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High antineoplastic activity of new heterocyclic compounds in cancer cells with resistance against classical DNA topoisomerase II-targeting drugs

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Twenty previously synthesized fused heterocyclic DNA-topoisomerase II (Topo II)-inhibiting compounds were investigated for their potential efficacy in various human cancer cell lines that were derived from different tumor entities. Moreover, different multidrug-resistant variants of these cancer cell lines with decreased Topo II expression were investigated. In parental, drug-sensitive cells merely the compounds BD3 and G35 showed efficacies, in terms of μM, which were similar to that of the classical Topo II inhibitor etoposide. On the other hand, most of the tested heterocyclic compounds were found more effective in drug-resistant cells than in the parental, drug-sensitive ones, and some of the compounds showed high antineoplastic efficacy in several drug-resistant cell models. Compounds BD13, BD14 and BD16 exhibited high antineoplastic activities against the drug-resistant sublines EPG85-257RNOV and EPG85-257RDB derived from gastric carcinoma, EPP85-181RNOV and EPP85-181RDB derived from pancreatic carcinoma, MCF-7/Adr derived from breast cancer, D79/86RNOV derived from fibrosarcoma, and MeWoETO1 derived from melanoma. Furthermore, compound D23 was found highly efficient in the multidrug-resistant variants HT-29RNOV and HT-29RDB derived from colon carcinoma, and compound D24 exhibited the highest antineoplastic activity among the tested compounds in the drug-resistant subline MDA-MB-231ROV derived from breast cancer. In conclusion, compounds BD 13, BD 14, BD 16, D 23 and D 24 may be useful for the treatment of different multidrug-resistant cancer cells with cross resistance against "classical" Topo II-targeting drugs.

Key words: benzoxazole; benzimidazole; benzoxazin; DNA topisomerase II inhibitors; multidrug resistance

DNA topoisomerases (Topo)[1] are enzymes that isomerise the tertiary structure of DNA without changing its primary structure. The high degree of conservation of these enzymes among prokaryotes and eukaryotes indicates an essential role in cell biology. Because its structure is a double helix, DNA is under tortional stress that results in multiplex twisting of the molecule. To be processed for replication or gene expression, the supercoiled DNA must become accessible to nucleic acids polymerases or components of the transcription machinery. This change requires relaxation and untangling of the intertwined DNA strands, which are the typical tasks of Topo.

In humans, 2 classes of Topo are well characterized, type I and type II. Topo type II (Topo II) are useful as drug target, since they have an indispensable function in cell biology and they lack biological redundancy. Inhibitors of these enzymes have become central parts of both primary and adjuvant chemotherapy regimens in neoplastic diseases, and they probably will remain so for the foreseeable future.[2] Two Topo II isoforms, the 170 kDa Topo IIα and the 180 kDa Topo IIβ, exist as homodimers and their amino acid sequences show homology at regions believed to be functionally significant (72% identical amino acid residues),[3] suggesting a comparable mode of action. The expression of Topo IIα varies during the cell cycle. It is low in quiescent cells, but maximal in the G_2-M phase, whereas the expression level of Topo IIβ remains constant throughout the cell cycle.[4]

Topo II binds to DNA, cleaves both strands, passes a second strand of DNA through the cleaved site in an ATP-depended manner and rejoins the strands at the original site of cleavage.[5] This Topo II-dependent reaction results in a DNA molecule altered in its topological configuration and represents an obligatory event during DNA replication, DNA transcription and segregation of chromosomes during mitosis. During breakage-reunion reaction, Topo II can form a cleavable complex with DNA with the covalent linking of each Topo II subunit to each 5′-phosphoryl end of the cleaved DNA molecule through a phosphotyrosyl bond.[6]

Classical Topo II-inhibiting agents such as epipodophyllotoxins or anthracyclines interfere with the breakage-reunion reaction of Topo II by stabilizing this cleavable complex. The stabilization of the cleavable complex and not the inhibition of the Topo II activity is supposed to play the decisive role in the cytotoxic effect of the classical Topo II interacting agents.[7] The stabilized cleavable complex leads to both single- and double-strand DNA breaks, which can trigger cellular signal transduction pathways leading to cell death.[8] Accordingly, resistance against classical Topo II-inhibiting agents can result from any process that leads to an altered binding of Topo II to drugs or DNA and a reduced formation of cleavable complexes. Indeed, it was demonstrated that decreased Topo II catalytic activity can mediate drug resistance to cancer cells.[9] Since these drug-resistant tumor cells showed cross resistance to other drugs, this phenotype was designated as altered Topo II multidrug resistance (at-MDR).[10] The decrease in Topo II activity can be caused by diminished expression levels of both Topo II isoforms[11] as well as by missense mutations within the Topo II isoenzyme encoding genes.[12]

Since drug resistance is a major obstacle in successful treatment of neoplastic diseases, it is important to design alternative therapy strategies that can be utilized for the treatment of drug-resistant cancer cells. One of such approaches is the development of alternative Topo II-targeting drugs without cross resistance in cancer cells exhibiting a drug-resistant phenotype against classical Topo II inhibitors. Thus, we previously tested several derivatives of benzoxazoles, benzimidazoles, and related fused heterocyclic compounds with antimicrobial and antiviral activities for their inhibitory properties on eukaryotic Topo II in a cell-free system.[13] Since some of these compounds showed Topo II-inhibiting activity under cell-free conditions, in this study, we analyzed the potential antineoplastic activity of these agents in various human cancer cell lines established from different tumor entities and derived multidrug-resistant sublines with decreased Topo II expression.

