1H), 8.16 (s, 1H), 8.20 (s, 1H), 9.86 (s, 1H); HRMS calcd for $C_{23}H_{22}N_2O_4H$: 391.1658; found 391.1653.

2.3.4. 12-(2-Nitrovinyl)-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (9)

A suspension of **7** (0.1 mmol), ammonium acetate (0.5 mmol) in nitromethane (2 mL) was stirred overnight at 80 °C. The resulting reaction mixture was triturated with a small amount of CH_2Cl_2 and the residue was pure enough and used for the next reaction without further purification; yield: 75%; mp 250–253 °C; IR (KBr) 1509; ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 4.10 (s, 3H), 4.16 (s, 3H), 6.18 (s, 2H), 7.39 (s, 1H), 7.53 (s, 1H), 7.80 (d, 1H, J = 13.2), 7.90 (s, 1H), 8.18 (s, 1H), 8.43 (s, 1H), 8.80 (d, 1H, J = 13.2), 9.86 (s, 1H); HRMS calcd for $C_{22}H_{16}N_2O_6H$: 405.1086; found 405.1079.

2.3.5. 12-(2-Nitroethyl)-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (10)

To a stirred suspension of $NaBH_4$ (0.5 mmol) in 1,4-dioxane/EtOH (2:1, 5 mL) was added a solution of **9** (0.1 mmol) in 1,4-dioxane (5 mL) dropwise at 0 °C. After this addition, the reaction mixture was stirred for an additional 30 min. The resulting reaction mixture was diluted with ice-water and quenched with 50% aqueous AcOH. The resulting suspension was concentrated and then partitioned between sat $NaHCO_3$ and CH_2Cl_2. The organic layer was again concentrated and the residue was chromatographed using 10:1 CH_2Cl_2/MeOH to provide a yellow powder in 61% yield; mp 260–264 °C; IR (KBr) 1554; ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 3.95 (t, 2H, J = 6.6), 4.09 (s, 3H), 4.11 (s, 3H), 4.18 (t, 2H, J = 6.6), 6.18 (s, 2H), 7.42 (s, 1H), 7.59 (s, 1H), 7.89 (s, 1H), 8.16 (s, 1H), 8.22 (s, 1H), 9.91 (s, 1H); HRMS calcd for $C_{22}H_{18}N_2O_6H$: 407.1243; found 407.1233.

2.3.6. 12-(2-Aminoethyl)-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (11)

To a stirred suspension of **10** (0.012 mmol) in acetic acid (1 mL) was added Zn power (0.24 mmol) portion wise over a period of 5 min at room temperature. The reaction mixture was stirred for an additional 3 h, and then filtered. The filtrate was diluted with saturated sodium bicarbonate, concentrated, and partitioned between satd $NaHCO_3$ and CH_2Cl_2. The organic layer was again concentrated and the residue was chromatographed using 10:1:0.1 CH_2Cl_2/MeOH/TEA to provide a yellow-green powder in 41% yield; mp 196–201 °C; ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 3.21 (t, 2H, J = 5.8), 3.40 (t, 2H, J = 5.8), 4.07 (s, 3H), 4.14 (s, 3H), 6.14 (s, 2H), 7.45 (s, 1H), 7.49 (s, 1H), 7.87 (s, 1H), 8.08 (s, 1H), 8.13 (s, 1H), 9.79 (s, 1H); HRMS calcd for $C_{22}H_{20}N_2O_4H$: 377.1501; found 377.1494.

