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Activities of Novel Nonglycosidic Epipodophyllotoxins in Etoposide-Sensitive and -Resistant Variants of Human KB Cells, P-388 Cells, and *In Vivo* Multidrug-Resistant Murine Leukemia Cells

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SUMMARY

Previous structure-activity studies of the antitumor compound etoposide (VP-16) have suggested that replacement of the glycoside moiety could afford therapeutically active analogues with different biochemical determinants for cellular accumulation and drug resistance. In the present report, 10 analogues of VP-16 in which the glycosidyl moiety was replaced with alkyl or arylamino substituents exhibited 5–10-fold better binding affinity for topoisomerase II/DNA complex in human KB cells. A similar increase in the binding affinity was observed in an isolated-nuclei model. The analogues displayed greater or comparable potency to VP-16 in cell growth-inhibition studies and were less affected by cell membrane-associated drug resistance mechanisms, as exemplified by overexpressions of P-glycoprotein multidrug-resistance gene or multidrug resistance-associated protein. Interestingly, in animal studies, analogues least affected by the membrane transport-deficiency phenotypes exhibited low therapeutic index values, thus suggesting that highly efficient modulation of cellular membrane transport defects could perturb the selectivity of antitumor agents for cancer cells. This report also suggests a new method of quantifying drug-induced protein-linked DNA breaks by graphically determining the apparent dissociation-inhibition constant (K_{cl}) for the inhibitors.

The epipodophyllotoxin group of compounds has yielded two commonly used antineoplastic agents, VP-16 and VM-26 (1, 2) (Fig. 1). The mechanism of action of this group of compounds is believed to primarily involve their ability to stabilize the reaction intermediate termed cleavable complex during the resealing of DNA by DNA topoisomerase II (3–6). During this action, the enzyme becomes covalently bound to the broken strands of DNA and fails to restore the intact DNA structure (7, 8). Like most anticancer agents, therapeutic uses of the epipodophyllotoxins are often hindered by acquired drug-resistance mechanisms developed by cancer cells. Two primary mechanisms of cellular resistance to this group of compounds are decreases in cellular drug accumulation and DNA topoisomerase II activity (9–11). The drug accumulation defect could be mediated either by P-glycoprotein-induced drug efflux (*MDR1*) (12) or by non-P-glycoprotein mechanism(s) such as that exemplified by the overexpression of MRP (13, 14).

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Classic attempts to circumvent membrane protein-mediated drug resistance by the use of modulators are often compromised by the secondary toxicities of the modulating agent (for a review, see Ref. 15). It is therefore of great clinical interest to generate active anticancer agents that could OK as is. evade the transmembrane protein-mediated drug resistance. During our previous attempts to discover novel epipodophyllotoxin analogues, we observed that the glycosidyl moiety on VP-16 could have a significant role in the cellular drug accumulation in VP-16-resistant cell lines (16, 17). The sugar unit, however, was not necessary for the cytotoxicity or the DNA topoisomerase II inhibition. It is therefore conceivable that replacement of the glycosidyl moiety could afford therapeutically active VP-16 analogues with different biochemical determinants for cellular uptake. Such compounds could also be expected to be less affected by the membrane-protein transport-mediated drug-resistance mechanisms that affect VP-16.

In the current study, we designed several epipodophyllotoxins in which the glycosidic unit of VP-16 was replaced with various amino substituents containing varying degrees

ABBREVIATIONS: VP-16, etoposide; MRP, multidrug resistance-associated protein; VM-26, teniposide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

