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Selective Cytotoxicity of Topoisomerase-Directed Protoberberines against Glioblastoma Cells

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ABSTRACT. Protoberberines are a new class of organic cations that are dual poisons of topoisomerases I and II. Certain protoberberines exhibit greater *in vitro* cytotoxicity against cell lines derived from solid tumors than from leukemias. Using a group of seventeen different protoberberine analogs, the structural basis for selective cytotoxicity toward sensitive SF-268 glioblastoma cells as compared with resistant RPMI 8402 lymphoblast cells was explored. The selective cytotoxicity is associated with the presence of an imminium ion and other structural features of protoberberines, and is not shared by drugs such as camptothecin, doxorubicin, vinblastine, and etoposide, which are either equally or more cytotoxic against RPMI 8402 cells than SF-268 cells. The selective cytotoxicity of protoberberines against SF-268 over RPMI 8402 cells is not due to differences in topoisomerase levels or known drug efflux systems such as multidrug resistance (*MDR1*) and multidrug-resistance protein (MRP). Comparative *in vitro* studies of the accumulation of coralyne, a fluorescent protoberberine, into sensitive and resistant cells demonstrated a correlation between drug accumulation and selective cytotoxicity. Inhibitors of coralyne uptake included several protoberberine-related compounds. Of these, palmatine, a minimally cytotoxic protoberberine, both inhibited coralyne accumulation and reduced cytotoxicity against SF-268 cells, but not against RPMI 8402 cells. Despite the structural resemblance of protoberberines to catecholamines, our experiments using inhibitors and cells expressing biogenic amine uptake systems have ruled out the involvement of biogenic amine uptake$_1$, uptake$_2$, and vesicular monoamine transport systems. Uptake systems remaining as candidates, supported by preliminary data, include transport via vesicles derived from specialized membrane invaginations and selected carrier-mediated organic amine transport systems. BIOCHEM PHARMACOL **56**;9: 1157–1166, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. DNA topoisomerase poison; biological transport; selective cytotoxicity; tumor cells, cultured; antineoplastic agents; berberines

Recent reports from our laboratories have established that synthetic protoberberine analogs are potent poisons of topoisomerases I and II [1–3]. These compounds are analogs of berberine, an isoquinoline plant alkaloid. Extracts of plants containing berberine have been used widely in Chinese and Indian folk medicine for many years, and they demonstrate weak antitumor activity in mice [4]. Members of the greater family of berberine-related isoquinolines include several naturally occurring and synthetic agents possessing significant and specific pharmacological activity [reviewed in Refs. 4 and 5].

Several of our protoberberine analogs have demonstrated significant cytotoxicity against cultured tumor cell lines [2, 3]. In the present study, we surprisingly found that some protoberberines, unlike many clinically used anticancer drugs tested, exhibited selective cytotoxicity against certain solid tumor-derived cells, including SF-268 glioblastoma cells, as compared with leukemia cells.

Previously identified selectively cytotoxic agents include certain ellipticinium salts, which constitute a class of topoisomerase II poisons [6, 7]. These agents exhibited CNS-tumor-specific killing in a screen of the NCI panel of 60 cell lines derived from tumors of various cell and tissue types [6–9]. Structure–activity relationship studies of the ellipticinium derivatives showed the substituent required for the selective cytotoxicity against the CNS-tumor cell group to be a quaternized nitrogen at the N^2 position [7]. The protoberberine analogs also contain a permanently charged nitrogen, an imminium ion located at the junction of the B- and C-rings. Another investigational compound exhibiting selective cytotoxicity against CNS-derived tu-

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mors is SarCNU[‖] [10]. In contrast to the ellipticinium salts, none of the nitrogens in SarCNU carries a permanent positive charge.