2.3.7. 12-(2-Dimethylamino)ethyl-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (12)

To a solution of **11** (0.02 mmol) and formalin (0.1 mmol) in MeOH (3 mL) was added a solution of $NaBH_3CN$ (in 0.1 mL MeOH, 0.15 mmol) at 0 °C. The resulting reaction solution was stirred for an additional 30 min at 0 °C, and then AcOH (two drops) was added. The reaction mixture was warmed up to room temperature with stirring for another 2 h. The resulting mixture was quenched by 1 N NaOH solution (0.1 mL), concentrated, and extracted by $CHCl_3$ (2 × 15 mL). The organic layer was concentrated and the res-

idue was chromatographed using 15:1 CH_2Cl_2/MeOH to provide a yellow powder in 58% yield; mp 195–198 °C; ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 2.45 (s, 6H), 2.79 (t, 2H, J = 8.0), 3.40 (t, 2H, J = 8.0), 4.09 (s, 3H), 4.16 (s, 3H), 6.17 (s, 2H), 7.52 (s, 1H), 7.56 (s, 1H), 7.93 (s, 1H), 8.15 (s, 1H), 8.20 (s, 1H), 9.89 (s, 1H); HRMS calcd for $C_{24}H_{24}N_2O_4H$: 405.1814; found 405.1805.

2.3.8. 2,3-Dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine-11-carboxylic acid (13)

To a suspension of ester, **5** (900 mg, 2.22 mmol) in ethanol (10 mL) was added 10% NaOH (20 mL) and refluxed for 4 h. Reaction mixture was cooled, acidified with Conc. HCl (≈pH 5) and filtered. The precipitate was further washed with methanol, chloroform and dried under vacuum to yield 605 mg of the acid as a yellow solid, in 72% yield; mp 277–278 °C; IR (KBr) 1706; ^{1}H NMR (DMSO-d_6): δ 3.84 (s, 3H), 4.01 (s, 3H), 6.19 (s, 2H), 7.47(s, 1H), 8.14 (s, 1H), 8.29 (s, 1H), 8.35 (s, 1H), 10.06 (s, 1H); HRMS (M^++Li) calcd for $C_{21}H_{15}NO_6Li$: 384.1059; found: 384.1061.

2.3.9. 12-(*N*-Aminocarbonyl)-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (14)

A mixture of carboxylic ethyl ester (0.1 mmol) in 10% NaOH (5 mL) and ethanol (10 mL) was heated to reflux with stirring for 1 h. The reaction mixture was acidified with 2 N HCl to pH 4, and then evaporated to dryness. The residue was suspended in dichloromethane (10 mL) and thionyl chloride (0.5 mL) was added. The resulting reaction mixture was refluxed for 2 h and then concentrated. The reaction residue was again suspended in dichloromethane and triethylamine (0.5 mL) was added. After 15 min, ammonia solution (0.5 mL, 2.0 M in tetrahydrofuran) was added and the resulting reaction mixture was refluxed for 1 h. The organic solvent and excess amine were removed under reduced pressure and the residue was chromatographed using 20:1 CH_2Cl_2/MeOH to provide an off-white powder in 15% yield; mp 291–295 °C; IR (KBr) 1649; ^{1}H NMR (DMSO-d_6) δ 3.91 (s, 3H), 4.09 (s, 3H), 6.27 (s, 2H), 7.56 (s, 1H), 7.79 (br, 1H), 7.90 (s, 1H), 8.25 (br, 1H), 8.33 (s, 1H), 8.46 (s, 1H), 8.64 (s, 1H), 10.16 (s, 1H); HRMS calcd for $C_{21}H_{16}N_2O_5H$: 377.1159; found 377.1149.

2.3.10. 12-(*N*-Methylaminocarbonyl)-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (15)

To a suspension of the acid, **13** (50 mg, 0.132 mmol) in chloroform (30 mL) was added thionyl chloride (3 mL, 41 mmol) and refluxed for 5 h and stirred at room temperature overnight. Reaction mixture was concentrated to dryness on a rotavap and dried under high vacuum. To this brown solid was added dry DCM (30 mL), TEA (3 mL, 21 mmol) and the mixture stirred for 15 min at room temperature. Then solid anhyd $MeNH_2$·HCl (200 mg, 29 mmol) was added and stirred for 2 h. The reaction mixture was concentrated under vacuum and the crude residue was purified by flash chromatography eluting with 2% methanol in chloroform to give a yellow solid in 23% yield; mp 341–343 °C (decomp.); IR (neat) 3438, 1638; ^{1}H NMR ($CDCl_3$): δ 3.24 (d, 3H, J = 5.2), 4.06 (s, 3H), 4.13 (s, 3H), 6.14 (s, 2H); 7.26 (s, 1H), 7.41 (br s, 1H), 7.61 (s, 1H), 7.73 (s, 1H), 7.95 (s, 1H), 8.07 (s, 1H), 9.67 (s, 1H), HRMS (M^++H) calcd for $C_{22}H_{18}N_2O_5H$: 391.1294; found: 391.1309.