Material and methods

Cell lines and cell culture

Human cancer cell lines and drug-resistant sublines (Table I) were grown in Leibovitz L-15 medium (Biowhittaker, Walkersville, MD) supplemented by 10% fetal calf serum (FCS) (GIBCO/BRL, Grand Island, NY), 1 mM L-glutamine, 6.25 mg/l fetuin, 80 IE/l insulin, 2.5 mg/ml transferrin, 0.5 g/l glucose, 1.1 g/l $NaHCO_3$, 1%

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TABLE I – CANCER CELL LINES WITH DRUG-RESISTANT SUBLINES

Cell line	Origin	Selection agent	Supposed resistance mechanisms	References
EPG85-257P	Gastric carcinoma	–	–	14
EPG85-257RNOV		Mitoxantrone	BCRP, GPC3, Topo II[1], TAP[1]	14, 15, 16, 17
EPG85-257RDB		Daunorubicin	MDR1/P-gp	18
EPP85-181P	Pancreatic carcinoma	–	–	19
EPP85-181RNOV		Mitoxantrone	Topo II	19
EPP85-181RDB		Daunorubicin	MDR1/P-gp	19
HT-29	Colon carcinoma	–	–	20
HT-29RNOV		Mitoxantrone	BCRP, MDR1/P-gp[1]	21, 22
HAT-29RDB		Daunorubicin	MDR1/P-gp	21
MCF-7	Breast cancer	–	–	23
MCF-7RNOV		Mitoxantrone	Topo II	–
MCF-7/Adr		Doxorubicin	MDR1/P-gp	24
MDA-MB-231	Breast cancer	–	–	25
MDA-MB-231RNOV		Mitoxantrone	Topo II, BCRP[1]	22
D79/86	Fibrosarcoma	–	–	21
D79/86RNOV		Mitoxantrone	BCRP	21, 22
MeWo	Melanoma	–	–	26
MeWo ETO1		Etoposide	Topo II, DFNA5[1]	11, 27, 28
HeLa[2]	Cervix carcinoma	–	–	29

[1]Minor contribution to the drug-resistant phenotype.–[2]HeLa was utilized as reference for relative mRNA expression levels.

minimal essential vitamins and 20,000 kIE/l trasylol in a humidified atmosphere of 5% CO_2 at 37°C. Drug-resistant cell lines were established from parental cell lines by continuous exposure of the cells to stepwise increasing concentrations of antineoplastic agents as described previously.[30] For maintenance of drug-resistant phenotypes, medium of drug-resistant sublines was supplemented with the selection agent.

DNA topoisomerase II-targeting compounds

DNA topoisomerase II-targeting derivatives of benzoxazoles, benzimidazoles, and related fused heterocyclic compounds (Fig. 1) were synthesized as reported previously.[13] As control, the classical DNA topoisomerase II inhibitor etoposide (Bristol-Myers, Munich, Germany) was used.

Cell proliferation assay

Resistance to etoposide and heterocyclic DNA topoisomerase II-inhibiting compounds was assessed using a proliferation assay based on sulforhodamine B (SRB) staining as described previously.[15,31] Briefly, 800 cells per well were seeded in 96-well plates in triplicates. After 24 hr attachment, the particular agent was added in dilution series for 5 days incubation. Cells were fixed by chilled 10% trichloroacetic acid for 1 hr at 4°C, washed 5 times with tap water before staining was performed with 0.4 % SRB in 1% acetic acid for 10 min at room temperature. After washing with 1% acetic acid, drying and resolubilization in 20 mM Tris-HCl (pH 10), absorbance was measured at 562 nm against the reference wavelength of 690 nm. IC_{50}-values were calculated from 3 independent experiments for each cell line.

Real-time quantitative

For analyzing Topo II mRNA levels, a real-time quantitative RT-PCR (QPCR) was carried out with total RNA by using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany). Oligodeoxynucleotide primers used for amplification were: TopoIIα-fw (5′-CACAACTGGCCCTCTCTTCTGCGAC -3), TopoIIα-rev (5′-GGCCAACCTTTACTTCTCGCTT -3′), TopoIIβ-fw (5′-ACCTGGCCAGCGGAAAGTTTTATT -3′), TopoIIβ-rev (5′-CCTGTTCTTTATATACCTGTGTCC -3), as control ald-fw (5′-ATCCTGGCTGCAGATGAGTC-3′), and ald-rev (5′-GCCCTTGTCTACCTTGATGC-3′). Amplified RT-PCR products were on-line detected via intercalation of the fluorescent dye SYBR-Green (LightCycler-FastStart DNA Master SYBR Green I kit, Roche Diagnostics). Quantification of the copy number was performed by serial dilutions of cDNA fragments (10^0 up to 10^7 cDNA copies), TA-cloned in the vector pCR2.1. RNA concentration was determined by spectrophotometry, and 2 μg RNA was reverse-transcribed by Superscript II enzyme (GIBCO BRL, Gaithersburg, MD) using arbitrary hexamers as random primers according to the manufacturer's instructions. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligodeoxynucleotide primers specific for the constitutively expressed aldolase encoding gene and normalized. Cycling conditions were as follows: initial enzyme activation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, T_H (T_H for Topo IIα, 54°C; Topo IIβ, 54°C; aldolase, 58°C) for 5 sec and 72°C for 15 sec. Gene-specific fluorescence was measured by T_M (T_M for Topo IIα, 78°C; Topo IIβ, 82°C; aldolase, 86°C). Specificity of amplification products was confirmed by melting curve analysis.

Western blot analyses

For detection of Topo IIα and Topo IIβ, Western blot analyses were performed as described previously.[11,19] In brief, samples of 20 μg nuclear proteins were loaded onto a 6% SDS-PA gel. Separated proteins were transferred to a 0.2 μm cellulose nitrate membrane (Schleicher and Schuell, Dassel, Germany). To avoid unspecific binding, the filters were incubated in 5% nonfat dry milk and 0.05% Tween-20 in TBS overnight. Finally, filters were incubated with mouse monoclonal antibody (mAb) directed against Topo IIα (DAKO, Glastrup, Danmark), or polyclonal rabbit antibodies directed against Topo IIβ (Biotrend, Cologne, Germany), diluted in the same solution (Topo IIα, 1:4000; Topo IIβ, 1:5000) for 2 hr and, afterward, with peroxidase-conjugated mouse anti-rabbit IgG (1:10,000) (Sigma, St. Louis, MO; # A-1949), or peroxidase-conjugated rabbit anti-mouse IgG (1:5,000) (Jackson ImmunoResearch Laboratories, West Grove, PA; # 315–035–003). As control, that equivalent protein amounts were blotted, the filters were stripped and incubated with a mouse mAb directed against laminin (Chemicon, Temecula, CA). The protein–antibody complexes were visualized by chemiluminescence (ECL system, Amersham) according to the manufacturer's protocol.