Mechanisms responsible for selective cytotoxicity include a number of documented possibilities. Those possibly relevant to the present observation include unequal expression of drug targets in sensitive and resistant cells [11, 12], cell-type specific expression of pleiotropic resistance mechanisms like multidrug resistance (*MDR1*) [13] or multidrug-resistance protein (MRP) [14], and cell-type specific expression of carrier systems responsible for fortuitous uptake of the drug. For example, the selective cytotoxicity of SarCNU is associated with cellular uptake of this drug through a catecholamine carrier known as uptake$_2$ [15]. For the CNS-selective ellipticiniums, preliminary studies revealed that a cellular uptake system may be responsible for the cell-specific cytotoxicity in the sensitive gliomas and/or astrocytomas [8, 16]. However, the selective accumulation of drug can be reversed by depolarizing the sensitive cells, and the specific uptake system has not been identified [8, 17].

We have explored the structural basis of and the general mechanism responsible for the selective cytotoxicity of protoberberine analogs against SF-268 glioblastoma compared with leukemia cells. The results identify the iminium nitrogen and A-ring substituents as important in determining selective cytotoxicity of protoberberine analogs. Furthermore, our studies have established that selective drug accumulation into SF-268 glioblastoma cells is responsible for their selective cytotoxicity.

MATERIALS AND METHODS

Materials

cDNAs for biogenic amine transporters were provided by Dr. George Uhl, NIDA Addiction Research Center (dopamine transporter); Dr. Susan Amara, Vollum Institute (norepinephrine transporter); and Dr. Beth Hoffman, National Institutes of Mental Health (serotonin transporter). The BCA protein assay system was purchased from Pierce, Empigen BB from Calbiochem, and the ECL system used for immunoblots from Amersham. Anti-topoisomerase I antibodies were prepared from sera from scleroderma patients [18].

Protoberberines and Related Compounds

Coralyne was purchased from the Sigma Chemical Co., and palmatine from the Aldrich Chemical Co. Both were dissolved at 1 mg/mL in water. Nitidine was synthesized as previously described [19]. All other protoberberine compounds were synthesized as the chloride or methylsulfate salts as reported previously [1, 3]. Compounds utilized for cytotoxicity assays were dissolved in DMSO. DMI was synthesized by oxidation of 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline in decalin in the presence of 10% palladium on carbon. Reaction of this product with methyl iodide provided the quaternary ammonium derivative, MDMI.

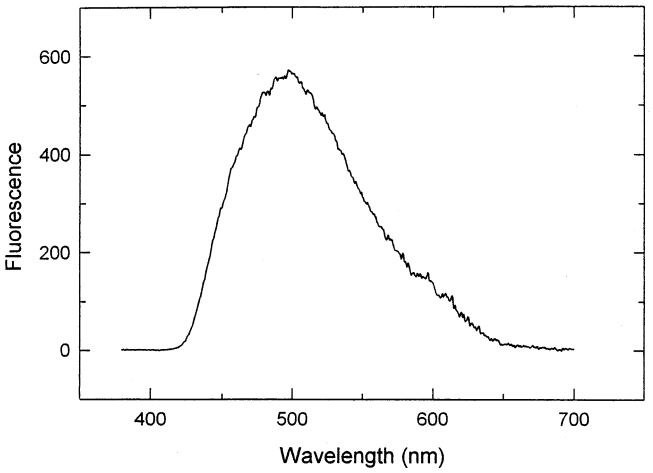


FIG. 1. Fluorescence emission spectrum of coralyne. Excitation was on the 322 nm shoulder of the excitation spectrum. Fluorescence was recorded in arbitrary units on a Perkin–Elmer MPF-66 spectrofluorometer.

The fluorescence intensity of coralyne is sufficient for assay of drug accumulation in cells. Figure 1 shows the emission spectrum of 5 μM coralyne dissolved in BSS (0.15 M NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM HEPES, pH 7.2), a simple balanced salt solution utilized for the accumulation studies. Excitation was at 322 nm on a shoulder of the absorption spectrum (not shown). Fluorescence of coralyne was not perturbed by pH, high salt, PEG, or cell extracts prepared as described below. In the concentration range utilized, coralyne fluorescence varied linearly with concentration (not shown). Two Perkin–Elmer MPF-66 spectrofluorometers were utilized for fluorescence spectra and for fixed wavelength measurements as described below.