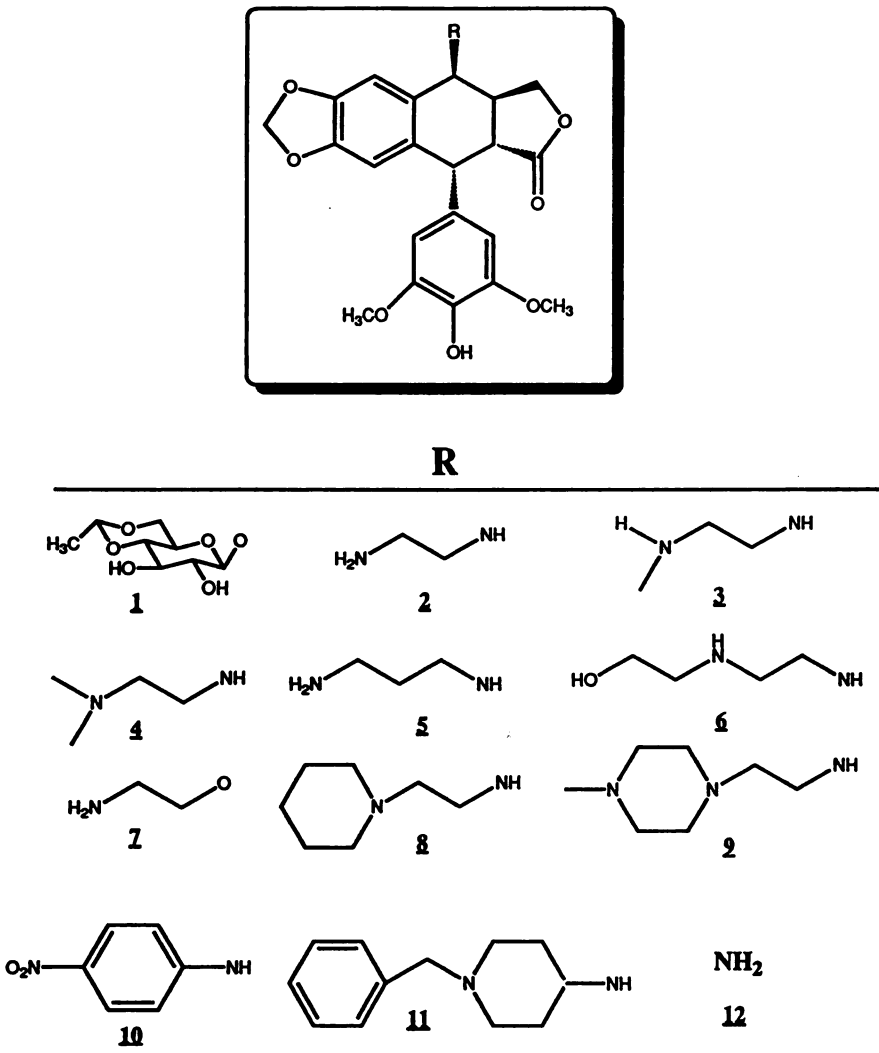

Fig. 1. Chemical structures of the nonglycosidic epipodophyllotoxin analogues (2-12) of VP-16.

of hydrophobicity and cationic charges. Their activities were evaluated in VP-16-sensitive human KB cells and the two resistant variants with deficient drug accumulation phenotypes. One of the cell lines has decreased DNA topoisomerase II activity. Similar studies were also performed with murine P-388 leukemia cells and the multidrug-resistant variant, P-388/Adr. Therapeutic efficacy of selected analogues was evaluated *in vivo* in mice implanted with the multidrug-resistant P-338 leukemia cells. The ability of the analogues to stabilize the topoisomerase II/DNA cleavable complex was studied in both whole-cell assays and an isolated-nuclei model. Furthermore, because the drug-stabilized topoisomerase II/DNA complex follows the classic Michaelis-Menten pattern with a hyperbolic saturation curve and linear double reciprocal plot, we suggest determination of apparent K_{di} values as a rational method of quantifying drug-induced protein-linked DNA breaks.