Results

DNA topoisomerase IIα and IIβ mRNA levels

Relative mRNA expression levels of the Topo IIα and Topo IIβ encoding mRNAs in various cancer cell lines was determined by QPCR. Each value represents the relative expression level in relation to the mRNA expression of the housekeeping enzyme aldolase and is given in relation to the Topo IIα or Topo IIβ: aldolase ratio in the cervix carcinoma cell line HeLa that was set to be 1.0, respectively. As shown in Figures 2*a* and 2*b*, most of the drug-resistant cell lines that were selected against the Topo II inhibitors mitoxantrone or etoposide decreased the mRNA expression levels

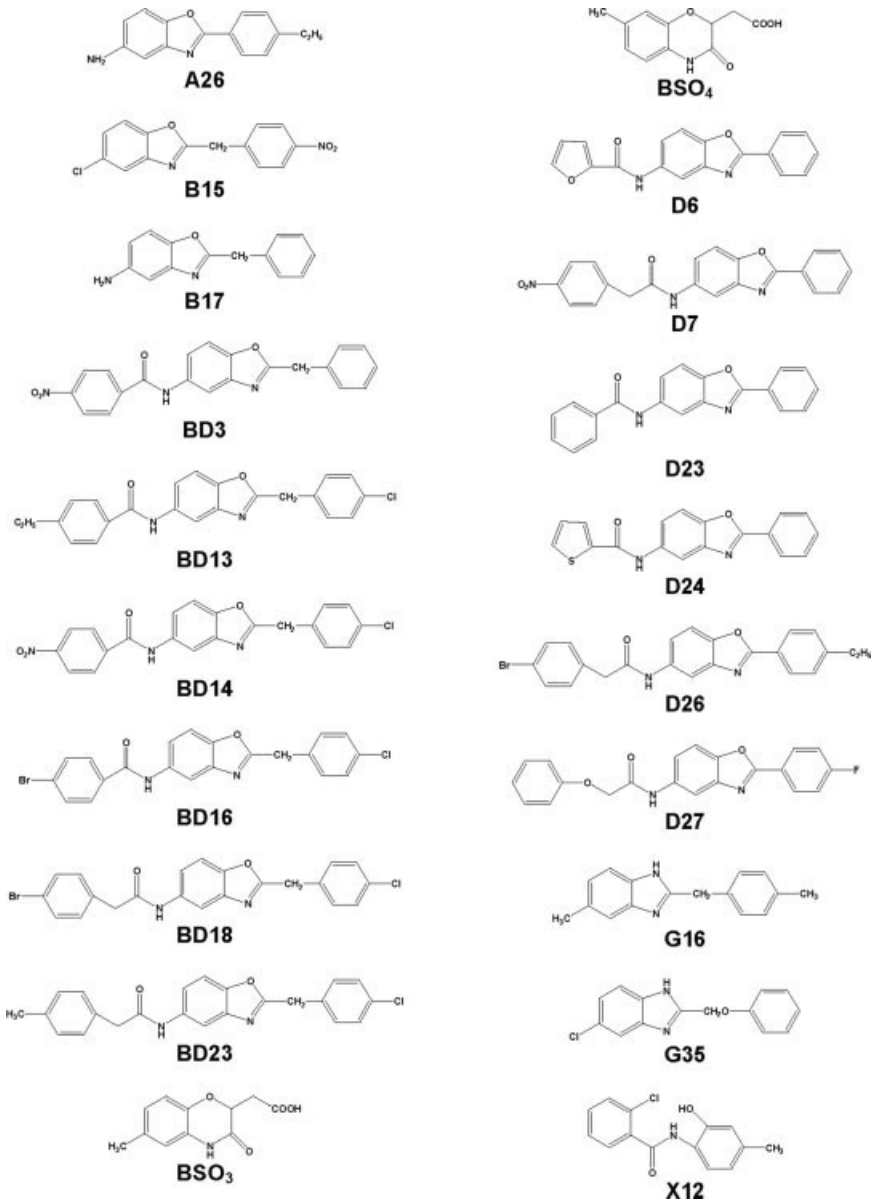

FIGURE 1 – Topo II-inhibiting compounds tested for cytotoxicity in human cancer cell lines and derived drug-resistant cell variants.

of both Topo II isoenzyme IIα and IIβ encoding mRNAs. A single exception is the mitoxantrone-selected fibrosarcoma cell line D79/86RNOV, which showed slightly elevated mRNA expression levels of both Topo II isoforms, IIα and IIβ, whereby the IIβ mRNA expression levels were extremely weak near the detection limit.

In contrast, the drug-resistant cell lines established by exposure to the anthracycline daunorubicin did not decrease Topo IIα mRNA expression levels, or in the case of the pancreatic carcinoma-derived cell line EPP85-181RDB merely showed very slight decrease in Topo IIα mRNA expression (Fig. 2*a*). The expression levels of Topo IIβ encoding mRNAs likewise were just decreased very weak in most of these cell models, or in the case of the colon carcinoma-derived cell variant HT-29RDB, were not diminished (Fig. 2*b*).

DNA topoisomerase IIα and IIβ protein levels

Western blot experiments using antibodies directed against Topo IIα (Fig. 2*a*) or Topo IIβ (Fig. 2*b*) demonstrated that most of the drug-resistant cell lines that were selected against the Topo II inhibitors mitoxantrone or etoposide decreased the nuclear protein expression levels of Topo IIα. In parental D79/86, as well as in mitoxantrone-resistant D79/86RNOV fibrosarcoma cells, Topo IIα expression could not be detected. In contrast, nuclear Topo IIα content was not significantly decreased in anthracycline-selected drug-resistant cell lines with 1 exception, in the breast cancer cell line MCF-7/Adr nuclear Topo IIα expression was reduced.