Cell Lines, Cell Culture, and Cytotoxicity Measurements

LLC-PK1 cells expressing cDNAs for the biogenic amine transporters (specific for dopamine, norepinephrine, or serotonin) were provided by Dr. G. Rudnick, Department of Pharmacology, Yale University [20]. SF-268 glioblastoma cells were obtained from the National Cancer Institute; KB3-1 epidermal cells from Dr. M. M. Gottesman, National Cancer Institute [21]; CEM lymphoblast cells from Dr. W. Beck, St. Jude Hospital [22]; and RPMI 8402 lymphoblast cells from Dr. T. Andoh, Gifu School of Medicine [23].

Culture of the human lymphoblast RPMI 8402 cells was as described previously [2]. All other cell lines were grown at 37° in 5% CO_2 and maintained by regular passage in

[‖] *Abbreviations:* SarCNU, (2-chloroethyl)-3-sarcosinamide-1-nitrosourea; DMI, 6,7-dimethoxyisoquinoline; MDMI, 6,7-dimethoxy-*N*-methylisoquinolinium iodide; BSS, balanced salt solution; ECL, enhanced chemiluminescence; PEG, polyethylene glycol; and MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

RPMI 1640 medium including penicillin/streptomycin and supplemented with 10% heat-inactivated fetal bovine serum.

The IC_{50} for each of the drugs was determined by the MTT-microtiter plate tetrazolium cytotoxicity assay [24, 25]. The cells were exposed continuously for 4 days to differing drug concentrations and were assayed at the end of day 4. Data for each concentration and the no-drug control were collected in at least two separate experiments using three or six replicate wells. The IC_{50} was determined either by inspection of log dose versus survival plots of the data, or by fitting the data to a sigmoidal inhibitory effect model, using GraphPad Prism Version 2.0 software.

Immunoblot Analysis of Topoisomerase I Protein in RPMI 8402 and SF-268 Cells

To quantify the amount of topoisomerase I protein in RPMI 8402 and SF-268 cells, 1×10^6 cells were pelleted and lysed with 1 × SDS sample buffer (50 mM Tris–Cl, pH 6.8, 15% sucrose, 2 mM EDTA, 3% SDS, 5% (w/v) β-mercaptoethanol, 0.01% bromophenol blue). The samples were heated for 5 min in a boiling water bath, and the protein from the equivalent of 3×10^5 cells was displayed on a 6% SDS gel. Proteins were transferred to nitrocellulose membranes. Topoisomerase I protein was detected after incubation with anti-topoisomerase I antibodies obtained from scleroderma patients followed by incubation with secondary sheep anti-human IG linked to horseradish peroxidase and the ECL protocol.

Coralyne Accumulation Studies

Coralyne (1 mg/mL in water) was added to the indicated final concentrations to 1.5-mL cultures of RPMI 8402 cells growing in tubes and to 3.0-mL cultures of SF-268 cells in 6-well plates. Following incubation at 37° for the indicated time, the RPMI 8402 cells were pelleted for 5 sec in a microcentrifuge, washed once with 1 mL BSS at room temperature, and immediately centrifuged again. SF-268 cells were washed once with 1 mL BSS (room temperature). Both SF-268 and RPMI 8402 cells were solubilized in 250 μL of 1% Empigen BB in BSS. After 10 min at room temperature, 125 μL of 15% PEG 8000 in 2.5 M NaCl was added to precipitate nucleic acids. The mixture was transferred to a microfuge tube and chilled on ice for 20 min followed by centrifugation in a microcentrifuge for 20 min at 4°. A 200-μL aliquot of the supernatant was added to 2.0 mL BSS for determination of fluorescence in a Perkin–Elmer MPF-66 spectrofluorometer, using excitation and emission at 322 and 478 nm, respectively.