2.3.11. 12-(*N*,*N*-Dimethylaminocarbonyl)-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (16)

Prepared from the acid, **13** (85 mg, 0.225 mmol) and Me_2NH (1.1 mL, 2.25 mmol, 2 M in THF) using the above procedure in 76% yield; mp 233–234 °C; IR (neat) 2969, 2932, 1620; ^{1}H NMR ($CDCl_3$): δ 2.88 (s, 3H), 3.33 (s, 3H), 4.02 (s, 3H), 4.16 (s, 3H), 6.17 (s, 2H); 7.20 (s, 1H), 7.62 (s, 1H), 7.82 (s, 1H), 8.11 (s, 1H), 8.17 (s, 1H), 9.86 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 34.1, 38.0, 55.1, 55.3, 98.4, 101.3, 101.5, 104.3, 105.2, 115.3, 119.3, 119.9, 122.5, 124.8, 129.5, 136.6, 140.0, 143.1, 148.0, 149.0, 149.5, 150.2, 169.4; HRMS (M^++H) calcd for $C_{23}H_{20}N_2O_5H$: 405.1432; found: 404.1439.

2.3.12. 12-(3-Dimethylamino)prop-1-enyl-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (17)

To a suspension of 2-(dimethylamino)ethyltriphenylphosphonium bromide (0.063 mmol) in THF (5 mL) was added LiHMDS (1.0 M in THF, 0.07 mmol) drop wise at room temperature. A solution of **7** (0.051 mmol) in THF was added to the reaction mixture drop wise and the resulting solution was stirred for 1.5 h, quenched by water (0.1 mL), concentrated and partitioned into CH_2Cl_2/water. The organic layer was concentrated under reduced pressure and the residue was chromatographed using 20:1 CH_2Cl_2/MeOH to provide a yellow powder in 60% yield; as a mixture 1:3 of *cis*/*trans* isomers mp 212–217 °C; ^{1}H NMR ($CDCl_3$ + CD_3OD) of the major isomers (*trans*) δ 2.44 (s, 6H), 3.33 (d, 2H, J = 6.2), 4.08 (s, 3H), 4.16 (s, 3H), 6.16 (s, 2H), 6.49 (dt, 1H, J = 15.8, 6.2), 7.29 (d, 1H, J = 15.8), 7.48 (s, 1H), 7.54 (s, 1H), 7.90 (s, 1H), 8.15 (s, 1H), 8.28 (s, 1H), 9.85 (s, 1H); HRMS calcd for $C_{25}H_{24}N_2O_4H$: 417.1810; found 417.1801.

2.3.13. 12-(3-Dimethylamino)propyl-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (18)

A suspension of **17** (0.035 mmol) and Pd–C (5 mg) in ethanol (10 mL) was shaken under hydrogen (40 psi) for 24 h. The mixture was filtered through Celite and concentrated under reduced pressure. The residue was chromatographed using 20:1 CH_2Cl_2/MeOH to provide a yellow powder in 50% yield; mp 195–200 °C; ^{1}H NMR ($CDCl_3$) δ 2.12 (m, 2H), 2.39 (s, 6H), 2.60 (t, 2H, J = 7.4), 3.27 (t, 2H, J = 8.0), 4.09 (s, 3H), 4.16 (s, 3H), 6.17 (s, 2H), 7.49 (s, 1H), 7.55 (s, 1H), 7.93 (s, 1H), 8.13 (s, 1H), 8.19 (s, 1H), 9.88 (s, 1H); HRMS calcd for $C_{25}H_{26}N_2O_4H$: 419.1971; found 419.1964.