The expression of the Topo IIβ isoform was markedly reduced in the mitoxantrone-resistant gastric carcinoma cell line EPG85-257RNOV and in the mitoxantrone-selected colon cancer cell line HT-29RNOV (Fig. 2*b*). A moderate down regulation of Topo IIβ expression could be observed in the mitoxantrone-resistant breast cancer cell line MDA-MB-231RNOV, the mitoxantrone-selected fibrosarcoma cell line D79/86RNOV and the etoposide-resistant malignant melanoma cell line MeWo ETO1. The mitoxantrone-resistant cell variants EPP85-181RNOV derived from pancreatic carcinoma and MCF-7RNOV established from breast carcinoma merely slightly or not decreased nuclear Topo IIβ content. In anthracyline-selected cell lines either no alteration in Topo IIβ expression could be observed, i.e., in the gastric carcinoma cell variant EPG85-257RDB and the breast cancer cell line MCF-7/

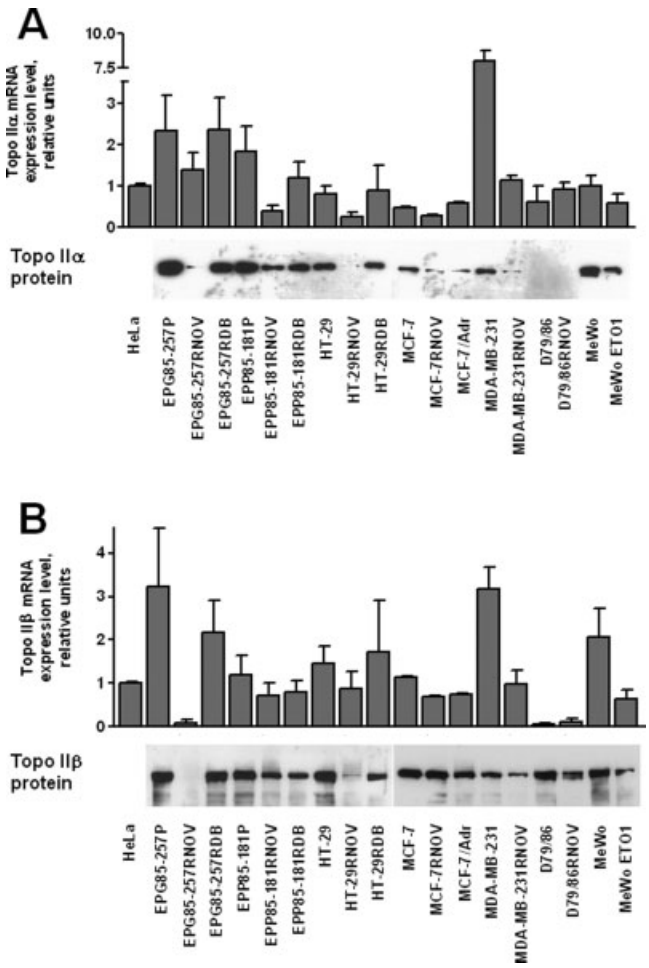

FIGURE 2 – Expression of (*a*) Topo IIα and (*b*) Topo IIβ on mRNA and protein levels. Relative mRNA expression levels were measured by QPCR. Each column represents the relative expression level in relation to the mRNA expression of the housekeeping enzyme aldolase and is given in relation to the Topo IIα or Topo IIβ: aldolase ratio in the cervix carcinoma cell line HeLa that was set to be 1.0, respectively. Protein expressions were detected by Western blot analyses of proteins prepared from crude nuclear extracts from various human parental cancer cell lines and drug-resistant derivatives harvested in the log-phase of growing. Samples of 20 μg nuclear protein were size-fractionated electrophoretically, transferred to a membrane, incubated with antibodies directed against Topo IIα and IIβ and visualized by chemiluminescence.

Adr, or a very slight decrease in nuclear Topo IIβ expression in the pancreatic carcinoma cell line EPP85-181RDB and HT-29RDB colon carcinoma cells.

Cytotoxicity of DNA topoisomerase II-targeting compounds in various drug-resistant cancer cells

For assessment of cytotoxicity of heterocyclic Topo II-targeting compounds, IC_{50}-values of each agent were determined by proliferation assays in the complete panel of human cancer cell lines. As control, the etoposide-specific IC_{50}-values were measured. The Topo II-inhibiting compound-specific IC_{50}-values and relative resistances of drug-resistant cell variants in comparison to the drug-sensitive parental cell lines are summarized in Table II for 3 EPG85-257 gastric carcinoma cell variants, in Table III for 3 EPP85-181 pancreatic carcinoma cells, in Table IV for 3 HT-29 colon carcinoma cell lines, in Table V for 3 MCF-7 breast cancer lines, in Table VI for a pair of MDA-MB-231 breast cancer cells, in Table VII for 2 D79/86 fibrosarcoma variants and in Table VIII for 2 MeWo melanoma cell lines.

TABLE II – CYTOTOXICITY OF HETEROCYCLIC COMPOUNDS IN DIFFERENT MULTIDRUG-RESISTANT EPG85-257 GASTRIC CARCINOMA CELLS

Compound	EPG85-257P	EPG85-257RNOV		EPG85-257RDB	
	IC_{50} (μM)	IC_{50} (μM)	RR[1]	IC_{50} (μM)	RR[1]
Etoposide	0.105	1.55	14.8	6.2	59.0
A 26	68	21	0.31	32	0.47
B 15	165	33	0.20	<10	**<0.06**
B 17	145	110	0.76	35	0.24
BD 3	2.2	0.46	0.21	0.33	0.15
BD 13	32	0.75	**0.02**	0.27	**0.008**
BD 14	195	1.5	**0.008**	0.43	**0.002**
BD 16	44	1.1	**0.025**	0.31	**0.007**
BD 18	16.5	4.8	0.29	1.55	**0.09**
BD 23	55	34	0.62	1.0	**0.02**
BSO 3	>200	>200	NC	195	0.98
BSO 4	>200	>200	NC	190	0.95
D 6	20	<0.5	**<0.025**	2.1	0.11
D 7	>200	42	<0.21	170	<0.85
D 23	15.5	<0.5	**<0.03**	1.35	**0.09**
D 24	16	<0.5	**<0.03**	1.8	0.11
D 26	>200	7.5	**<0.04**	15	**<0.08**
D 27	14.5	<0.5	**<0.03**	3.9	0.27
G 16	18	14.5	0.81	6.2	0.34
G 35	6	1.35	0.23	3.8	0.63
X 12	32	3.9	0.12	3.7	0.12

NC, not calculated.
[1]RR, relative resistance in relation to the parental, drug-sensitive cell line EPG85-257P. Relative resistances of highly efficient compounds are in bold.