A 25-μL aliquot of each supernatant was utilized to measure the protein content with the BCA assay (Pierce). Protein content was used to normalize the fluorescence data for each experiment. The coralyne accumulation data are reported as arbitrary fluorescence units normalized to the quantity of protein noted in the figure legends.

TABLE 1. IC_{50} Values for selected anticancer drugs against SF-268 and RPMI 8402 cells

Compound	IC_{50} (μg/mL) for: SF-268	RPMI 8402
Camptothecin	0.003 ± 0.0006	0.003 ± 0.0007
VP-16	0.40 ± 0.09	0.19 ± 0.02
Doxorubicin	0.064 ± 0.008	0.030 ± 0.006
Vinblastine	0.005 ± 0.0002	0.003 ± 0.0001
Coralyne	0.15 ± 0.02	1.68 ± 0.2*
Nitidine	0.015 ± 0.003	0.2 ± 0.03*
DM-II-32	0.03 ± 0.01	0.4 ± 0.1*

Values are means ± SEM from a single experiment (4 replicates). Data are representative of 2–10 experiments.

* Significantly different from SF-268 ($P < 0.01$).

RESULTS

Selective Cytotoxicity of Certain Protoberberines Toward Nonlymphoblastic Leukemia Tumor Cell Lines

Table 1 shows the IC_{50} values for several cytotoxic drugs that act by poisoning either topoisomerase I or II against SF-268 glioblastoma cells [26] and RPMI 8402 leukemia cells [23]. These drugs exhibited three different patterns of relative cytotoxicity against the two cell lines. Camptothecin, a topoisomerase I poison, showed similar cytotoxic activity toward both cell lines. The topoisomerase II poisons VP-16 and doxorubicin and the tubulin-targeted drug vinblastine were all 2- to 4-fold more cytotoxic toward RPMI 8402 cells than toward the SF-268 cells. These drugs are known substrates of multidrug-resistance systems mediated by P-glycoprotein or multidrug-resistance protein [13, 14]. It is possible that SF-268 cells express more P-glycoprotein than RPMI 8402 cells, since they demonstrate resistance to known substrates of P-glycoprotein and may express P-glycoprotein mRNA [27]. In contrast, coralyne, DM-II-32, and nitidine are *ca.* 10-fold *more* cytotoxic to SF-268 than to RPMI 8402. Coralyne and DM-II-32 are synthetic isoquinoline alkaloids (see Table 3) structurally related to berberine. They contain quaternary (imminium) nitrogens and are potent human topoisomerase I poisons *in vitro* [2]. Nitidine is a benzo[*c*]phenanthridine alkaloid sharing significant structural similarity to coralyne. Nitidine poisons both human topoisomerases I and II *in vitro* [2] and demonstrates topoisomerase I-targeted cytotoxicity in yeast expressing human *TOP1* [2].

We have reported previously the synthesis and *in vitro* topoisomerase poisoning activity of a series of protoberberine compounds [1, 3]. DM-II-32 is one of the most potent human topoisomerase I poisons within this series (see Table 3 for structure) [1]. The cytotoxic activity of DM-II-32 was compared with that of camptothecin against four different tumor cell lines (Table 2). Camptothecin demonstrated IC_{50} values ranging from 0.0009 to 0.004 μg/mL for all the cell lines, while the IC_{50} values for DM-II-32 against these lines fell into two groups. In the first pair of lines, the range was from 0.02 to 0.05 μg/mL, while in the second pair the IC_{50} values were *ca.* 10-fold higher. The sensitive tumor cell

TABLE 2. Comparison of IC_{50} values for camptothecin and DM-II-32 in a series of tumor cell lines