Materials and Methods

Cells. Human nasopharyngeal carcinoma KB cells were obtained from American Type Culture Collection (Bethesda, MD). The VP-16-resistant cell lines KB7D and KBV20C were obtained by sequential selection in culture to increasing concentrations of VP-16 and vincristine, respectively (18, 19). Cell lines were maintained in RPMI 1640 medium containing 5% fetal bovine serum and 100 μg/ml kanamycin. The culture media for the resistant lines were also supplemented with 7.0 μm VP-16 for KB7D and 20 nM vincristine for KBV20C. The murine leukemia cells P-388 and the P-388/Adr, which were selected for resistance to adriamycin, were obtained from Dr. W. H. Hait (Yale School of Medicine, New Haven, CT). Both leukemia cell lines were cultured as suspensions in RPMI 1640 medium containing 10% fetal bovine serum and 100 μg/mL kanamycin. All cells were grown under humidified air containing 5% CO_2 at 37°. Cells were tested periodically for *Mycoplasma* with the Gen-Probe Rapid Detection System (San Diego, CA). All drug-resistant cell lines were maintained in drug-free medium for ≥3 days before biological activity assays.

Animals. Adult female BDF_1 mice (8–10 weeks old) weighing 18–20 g were obtained from the National Institutes of Health (Bethesda, MD). Animals used in the antitumor activity studies were implanted with tumor cells by intraperitoneal injection.

Chemicals. VP-16 was purchased from Sigma Chemical Co. (St. Louis, MO). The nonglycosidic epipodophyllotoxin analogues were synthesized (Dr. K. H. Lee, School of Pharmacy at University of North Carolina at Chapel Hill, NC). Drugs were stored as 20 mM stock solutions in dimethylsulfoxide at −20° and diluted in sterile water immediately before use. Radiolabeled thymidine ([2-^{14}C]thymidine; specific activity, 55 mCi/mmol) was obtained from ICN Radiochemicals (Costa Mesa, CA). Other chemicals were of the highest reagent grade available.

Cytotoxicity studies. KB cells and the resistant variants were seeded at a density of 10^4 cells/2 ml drug-free medium in 16-mm^2

wells and incubated overnight before drug treatment. Subsequently, cells were continuously exposed to various concentrations of drugs for 72 hr at 37°. The cell growth inhibition was determined with the methylene blue assay according to the method of Finlay *et al.* (20). P-388 and P-388/Adr were seeded at a density of 10^5 cells/10 ml drug-free medium in 25-cm^3 flasks and incubated overnight before drug treatment. Initial and final cell numbers were determined with a Coulter electronic particle counter (Hialeah, FL). IC_{50} values were determined from the interpolation of plotted data.

Therapeutic activity studies. Six BDF_1 mice implanted 1 day earlier with 10^6 P388 or P388/Adr cells were treated by intraperitoneal injection with various doses of the anticancer compounds. Animals were observed and weighed daily. Therapeutic efficacy was determined from the survival time of the mice bearing leukemia and the increase in the life spans of the drug-treated animals compared with the untreated animals.

***In vivo* toxicity studies.** Five or six normal (nontumor-bearing) BDF_1 mice were administered various doses of drugs by intraperitoneal injection at a schedule of once daily for 5 consecutive days. Treated and untreated control animals were observed and weighed daily until the termination of the experiment on day 40 after drug treatment. At the end of the experiment, treated animals were evaluated for gross pathological changes in the main organs. Toxicity was determined from death/survival ratio of the treated versus control animals. The LD_{50} also was calculated.

Cellular protein-linked DNA breaks. Approximately 5×10^6 KB cells logarithmically growing in 20 ml drug-free medium were labeled with [^{14}C]thymidine (0.05 μCi/μl) for 18–24 hr. Labeled cells were washed twice with 10 ml phosphate-buffered saline, trypsinized, and resuspended in a nonradioactive culture medium at a density of 2×10^5 cells/ml. After ≥1-hr incubation at 37°, cells were treated in triplicate with various concentrations of drugs for 60 min. Drug-treated cell and control cells were collected by centrifugation and analyzed for protein-linked DNA breaks according to the potassium/sodium dodecyl sulfate method of Rowe *et al.* (21). Apparent K_{di} values were determined graphically from the double-reciprocal plots of the saturation curves. Maximal topoisomerase II/DNA B_{max} values were 20% of the labeled DNA.