2.3.14. 12-(4-Dimethylamino)but-1-enyl-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (19)

To a suspension of 3-(dimethylamino)propyltriphenylphosphonium bromide (0.1 mmol) in THF (5 mL) was added LiHMDS (1.0 M in THF, 0.1 mmol) drop wise at room temperature. A solution of **7** (0.075 mmol) in THF was added to the reaction mixture drop wise and the resulting solution was stirred for 1.5 h, quenched by water (0.1 mL), concentrated and partitioned into CH_2Cl_2/water. The organic layer was concentrated under reduced pressure and the residue was chromatographed using 20:1 CH_2Cl_2/MeOH to provide a yellow powder in 62% yield of a 1:3 mixture of the *cis*/*trans* isomers: mp 215–219 °C; ^{1}H NMR ($CDCl_3$ + CD_3OD) the major isomer (*trans*) had: δ 2.45 (s, 6H), 2.72 (m, 4H), 4.01 (s, 3H), 4.09 (s, 3H), 6.10 (s, 2H), 6.38 (dt, 1H, J = 15.4, 6.2), 7.17 (d, 1H, J = 15.4), 7.26 (s, 1H), 7.41 (s, 1H), 7.94 (s, 1H), 8.07 (s, 1H), 8.25 (s, 1H), 9.70 (s, 1H); HRMS calcd for $C_{26}H_{26}N_2O_4H$: 431.1971; found 431.1967.

2.3.15. 12-(4-Dimethylamino)butyl-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (20)

A suspension of **19** (0.035 mmol) and Pd–C (5 mg) in ethanol (10 mL) was shaken under hydrogen (40 psi) for 24 h. The mixture was filtered through Celite and concentrated under reduced pressure. The residue was chromatographed using 20:1 CH_2Cl_2/MeOH to provide a yellow powder in 50% yield; mp 213–217 °C; ^{1}H NMR (DMSO-d_6) δ 1.85 (m, 4H), 2.75 (s, 6H), 3.38 (m, 4H), 3.99 (s, 3H), 4.07 (s, 3H), 6.29 (m, 2H), 7.44 (s, 1H), 7.63 (s, 1H), 8.31 (s, 1H), 8.32 (s, 1H), 8.37 (s, 1H), 10.13 (s, 1H); HRMS calcd for $C_{26}H_{28}N_2O_4H$: 433.2123; found 433.2130.

2.3.16. 12-(4-Dimethylamino-1-hydroxy)butyl-2,3-dimethoxy-8,9-methylenedioxybenzo[*i*]phenanthridine (21)

To a solution of **7** (0.2 mmol) in THF (8 mL) was added drop wise freshly prepared dimethylaminopropyl magnesium chloride

THF solution (0.8 M, 0.3 mL) at 0 °C. The resulting reaction mixture was stirred at the same temperature for 15 min and then warmed up to room temperature with stirring overnight. The reaction was quenched with water (0.1 mL), concentrated and the residue was chromatographed using 15:1 CH_2Cl_2/MeOH to provide a yellow powder in 32% yield; mp 213–217 °C; IR (KBr) 3440; ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 2.11 (m, 4H), 2.84 (s, 6H), 3.17 (t, 2H, J = 6.0), 4.11(s, 3H), 4.15 (s, 3H), 5.57 (t, 2H, J = 6.0), 6.15 (m, 2H), 7.36 (s, 1H), 7.46 (s, 1H), 7.88 (s, 1H), 7.96 (s, 1H), 8.37 (s, 1H), 9.88 (s, 1H); HRMS calcd for $C_{26}H_{28}N_2O_5H$: 449.2076; found 449.2069.