Cell line	IC_{50} (μg/mL) for Camptothecin	DM-II-32
SF-268 (glioblastoma)	0.002 ± 0.0009*	0.02 ± 0.001†
KB3-I(epidermal, oral)	0.004 ± 0.0009*	0.05 ± 0.01‡
RPMI 8402 (lymphoblast)	0.003 ± 0.0009	0.3 ± 0.1
CEM (lymphoblast)	0.0009 ± 0.0006*	0.26 ± 0.1*

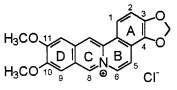
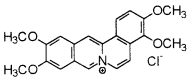
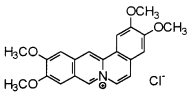
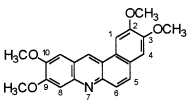
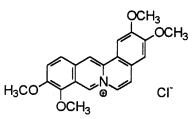
Values are means ± SD for three independent experiments.
* Not significantly different from RPMI 8402 for this drug.
† Significantly different from RPMI 8402 ($P = 0.010$).
‡ Significantly different from RPMI 8402 ($P = 0.026$).

lines included a glioblastoma and an epidermal line, both derived from solid tumors, while the less sensitive cell lines were leukemias. The observation that these solid tumors exhibit enhanced sensitivity over leukemias to killing by protoberberines corroborates results obtained for several related compounds tested against the National Cancer Institute panel of 60 cell lines. These data show that some cell lines derived from solid tumors are quite sensitive to nitidine and coralyne derivatives, while leukemia lines are consistently and uniformly more resistant (data communicated from the Drug Synthesis and Chemistry Branch, NCI).

Table 3 compares the IC_{50} values of seventeen compounds against SF-268 and RPMI 8402 cells. The compounds are grouped according to their core structures (column 2) and substituents (columns 4–6). Core structures I–III all have an imminium ion at position 7 and differ only in the identity and position of substituents on the A ring. As a group, the 3,4-methylenedioxy compounds (based on core structure I) showed both the highest potency against SF-268 and the most prominent *selective cytotoxicity* (IC_{50} ratios all < 0.1). Compounds based on core structures II (3,4-dimethoxy) or III (2,3-dimethoxy) demonstrated variable cytotoxicity as well as variable *selective* cytotoxicity. Most of the core II and III compounds had diminished selective cytotoxicity compared with the core structure I group.

The compounds based on core structure IV (DM-II-98) have their nitrogen moved away from the ring junction position, but possess A and D ring structures identical to those associated with core structure III. The nitrogen in DM-II-98 and DM-II-96 no longer carries a permanent positive charge, but addition of the 7-methyl group in DM-II-100 and DM-II-97a converts it to a quaternary ammonium ion. The entire core structure IV group exhibited a significant decrease in cytotoxic activity, as well as a loss in selective cytotoxicity. The single core V structure shown (palmatine) differs from DM-II-24 (a core III compound) only in the position of the methoxy groups on the

TABLE 3. Protoberberine structures and cytotoxicities for SF268 and RPMI 8402 cells

Core* No.	Structure	Compound	Substituents† 5,6-Dihydro	8-Me	7-Me	IC_{50} (μg/mL) for: SF-268	RPMI 8402	Ratio SF-268: RPMI 8402
I		DM-II-21				0.04‡	0.3‡	0.13
		DM-II-20		+		0.04	1	0.04
		DM-II-33	+			0.08	2.25	0.04
		DM-II-32	+	+		0.02	0.25	0.08
II		DM-II-18				0.04	1	0.04
		DM-II-7		+		10	>10	<1
		DM-II-23	+			0.6	3.5	0.17
		DM-II-22	+	+		2	3.5	0.57
III		DM-I-154				1.5	5	0.3
		Coralyne		+		0.15	1.68	0.09
		DM-II-24	+			1.5	4	0.38
		DM-I-174	+	+		0.27	3	0.09
IV		DM-II-98				10	1.5	6.67
		DM-II-100			+	50	12	4.17
		DM-II-96	+			15	2.5	6.00
		DM-II-97a	+		+	65	>10	<6.5
V		Palmatine	+			3	10	0.3

* *Core* refers to the backbone structures numbered in column 1 and shown in column 2. Note that movement of the nitrogen in core IV requires a changed numbering system.
† *Substituents* refers to the presence of saturation at the 5,6-position (5,6-dihydro) and/or methylation at the 8- or 7-position (8-Me or 7-Me) of the core structures. The plus (+) indicates the presence of the substituent.
‡ Mean of ≥3.