Nuclear protein-linked DNA breaks. Logarithmic phase growing KB cells labeled with thymidine as previously described were used for nuclei preparation. Nuclei were isolated under isotonic conditions according to the method of Glisson *et al.* (22). The isolated nuclei were suspended in the reaction buffer (40 mM Tris, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM EGTA) at density of 2×10^5 nuclei/ml. Nuclei were exposed to various concentrations of drug for 30 min at 37°. Protein-linked DNA breaks were assessed as previously described. The K_{di} values were also determined as previously described. The maximum topoisomerase II/DNA association constant was 25% of labeled DNA.

Results and Discussion

Target-enzyme inhibition. DNA topoisomerase II has long been suggested to be the primary mechanistic target for the anticancer epipodophyllotoxins VP-16 and VP-26 (23, 24). The enzyme causes transient double-stranded DNA breaks (cleavable complex) during the topological rearrangement of DNA and in the process is covalently bound to 5′ end of the broken DNA (7, 8). Epipodophyllotoxins and other drugs interfere with this process by stabilizing the cleavable complex, presumably via a mechanism that involves formation of a ternary complex (8). With the use of protein-denaturing agents, this complex can be permanently trapped and analyzed (6, 21). A steady state equilibrium plot of the protein/DNA complex versus epipodophyllotoxin concentrations yields a hyperbolic saturation curve with a straight line double-reciprocal plot analogous to Michaelis-Menten kinetics (Fig. 2). It is therefore possible to determine the maximal protein/DNA B_{max} and apparent K_{di} values for the inhibitors.