TABLE III – CYTOTOXICITY OF HETEROCYCLIC COMPOUNDS IN DIFFERENT MULTIDRUG-RESISTANT EPP85-181 PANCREATIC CARCINOMA CELLS

Compound	EPP85-181P	EPP85-181RNOV		EPP85-181RDB	
	IC_{50} (μM)	IC_{50} (μM)	RR[1]	IC_{50} (μM)	RR[1]
Etoposide	0.58	4.5	7.8	62	106.9
A 26	82	75	0.91	70	0.85
B 15	195	180	0.92	65	0.33
B 17	199	195	0.98	185	0.93
BD 3	2.5	1.5	0.6	1.1	0.44
BD 13	195	2.38	**0.012**	1.35	**0.007**
BD 14	>200	3.7	**<0.019**	ND	NC
BD 16	>200	2.05	**<0.010**	1.5	**<0.008**
BD 18	>200	90	<0.45	>200	NC
BD 23	>200	>200	NC	>200	NC
BSO 3	>200	>200	NC	>200	NC
BSO 4	>200	180	<0.90	175	<0.88
D 6	46	23	0.50	18.5	0.40
D 7	>200	>200	NC	>200	NC
D 23	>100	>50	NC	>50	NC
D 24	>200	7.4	**<0.04**	9.3	**<0.05**
D 26	>200	>200	NC	>200	NC
D 27	>200	>100	NC	>100	NC
G 16	31	18	0.58	21.5	0.69
G 35	4.2	2.0	0.48	2.6	0.62
X 12	148	87	0.59	66	0.45

NC, not calculated; ND, not determined.
[1]RR, relative resistance in relation to the parental, drug-sensitive cell line EPP85-181P. Relative resistances of highly efficient compounds are in bold.

In parental, drug-sensitive cell lines, none of the heterocyclic compounds was more efficient, in terms of μM, than the classical Topo II inhibitor etoposide. However, in most of the parental cell lines compound BD 3 and compound G 35 showed the most pronounced efficacies that were in part near that of etoposide. For example, in HT-29 cells, the etoposide-specific IC_{50}-value was 2.3 μM, the BD 3-specific IC_{50}-value was 2.6 μM and the G 35-specific IC_{50}-value was 5.7 μM. In parental, D79/86P cells and parental MDA-

TABLE IV – CYTOTOXICITY OF HETEROCYCLIC COMPOUNDS IN DIFFERENT MULTIDRUG-RESISTANT HT-29 COLON CARCINOMA CELLS

Compound	HT-29	HT-29RNOV		HT-29RDB	
	IC_{50} (μM)	IC_{50} (μM)	RR[1]	IC_{50} (μM)	RR[1]
Etoposide	2.3	35	15.2	26	11.3
A 26	85	31	0.36	52	0.61
B 15	>200	140	<0.70	165	<0.83
B 17	>200	125	<0.63	180	<0.90
BD 3	2.6	2.1	0.81	2.0	0.77
BD 13	>200	>200	NC	>200	NC
BD 14	>200	>200	NC	>200	NC
BD 16	>200	68	<0.34	185	<0.93
BD 18	>200	>200	NC	>200	NC
BD 23	>200	>200	NC	>200	NC
BSO 3	>200	195	<0.98	>200	NC
BSO 4	>200	>200	NC	>200	NC
D 6	33	25	0.76	23	0.70
D 7	>200	>200	NC	>200	NC
D 23	>150	7.5	**<0.05**	8.8	**<0.06**
D 24	>200	72	<0.36	16.5	**<0.08**
D 26	>200	>200	NC	>200	NC
D 27	>150	>130	NC	>130	NC
G 16	23	12.5	0.54	21	0.91
G 35	5.7	1.8	0.32	4.1	0.72
X 12	>150	36	<0.24	68	<0.45

NC, not calculated.
[1]RR, relative resistance in relation to the parental, drug-sensitive cell line HT-29. Relative resistances of highly efficient compounds are in bold.

TABLE V – CYTOTOXICITY OF HETEROCYCLIC COMPOUNDS IN DIFFERENT MULTIDRUG-RESISTANT MCF-7 BREAST CANCER CELLS

Compound	MCF-7	MCF-7RNOV		MCF-7/Adr	
	IC_{50} (μM)	IC_{50} (μM)	RR[1]	IC_{50} (μM)	RR[1]
Etoposide	1.6	0.58	0.36	14	8.8
A 26	88	44	0.5	65	0.74
B 15	155	>100	>0.65	>100	>0.65
B 17	148	165	1.1	140	0.95
BD 3	5.1	2.3	0.45	0.85	0.17
BD 13	60	98	1.6	<1	**<0.017**
BD 14	>150	115	<0.77	<5	**<0.033**
BD 16	75	60	0.8	1	**0.013**
BD 18	>200	>200	NC	66	NC
BD 23	>200	>100	NC	>100	NC
BSO 3	>200	160	<0.8	>200	NC
BSO 4	155	145	0.94	185	1.2
D 6	18.5	27	1.5	14	0.76
D 7	>200	>100	NC	>100	NC
D 23	7.6	68	8.9	7.0	0.92
D 24	7.5	17	2.3	7.8	1.0
D 26	>200	49.5	<0.25	35	<0.18
D 27	46	27	0.59	14	0.30
G 16	22	24	1.1	16	0.73
G 35	4.1	3.8	0.93	2.7	0.66
X 12	185	73	0.39	46	0.25

NC, not calculated.
[1]RR, relative resistance in relation to the parental, drug-sensitive cell line MCF-7. Relative resistances of highly efficient compounds are in bold.