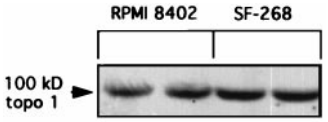


FIG. 2. Immunodetection of topoisomerase I protein in RPMI 8402 and SF-268 cells. Cells were pelleted and lysed directly with SDS sample buffer. Lysates from equal numbers of cells were then subjected to SDS–PAGE followed by transfer to a nitrocellulose membrane and immunoblotting as described in Materials and Methods. Duplicate samples from each cell line were analyzed.

D ring. This structural alteration has a minimal effect on selective cytotoxicity.

Thus, we conclude that the core structure itself is important in determining selective cytotoxicity. Changes in the tested core structures that diminish selective cytotoxicity include: (1) the relocation of the nitrogen atom away from the ring junction position as in core structure IV, and (2) the substituents on the A-ring. All of the selectively cytotoxic structures have the imminium ion at the B–C ring junction. Our data demonstrated that relocation of the nitrogen correlates with a significant loss of selective cytotoxicity regardless of the presence of a quaternary amine, and thus a permanent positive charge, in the new position. On the A-ring, the 3,4-methylenedioxy substitution (core I) exhibited consistently high potency and selective cytotoxicity, while cores II and III were only variably selectively cytotoxic.

We have reported previously that the introduction of either an 8-methyl substituent or saturation at the 5,6-positions of core structures I–III alters topoisomerase I or II poisoning activity [3]. Thus, the seventeen compounds tested here have significantly different specificities for topoisomerase I as compared with topoisomerase II, and their potencies as poisons for the two enzymes differ over a 200-fold range [1]. However, the data reported in Table 3 show that the 5,6 saturation and/or the 8-methyl substitution did not produce consistent effects on IC_{50} values or selective cytotoxicity.

Selective Accumulation of Coralyne into SF-268 Cells

One possible reason for selective cytotoxicity of certain protoberberines (e.g. DM-II-32) is that the levels of the drug targets (i.e. topoisomerase I) may be higher in the sensitive cells. We have compared the topoisomerase I levels in SF-268 and RPMI 8402 cells and found no difference (Fig. 2). The fact that SF-268 and RPMI 8402 cells are equally sensitive to the prototypic topoisomerase I poison camptothecin further supports this conclusion (see Table 1).

Another simple explanation for the selective cytotoxicity could be differences in drug uptake systems between the various cell types. To determine whether uptake differs in SF-268 and RPMI 8402 cells, we have characterized the accumulation of coralyne into SF-268 and RPMI 8402