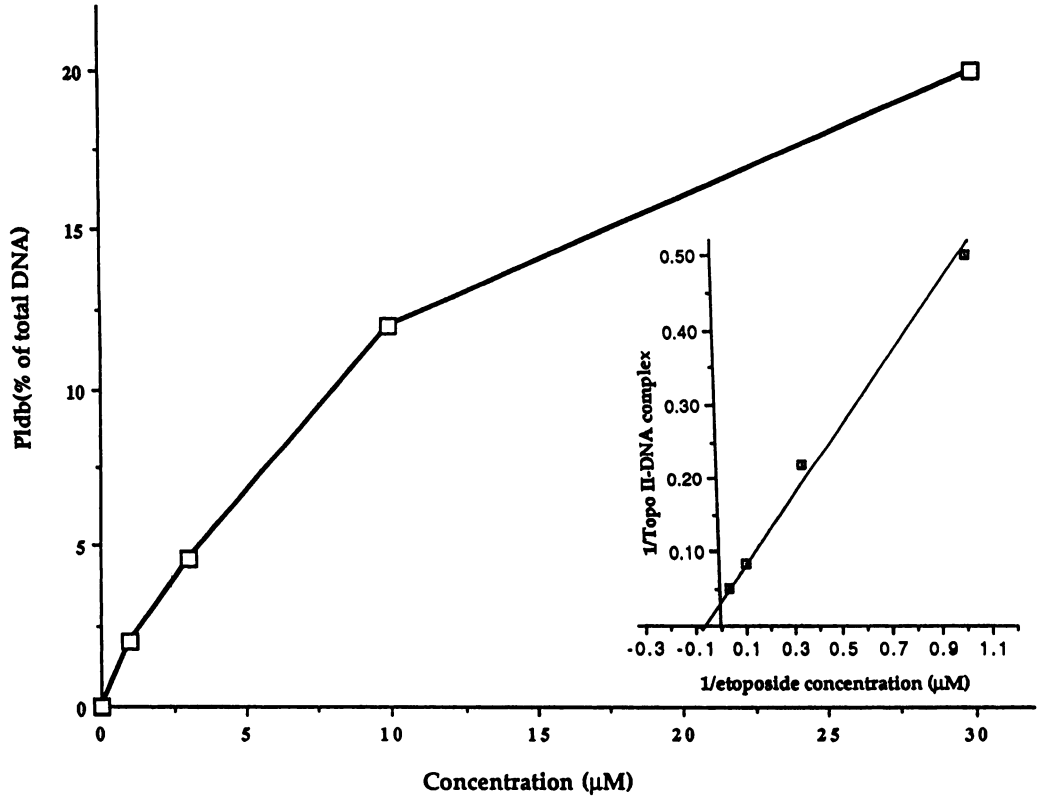

Fig. 2. Typical saturation curve of VP-16-induced stabilization of topoisomerase II/DNA complex as determined by protein-linked DNA breaks (*Pldb*) assay in human KB cells. *Inset*, double-reciprocal plot of the saturation curve. *X*-axis intercept of the linear line, apparent K_{di} for the tested compound. *Y*-axis intercept, maximum protein-linked DNA breaks (percent of labeled DNA) possible for the particular experiment (B_{max}).

TABLE 1
Activities of epipodophyllotoxin analogues in human KB cell variants

Compound	Apparent K_{di}[a]		IC_{50} values for human KB cell variants		
	Whole-cell	Isolated-nuclei	KB	KB7D	KBV20C
	μM			μM	
1	20 ± 5.0	30 ± 10	0.32 ± 0.02	18 ± 1.60	1.53 ± 0.20
2	5.8 ± 1.1	16.3 ± 10	0.13 ± 0.01	4.9 ± 2.4	0.77 ± 0.03
3	4.4 ± 0.1	13.3 ± 9.4	0.20 ± 0.03	1.31 ± 0.72	0.31 ± 0.10
4	1.9 ± 1.2	10 ± 4.7	0.29 ± 0.08	0.61 ± 0.2	0.37 ± 0.11
5	4.0 ± 1.7	8.3 ± 2.4	0.57 ± 0.24	4.3 ± 0.7	2.1 ± 0.35
6	5.8 ± 0.8	2.7 ± 0.9	0.59 ± 0.02	4.6 ± 1.1	3.0 ± 0.8
7	9.7 ± 0.6	15 ± 1.1	0.20 ± 0.02	1.9 ± 0.9	0.26
8	4.4 ± 3.1	7.5 ± 3.2	0.34 ± 0.04	0.31 ± 0.16	0.52 ± 0.15
9	3.2 ± 2.5	8.1 ± 2.7	0.35 ± 0.05	1.06 ± 0.4	0.3
10	5.0 ± 0.0	3.5 ± 2.1	0.49 ± 0.19	7.7 ± 1.2	0.29 ± 0.1
11	1.7 ± 0.7	4.0 ± 0.0	0.03 ± 0.0	0.06 ± 0.01	0.02 ± 0.01
12	90 ± 10	93 ± 12			

[a] K_{di} values were derived from the double reciprocal plots of drug-induced protein-linked DNA breaks based on the maximal topoisomerase II DNA association constants of 20% and 25% total DNA for whole-cell and isolated-nuclei assays, respectively. Reported data represent average ± standard deviations values determined from at least two experiments conducted in triplicate.