TABLE VI – CYTOTOXICITY OF HETEROCYCLIC COMPOUNDS IN ATYPICAL MULTIDRUG-RESISTANT MDA-MB-231 BREAST CANCER CELLS

Compound	MDA-MB-231	MDA-MB-231RNOV	
	IC_{50} (μM)	IC_{50} (μM)	RR[1]
Etoposide	0.36	50	138.9
A 26	110	70	0.64
B 15	>200	>100	NC
B 17	180	145	0.81
BD 3	>10	2.6	<0.26
BD 13	67	>100	>1.5
BD 14	66	18	0.27
BD 16	88	12.5	0.14
BD 18	>200	99	>0.5
BD 23	>200	>100	NC
BSO 3	>200	>200	NC
BSO 4	>200	190	<0.95
D 6	44	21	0.47
D 7	>200	>100	NC
D 23	47	64	1.4
D 24	>200	12.5	**<0.06**
D 26	135	33	0.24
D 27	23	16.5	0.72
G 16	37	42	1.1
G 35	2.2	2.6	1.2
X 12	93	85	0.91

NC, not calculated.
[1]RR, relative resistance in relation to the parental, drug-sensitive cell line MDA-MB-231. Relative resistances of highly efficient compounds are in bold.

TABLE VII – CYTOTOXICITY OF HETEROCYCLIC COMPOUNDS IN ATYPICAL MULTIDRUG-RESISTANT D79/86 FIBROSARCOMA CELLS

Compound	D79/86P	D79/86RNOV	
	IC_{50} (μM)	IC_{50} (μM)	RR[1]
Etoposide	0.21	0.47	2.2
A 26	140	44	0.31
B 15	127	23	0.18
B 17	>200	115	<0.58
BD 3	>10	7	<0.7
BD 13	53	4.7	**0.09**
BD 14	9.8	5.5	0.56
BD 16	21	4.4	0.21
BD 18	65	21	0.32
BD 23	70	55	0.79
BSO 3	>200	>200	NC
BSO 4	170	135	0.79
D 6	35	16.5	0.47
D 7	44	24	0.55
D 23	55	13	0.24
D 24	21	8	0.38
D 26	23	6.7	0.29
D 27	18	7.7	0.43
G 16	21	14.5	0.69
G 35	3	<1	<0.33
X 12	46	21	0.46

NC, not calculated.
[1]RR, relative resistance in relation to the parental, drug-sensitive cell line D79/86P. Relative resistances of highly efficient compounds are in bold.

MB-231 cells merely compound G 35 was highly efficient, whereas compound BD 3 was less biological active. Furthermore, in parental MCF-7 breast cancer cells beside compound BD 3 and compound G 35 also compounds D 23 and D24 showed high efficacies, i.e., IC_{50}-value of etoposide was 1.6 μM, of compound BD 3 was 5.1 μM, of compound G 35 was 4.1 μM, of compound D23 was 7.6 μM and of compound D24 was 7.5 μM.

As expected, most of the 11 drug-resistant cell variants, selected for anthracyclines, mitoxantrone, or etoposide, showed cross resistance against the classical Topo II inhibitor etoposide. A single exception of these drug-resistant cell lines was the breast cancer cell variant MCF-7RNOV that was slightly more sensitive to etoposide than the parental line MCF-7, i.e., 36% of the MCF-7-specific IC_{50}-value.

In contrast, in infrequent cases, drug-resistant cell variants exhibited a cross-resistant phenotype or no changes in resistance against heterocyclic Topo II-targeting compounds. Merely, the drug-resistant breast carcinoma cell lines, i.e., MCF-7RNOV, MCF7/Adr and MDA-MB-231RNOV, and the etoposide-resistant melanoma cell line MeWo ETO1 showed cross resistance against some of the heterocyclic compounds.

TABLE VIII – CYTOTOXICITY OF HETEROCYCLIC COMPOUNDS IN ETOPOSIDE-RESISTANT MEWO MELANOMA CELLS

Compound	MeWo	MeWoETO1	
	IC_{50} (μM)	IC_{50} (μM)	RR[1]
Etoposide	0.2	8.6	43
A 26	90	77	0.86
B 15	160	44	0.28
B 17	152	175	1.2
BD 3	2.2	2.4	1.1
BD 13	155	156	1
BD 14	89	67	0.75
BD 16	58	70	1.2
BD 18	190	>200	>1
BD 23	>200	>200	NC
BSO 3	195	>200	>1
BSO 4	>200	>200	NC
D 6	18.5	18	0.97
D 7	>200	>200	NC
D 23	148	160	1.1
D 24	69	80	1.1
D 26	54	>100	>1.9
D 27	155	>100	>0.65
G 16	27	27	1
G 35	5.3	5.2	0.98
X 12	57	76	1.3

NC, not calculated.
[1]RR, relative resistance in relation to the parental, drug-sensitive cell line MeWo.

In all cases, drug-resistant cancer sublines derived from gastrointestinal neoplasms, i.e., EPG85-257RNOV, EPG85-257RDB, EPP85-181RNOV, EPP85-181RDB, HT-29RNOV, HT-29RDB, and the mitoxantrone-selected fibrosarcoma cell line D79/86RNOV showed increased sensitivities to the complete set of heterocyclic Topo II-inhibiting compounds. In cases where the sensitivity against a given compound was less than 10% of the corresponding parental cell line, the heterocyclic compound was assessed to be highly efficient in this drug-resistant cell line. In the tables, these values are marked by bold. Furthermore, in these cases of high cytotoxic efficacy in drug-resistant cancer cells, the demandable molar concentrations to inhibit cell growth to 50% of control (IC_{50}) of the heterocyclic compounds were lower than the needful molar concentrations of etoposide in the corresponding parental, drug-sensitive cancer cells. None of these compounds showed high efficacy in all of these drug-resistant cell models. However, compound BD 13 was highly efficient in 6 of these 7 cell lines; compounds BD 16 and D 24 in 5 of 7 cell lines and compounds B14 and D 23 in 4 of 7 drug resistance cell models.