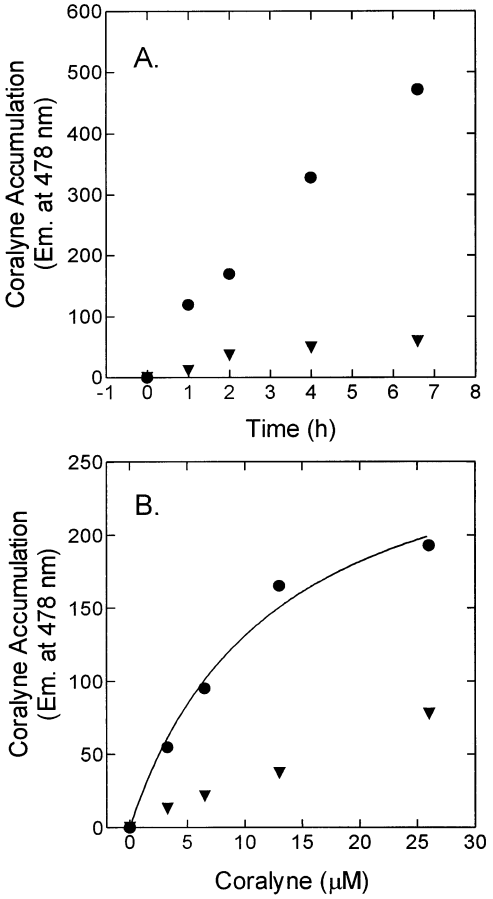


FIG. 3. Accumulation of coralyne in SF-268 and RPMI 8402 cells. Coralyne accumulation was measured as described in Materials and Methods, and is reported in arbitrary fluorescence units. Panel A shows accumulation of 3.25 μM coralyne into SF-268 (●) and RPMI 8402 (▼) cells as a function of time. The data were normalized to 30 μg protein/25-μL sample. Panel B shows a plot of coralyne accumulation at 2 hr as a function of concentration. The data were normalized to 20 μg protein/25-μL sample. The SF-268 data were fit to a simple rectangular hyperbola (solid line). Both experiments were performed three times with qualitatively similar results; the data shown in panels A and B were from different experiments using different MPF-66 spectrofluorometers.

cells. Coralyne was selected for initial quantitative accumulation studies because it is the most fluorescent of the selectively cytotoxic protoberberines (see emission spectrum in Fig. 1, Materials and Methods).

A time course of coralyne accumulation into SF-268 and RPMI 8402 cells at concentrations close to the IC_{50} for RPMI 8402 cells is shown in Fig. 3A. Under these conditions, coralyne accumulation into SF-268 cells continued to increase for 7 hr, while accumulation into RPMI 8402 lymphoblast cells was approximately 8-fold less. A plot of accumulation of coralyne at 2 hr (a steady-state time point at higher coralyne concentrations) as a function of concentration (Fig. 3B) further demonstrated the differences in levels of accumulation of coralyn in these two cell types.

To investigate the structural specificity of the accumulation system(s), a series of inhibition experiments were