Unlike other groups of topoisomerase II inhibitors, epipodophyllotoxins bind neither the enzyme nor DNA alone; the K_{di} values thus reflect the apparent binding affinity of the compounds for the topoisomerase II/DNA complex. We used this method to determine the K_{di} values for VP-16 (**1**) and the novel nonglycosidic epipodophyllotoxins in human KB cell and isolated nuclei (Table 1). The nuclear assay was necessary to account for the differences in cellular uptake of the compounds. Most of the tested compounds exhibited 3–10-fold better binding affinity for the topoisomerase II/DNA complex than did **1** (K_{di} = 20 ± 5 μM) in the whole-cell assay. Similar differences in the binding affinity were observed in the nuclear model. The analogues with tertiary amine substituents, such as compounds **4**, **8**, **9**, and **11**, were more potent inhibitors, with K_{di} values of ~2.0–4.0 μM in the cell assay. Interestingly, the drug-induced stabilization of the topoisomerase II/DNA complex was less in the nuclear assay than in the whole-cell studies; a similar observation has been reported (25). We suggest that determinations of the maximal topoisomerase II/DNA B_{max} and apparent K_{di} values for the topoisomerase II poisons provide a rational method of comparing the inhibitory activities of each group of compounds in different experiments.

Cytotoxicity studies. The tumor cell growth-inhibitory activities of the nonglycosidic compounds with K_{di} values better than or comparable to that of **1** were evaluated in the VP-16-sensitive human KB cells and the two resistant variants, KB7D and KBV20C (Table 1). Phenotypic characteristics of KB7D cells include a two fold decrease in topoisomerase II/DNA activity and protein level, collateral increase in topoisomerase I level, and MRP-mediated decrease in drug accumulation (18).[1] KBV20C is primarily characterized by the overexpression of *MDR* and the concomitant increase in drug efflux (19). The novel compounds displayed greater or comparable potency to that of **1** in the studies with KB cell and were less affected by resistance phenotypes exhibited by the resistant lines. Although KB7D and KBV20C exhibited ~60- and 5-fold resistance, respectively, to **1**, the IC_{50} values for most of the nonglycosidic compounds were virtually unchanged for KBV20C and moderately increased for KB7D (1.2–10-fold). The moderate level of drug-resistance displayed by KB7D was most likely due to the decreased activity of the target enzyme topoisomerase II in that cell line. The structure-activity pattern exhibited by the tested compounds toward the resistant lines were in the following order: tertiary amino substituents ≫ secondary amino ≫ primary amino ≫ arylamino ≫ VP-16. It thus appears that a increase in cationic charge of the substituents decreases the affinity for the drug resistance-associated transmembrane proteins, as exemplified by P-glycoprotein in KBV20C and MRP in KB7D. Drug accumulation studies based on the relative amount of protein-linked DNA breaks induced by **1**, **4**, and **10** in whole-cell assays in the presence and absence of membrane-transport deficiency modulators verapamil or BIBW in the three KB cell lines attest to this hypothesis (data not shown).

Comparative studies of the activities of **1**, **10**, and **11** were also conducted in murine P-388 leukemia cells and the adriamycin-selected multidrug-resistant variant P-388/Adr. The mechanism for drug resistance in P-388/ADR is multifactorial and includes P-glycoprotein-mediated drug efflux, decreased topoisomerase II activity, enhanced DNA repair, and increased glutathione transferase activity (26–28). In our study, the cell line exhibited cross-resistance to different classes of agents (Table 2), including adriamycin (67-fold), vincristine (27-fold), **1** (397-fold), **10** (12-fold), and **11** (14-fold). Cotreatment with multidrug-resistance reversal agent verapamil completely abolished the resistance to vincristine and caused ~6-fold decrease in the resistance to adriamycin. However, the resistance to epipodophyllotoxin analogues was only slightly diminished; also noteworthy is that **10** and **11** were ~40- and 70-fold more potent than **1**, respectively, in growth-inhibitory activities toward the resistant P-388/Adr. The tertiary amino-substituted analogous **11** was ~5-fold more potent than **1** and **10** against the sensitive parental leukemia line, which was also susceptible to chemosensitization by verapamil.