In the malignant melanoma-derived etoposide-resistant cell line MeWo ETO1 and the breast cancer cell line MCF-7RNOV, none of the heterocyclic compounds was highly efficient, and in the breast cancer cell line MDA-MB-231RNOV as well as in D79/86RNOV fibrosarcoma cells merely a single compound showed high efficacy.

Discussion

In previous studies, we reported that some derivatives of benzoxazoles, benzimidazoles, and related fused heterocyclic compounds exhibited antimicrobial,[32] antiviral activities[33] and inhibiting activity on eukaryotic Topo II in a cell-free system.[13] The compounds possess 3 different fused ring systems such as benzoxazole, benzimidazole, benzoxazin and an amide derivative (X12), which is the hydrolyzed form of the corresponding benzoxazole structure consisting of a cleavage of the oxazolo ring at the (C—O) linkage that revealed at the phase I possible metabolites of benzoxazoles in the rabbit by mild hydrolysis.[34,35] In this study, we investigated the potential efficacy of these compounds in various human cancer cell lines that were derived from different tumor entities. Moreover, different multidrug-resistant variants of these cells with decreased Topo II expression were analyzed. If the MDR phenotype was associated with expression of the ABC-transporter MDR1/P-glycoprotein (MDR1/P-gp),[36] the cells showed a "classical" MDR phenotype. This was true for anthracycline-selected cell lines EPG85-257RDB,[18] EPP85-181RDB,[19] HT-29RDB[21] and MCF7/Adr.[24] In the case of altered Topo II expression, the MDR phenotype was an at-MDR. Cell lines with an at-MDR phenotype were the mitoxantrone- and etoposide-selected cell lines EPG85-257RNOV, EPP85-181RNOV, HT29-RNOV, MCF-7RNOV, MDA-MB-231RNOV and MeWo ETO1 (overview in Table I).

Interestingly, only few drug-resistant cell lines derived from breast cancer or melanoma, MCF-7RNOV, MCF7/Adr, MDA-MB-231RNOV and MeWo ETO1, showed cross resistance against some of the heterocyclic compounds. Furthermore, most of the heterocyclic compounds were more effective in drug-resistant cells than in the parental, drug-sensitive ones, and some of the compounds showed high antineoplastic efficacy in several drug-resistant cell models. In the cell lines with a cross-resistant phenotype, commonly the heterocyclic compounds showed only few activity. These observations indicate that the antiproliferative effect of the compounds is strong tissue-dependent. Moreover, the type of MDR has influence of the biological activity of the high-efficient heterocyclic compounds.

During DNA replication and DNA transcription, Topo II binds to DNA, cleaves both strands, passes a second strand of DNA through the cleaved site in an ATP-depended manner and rejoins the strands at the original site of cleavage.[14] This Topo II-dependent reaction results in a DNA molecule altered in its topological configuration. During breakage-reunion reaction, Topo II can form a cleavable complex with DNA with the covalent linking of each Topo II subunit to each 5′-phosphoryl end of the cleaved DNA molecule through a phosphotyrosyl bond.[20] "Classical" Topo II-interacting agents like etoposide or mitoxantrone then interfere with the breakage-reunion reaction of Topo II by stabilizing this cleavable complex. The stabilization of the cleavable complex and not the inhibition of the Topo II activity is supposed to play the decisive role in the cytotoxic effect of these Topo II interacting agents.[23] The stabilized cleavable complex leads to both single- and double-strand DNA breaks, which can trigger pathways leading to cell death. Since the used drug-resistant cell models showed cross resistance against the "classical" Topo II inhibitors, but more evolved sensitivity against some of the fused heterocyclic compounds, it is obvious that these agents interact with the Topo II molecule in a different way.

Since it was demonstrated in a cell-free system that the heterocyclic compounds have Topo II-inhibiting properties,[13] it is likely that the antiproliferative activities these of agents in cancer cells are the result of interaction with Topo II. Consequently, the more pronounced antineoplastic efficacies of these substances in drug-resistant cancer cells may be caused: in parental, non-resistant cells with relative high amounts of Topo II, the inhibiting effects may lead merely to a decelerated proliferation rate. In contrast, in drug-resistant cell variants with very low amounts of Topo II, the residual Topo II activity may be blocked nearly completely. The result is a much more pronounced cytostatic effect. However, fused heterocyclic compounds were also efficient in "classical" MDR1/P-gp producing MDR cells without significant Topo II decrease. Thus, in these tumor cells alternative mechanisms may mediate the antiproliferative effects, e.g., a facilitated cellular condition to trigger apoptotic pathways. This point of view is supported by the observation that similar benzoxazoles derivatives were able to induce apoptosis in multidrug-resistant mouse lymphoma cells.[37]

For the tested drug-resistant cancer cell lines EPG85-257RNOV and EPG85-257RDB, the benzoxazole derivative BD14 exhibited the most effective antineoplastic activity as showing relative resistance (RR) values of 0.008 and 0.002, respectively. In addition, benzoxazole derivatives B13 and B16 were also found to be highly effective in these drug-resistant gastric carcinoma cancer cell lines. When the activity data (Table II) are compared, it reveals that the compounds consisting of 2-*p*-chlorobenzylbenzoxazole structure holding a benzamido group on position 5 of the benzoxazole ring system are significant for the antineoplastic activity against the tested drug-resistant sublines derived from gas-

tric carcinoma. If the para position of phenyl moiety of benzamido group on position 5 is substituted with a nitro group, the antineoplastic activity is increasing for both MDR gastric carcinoma sublines. However, a phenylacetamido group, instead of benzamido, including a methylene group to increase the length of substituent at the 5th position of benzoxazole structure, is diminishing the biological activity (BD16 and BD18 in Table II).