2.3.17. 12-[(2-*N*,*N*-Dimethylaminoethyl)]aminomethyl-2,3-dimethoxy-8,9-methylenedioxy-benzo[*i*]phenanthridine (22)

To a solution of aldehyde, **7** (12 mg, 0.033 mmol) in DCM (30 mL) was added *N*,*N*-dimethylethylenediamine (18 μL, 0.166 mmol) and stirred for 10 min at rt. To this $NaCNBH_3$ (6 mg, 0.099 mmol) was added and stirred for 2 h. Reaction mixture was diluted with 10% NaOH solution (0.5 mL) and extracted with DCM. Organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated. The crude was purified by flash chromatography eluting with 1% methanol in chloroform to get a pale yellow solid in 28% yield; mp 255–256 °C; IR (neat) 3448; ^{1}H NMR ($CDCl_3$): δ 2.27 (s, 6H), 2.59 (t, 2H, J = 6.0), 2.90 (t, 2H, J = 6.0) 4.06 (s, 3H), 4.14 (s, 3H), 4.35 (s, 2H), 6.14 (s, 2H); 7.50 (s, 1H), 7.54 (s, 1H), 7.99 (s, 1H), 8.15 (s, 1H), 8.31 (s, 1H), 9.81 (s, 1H); HRMS (M^++H) calcd for $C_{25}H_{27}N_3O_4H$: 434.2091; found: 434.2093.

2.4. Cytotoxicity assays

The cytotoxicity was determined using the MTT-microtiter plate tetrazolinium cytotoxicity assay (MTA). The human lymphoblast RPMI 8402 and its camptothecin-resistant variant cell line, CPT-K5 was provided by Dr. Toshiwo Andoh (Aichi Cancer Center Research Institute, Nagoya, Japan).[28] The P388 mouse leukemia cell line and its CPT-resistant TOP1-deficient variant P388/CPT45 were obtained from Michael R. Mattern and Randal K. Johnson (GlaxoSmithKline, King of Prussia, PA).[29] The KB3-1 cell line and its multidrug-resistant variant KBV-1 were obtained from K.V. Chin (The Cancer Institute of New Jersey, New Brunswick, NJ).[30] The KBH5.0 cell line as noted previously was derived from KB3-1 by stepwise selection against Hoechst 33342.[18] The cytotoxicity assay was performed using 96-well microtiter plates. Cells were grown in suspension at 37 °C in 5% CO_2 and maintained by regular passage in RPMI medium supplemented with 10% heat inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/mL), and Streptomycin (0.1 mg/mL). For determination of IC_{50}, cells were exposed continuously for four days to varying concentrations of the drug, and MTT assays were performed at the end of the fourth day. Each assay was performed with a control that did not contain any drug. All assays were performed at least twice in six replicate wells.

2.5. Topoisomerase-mediated DNA cleavage assays

Human topoisomerase I was purified using the baculovirus system as described with slight modification.[31] Human topoisomerase I was expressed in SF9 insect cells and purified from nuclear extracts by hydroxyapatite chromatography as described.[32] Plasmid YEpG was also purified by the alkali lysis method followed by phenol deproteination and CsCl/ethidium isopycnic centrifugation method as described.[33,34] The 3′ endlabeling of the plasmid was accomplished by digestion with a restriction enzyme followed by end filling with Klenow polymerase as previously described.[35] The cleavage assays were performed as previously reported.[32,36] The drug and the DNA in the presence of topoisomerase I was incubated for 30 min at room temperature. Compounds were evaluated at concentrations ranging from 0.01 to 10 μg/mL. The reactions were terminated by the addition of 5 μL of 5% SDS and 1 mg/mL protein kinase K with an additional 1 h of incubation at 37 °C. Samples were then alkali denatured by the addition of NaOH, EDTA, sucrose, and bromophenol blue to final concentrations of 75 mM, 2.5%, and 0.05 mg/mL, respectively, prior to loading onto a neutral agarose gel. After development of the gels, typically 24-h exposure was used to obtain autoradiograms outlining the extent of DNA fragmentation. Topoisomerase I-mediated DNA cleavage values are reported as Relative Effective Concentration (REC), which represents concentrations relative to topotecan, whose value is arbitrarily assumed as 1.0, that are able to produce the same 10% cleavage on the plasmid DNA in the presence of human topoisomerase I.

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