Animal studies. Therapeutic activities of the epipodophyllotoxin analogues **1**, **10**, and **11** were also compared *in vivo* in BDF_1 mice with actively growing multidrug-resistant P-388/ADR tumor cells, and toxicity studies were conducted with non-tumor-bearing animals. As depicted in Table 3, **10**

[1] C. L. Gaj, I. O. Anyanwutaku, Y. H. Chang, and Y.-C. Cheng, unpublished observations.

TABLE 2

IC_{50} values of epipodophyllotoxin analogues against P-388 and the multidrug-resistant P-388/Adr cells

Compound	P-388	P-388/Adr	Relative resistance[a]
	nM		
Adr	45 ± 3.0	3020 ± 383	67
Adr + VPL[b]	9.4 ± 2.6	113 ± 20	12
VCR	3.1 ± 0.4	83 ± 3.0	27
VCR + VPL	0.6 ± 0.1	0.7 ± 0.2	1
1	33 ± 1.0	13,100 ± 120	397
1 + VPL	9.4 ± 1.2	2300 ± 110	245
10	28 ± 1.0	343 ± 20	12
10 + VPL	23 ± 3.3	138 ± 26	6
11	6.1 ± 2.6	150 ± 43	24.5
11 + VPL	8.2 ± 2.8	162 ± 94	19.7

[a] Relative resistance was determined from the ratio of the IC_{50} values for P-388/Adr and P-388 cells.
[b] Verapamil is 10 μM. Adr, adriamycin; VPL, verapamil; VCR, vincristine.

TABLE 3

Therapeutic activity of epipodophyllotoxin analogues in P-388/Adr tumor cell-bearing mice

Compound	LD_{50}	Increase in life span[a]	p[b]
	mg/kg	%	
1	32.5	32	<0.05
10	30.0	46	
1	32.0	22	>0.5
11	6.5	20	

[a] Reported increase in life span data represent values obtained at maximum tolerated doses (approximately one third of the LD_{50}) for the tested compounds and include doses of 10 mg/kg for 1 and 10 and 2.3 mg/kg for 11.
[b] Reported values are significant as verified by Student's t test.

increased the life span of the tumor-bearing mice significantly (increase in life span, 46.4%; $p < 0.05$) better than did **1** (increase in life span, 32.0%). Interestingly, the tertiary amino-substituted analogue **11**, which was very potent against varieties of tumor cells in culture and was unaffected by the *MDR*- or MRP-mediated drug-resistance mechanisms, did not significantly increase the life span of the tumor-bearing animals (increase in life span, 22.0%; $p > 0.5$). The analogue **11**, however, was ~3-fold more toxic to the animals (LD_{50}, 6.5 mg/kg) than either **1** or **10**. This finding confirms that extreme modulation of transmembrane protein-mediated drug resistance mechanism(s) perturbs the selectivity of anticancer drug for tumor cells. Even more significantly, it suggests that evasion of MRP and not necessarily of P-glycoprotein (as with **11** and **10**, respectively; Table 1) predisposes normal cells to toxicity of xenobiotics. Both P-glycoprotein and MRP are membrane proteins that are commonly expressed in both normal and tumor cells (29–32). Although the functional role of MRP has not been elucidated, P-glycoprotein is suggested to protect normal cells from cytotoxic agents by excluding these agents from the cells (33). Based on our findings, it is tempting to speculate that MRP may be even more significant than P-glycoprotein in protecting normal cells from toxic agents. Therefore, aggressive efforts to modulate this activity in tumor cells with either membrane transport modulators such as verapamil (34) or cytotoxic anticancer agents that completely evade the proteins, such as **11**, could be countercontributory as such efforts also expose normal cells to the toxicity of the therapeutic agents. This report is the first demonstration of toxicity to normal tissues by anticancer agents that evade membrane protein-associated multidrug resistance. Our findings also suggest that only moderate evasion of these resistant phenotypes, as exemplified by the analogue **10**, or moderate modulation with carefully determined doses of modulating agents could yield better therapeutic efficacy against P-glycoprotein- and MRP-overexpressing tumors.

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