Furthermore, compounds BD16 and BD13 exhibited the most effective antineoplastic activity in the drug-resistant pancreatic carcinoma cell lines EPP85-181RNOV and EPP85-181RDB and the classical MDR breast cancer cell line MCF7/Adr, showing RR values of <0.01, <0.008, 0.013 and 0.012, 0.007, <0.017, respectively. However, in the case of compound BD14, a lower efficiency was shown in the drug-resistant cell lines EPP85-181RNOV and MCF7/Adr, and no biological activity was demonstrated in the classical MDR cell variant EPP85-181RDB. Activity data for these drug-resistant cancer cell lines (Tables III and V) revealed that substituent at the para position of phenyl moiety in the benzamido group on position 5 of the benzoxazole ring is important for the activity. If this position substituted with a group having less lipophilic and high-resonance effects such as a nitro group, a diminishing and/or eliminating of the antineoplastic activity in these MDR cell lines is the result (BD14, BD13 and BD16, in Tables III and V).

On the other hand, 5-benzamido-2-phenylbenzoxazole (D23) was found to be the most efficient compound in the drug-resistant colon carcinoma cell lines HT-29RNOV and HT-29RDB, showing RR values of <0.05 and <0.06, respectively. If the activity data (Table IV) are compared, it reveals that either having a methylene bridge between the benzoxazole structure and phenyl ring on position 2 or substituting the para positions of the phenyl moieties on the 2nd and 5th positions of the benzoxazole structure are markedly reducing and/or eliminating the antineoplastic activity in these drug-resistant colon carcinoma cell lines (D23, BD3, BD13, BD14 and BD16 in Table IV).

In general, structure–activity relationships on this set of DNA topoisomerase II-targeting compounds in various drug-resistant cancer cell lines indicate that 2-*p*-chlorobenzylbenzoxazole derivatives holding a benzamido group instead of phenylacetamido moiety on the position 5 of the fused heterocyclic system is more favorable for the antineoplastic activity in several drug-resistant cancer cell lines derived from gastric, pancreatic and breast cancer. In some cases, substituting the para position of phenyl moiety at the benzamido group on position 5 of the 2-*p*-chlorobenzylbenzoxazole structure with a nitro group is enhancing the activity such as seen in drug-resistant cancer cell lines EPG85-257RNOV and EPG85-257RDB derived from gastric carcinoma and the melanoma line MeWoETO1. On the contrary, a nitro group on this para position, instead of a brome atom or an ethyl group, which is more lipophilic and have low-resonance effects than a nitro group, is diminishing and/or eliminating the antineoplastic activity in the drug-resistant cell lines EPP85-181RNOV, EPP85-181RDB and MCF7/Adr. However, in the drug-resistant colon carcinoma cell lines HT-29RNOV and HT-29RDB the coplanar conformation of 2-phenylbenzoxazole, holding a benzamido group on position 5, was found to be more effective than the 2-benzylbenzoxazole analogous, which is providing flexibility to the structure having a methylene bridge between the benzoxazole structure and phenyl ring on position 2.

However, there is no meaningful structure–activity relationships found on this set of training compounds against the other tested drug-resistant sublines MCF-7RNOV and MDA-MB-231RNOV derived from breast cancer, and D79/86RNOV derived from fibrosarcoma, which exhibited certain resistances to the most of the tested compounds.

The observed antineoplastic activity data on this set of compounds against drug-resistant cell lines derived from different human cancer cell lines indicated that the compounds containing an aromatic fused heterocyclic system possessing a 5 member ring moiety in their structure, such as benzoxazoles, are more preferable than the compounds having a 6 member no aromatic ring system, such as benzoxazines (BSO3 and BSO4 in Tables II–VIII). Compounds, consisting of 2-*p*-chlorobenzylbenzoxazole structure holding a para-substituted-benzamido group on position 5 at the fused ring system such as BD13, BD14 and BD16 exhibited high antineoplastic activities against the drug-resistant gastric carcinoma sublines EPG85-257RNOV and EPG85-257RDB, the pancreatic carcinoma lines EPP85-181RNOV and EPP85-181RDB, the breast cancer line MCF-7Adr, the fibrosarcoma line D79/86RNOV and the melanoma line MeWoETO1. Compound D23 with a 2-phenylbenzoxazole structure holding a benzamido group on the 5th position of benzoxazole was found to be highly efficient in the multidrug-resistant variants HT-29RNOV and HT-29RDB derived from colon carcinoma. On the other hand, compound D24, which is holding a 2-thienyl ring instead of phenyl in the benzamide function on position 5 of the 2-phenylbenzoxazol structure, exhibited the highest antineoplastic activity among the tested compounds in the drug-resistant breast cancer cell line MDA-MB-231ROV. The opened ring compound X12, which is the possible metabolite form of the benzoxazole structure, was also exhibited some antineoplastic activities against the tested drug-resistant cancer cell lines. This observation suggests that some prolonged antineoplastic activities for the metabolite forms of the benzoxazoles can be expected.

In general, the expression levels of Topo IIα and IIβ encoding mRNAs and proteins showed a good correlation in a given cell model. However, in a few cases, there was not a strong correlation. For example, the cell line HT-29RNOV showed a marked down regulation of Topo IIβ on protein level, but merely a moderate decrease in the expression level of the Topo IIβ encoding mRNA; or the parental, drug-sensitive gastric carcinoma cell line EPG85-257P showed a 2-fold higher expression of the Topo IIβ encoding mRNA than the parental colon carcinoma cell line HT-29, but the detectable amounts of Topo IIβ proteins were similar. These observations indicate that the Topo II isoenzymes are differentially posttranscriptional or posttranslational regulated in different cell systems. Since previous studies already reported the same phenomenon of disaccording Topo II mRNA and protein levels in drug-resistant cell models derived from different tumor entities, e.g., from gastric carcinoma,[38] pancreatic carcinoma[19] or melanoma,[11] these observations are not astonishing.

In conclusion, some of the compounds, in particular, compounds BD 13, BD 14, BD 16, D 23, and D 24, may be useful for the treatment of multidrug-resistant cancer cells with cross resistance against "classical" Topo II-targeting drugs such as epipodophyllotoxins, mitoxantrone or anthracyclines. However, for further consideration to use these compounds as drugs, additional experiments including *in vivo* studies are necessary.

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