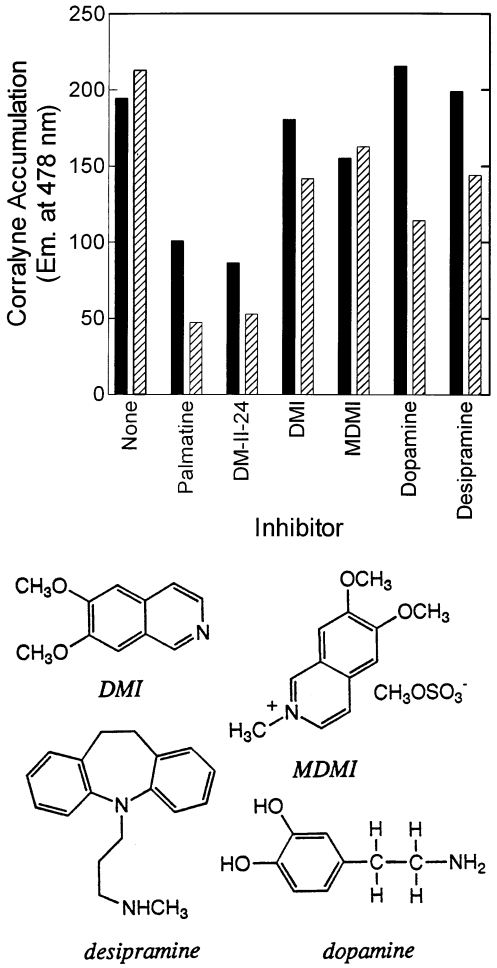


FIG. 4. Inhibition of accumulation of coralyne into SF-268 cells. Top panel: SF-268 cells were preincubated for 30 min with the concentration of inhibitors listed below. Coralyne was then added to each well to 3.25 μM. After 2 hr the cells were harvested and solubilized as described in Materials and Methods. The inhibitors palmatine, DM-II-24, DMI, MDMI and desipramine were present at 30 μM (black bars) or 90 μM (striped bars). Dopamine was present at 1 mM (black bar) or 5 mM (striped bar). The data were normalized to 25 μg protein/25-μL sample. Experiments were performed three times. Bottom panel: Structures of the inhibitors. MDMI is shown rotated 120° to the right of DMI to illustrate the structural relationship with the A and B rings, as well as the C and D rings of protoberberines. The core structures and substituents on palmatine and DM-II-24 are described in Table 3.

performed using various protoberberine compounds as well as other drugs. The top panel of Fig. 4 shows the effects of selected compounds upon accumulation of coralyne into SF-268 cells after a 2-hr exposure. Of the various protoberberines tested, palmatine and DM-II-24 at 30 or 90 μM were the more effective inhibitors. Nitidine and DM-II-32 also reduced coralyne accumulation significantly (data not shown). Palmatine and DM-II-24 share the same A-ring substituents but differ in the position of the methoxy substitutions on the D-ring (Table 3).

DMI and MDMI (Fig. 4, bottom panel) were synthesized to determine whether structural analogs of only two rings of coralyne were capable of blocking coralyne accumulation. As illustrated in the figure, these compounds can be viewed as being analogous to either the A and B rings (compare MDMI to core III, Table 3), or the C and D rings of coralyne (compare DMI to core III, Table 3). Neither of these isoquinoline derivatives, however, inhibited coralyne accumulation. Thus, analogs of these protoberberines that are larger than two rings appear to be required to block cellular accumulation effectively.

We have also tested dopamine and desipramine for inhibition of coralyne accumulation. Berberine is synthesized in numerous plant species from dopamine [5, 28], and the phenylethylamine backbone of dopamine can be superimposed on coralyne, berberine, and palmatine. Desipramine is a well-characterized antidepressant and a potent inhibitor of some biogenic amine transport systems [see, for example, Refs. 20 and 29]. Figure 4 (top panel) shows that both dopamine and desipramine inhibited coralyne accumulation into SF-268 cells, but the concentration required for dopamine inhibition was quite high. A number of other organic amines have been tested for inhibition of coralyne accumulation, including chloroquine, reserpine, verapamil, isoproterenol, phenylephrine, and choline (data not shown). None of these compounds inhibited accumulation.

Palmatine Effects on DM-II-32 Cytotoxicity in SF-268 and RPMI 8402 Cells

The accumulation and inhibition data reported in the previous section raise the question of whether preferential protoberberine uptake in SF-268 cells is responsible for the selective cytotoxicity of certain protoberberines. The fact that palmatine is a potent inhibitor of coralyne accumulation but is only marginally cytotoxic itself makes it possible to test this question. Thus, the IC_{50} value of DM-II-32, the most potent protoberberine, was compared with the IC_{50} values of palmatine alone and of the mixture of DM-II-32 plus palmatine. If palmatine inhibits the protoberberine uptake system, the concentration–response curve for DM-II-32 should be shifted to the right. Figure 5A shows that palmatine (DM-I-159) did not affect the IC_{50} of DM-II-32 in RPMI 8402 cells. However, in SF-268 cells, palmatine shifted the IC_{50} of DM-II-32 significantly to a value similar to that seen in RPMI 8402 cells (Fig. 5B). The same result was observed when nitidine was used as the cytotoxic agent (data not shown).

Role of Biogenic Amine Uptake Systems in Mediating Accumulation of Protoberberines into Sensitive Cells

The fact that dopamine is a precursor in the biosynthesis of berberine and the preservation of the phenylethylamine backbone in protoberberines suggested that the accumulation of these agents into cells could be mediated by a biogenic amine uptake system. The finding that dopamine and desipramine did inhibit accumulation appears to strengthen this possibility. Stably transfected cell lines [20]

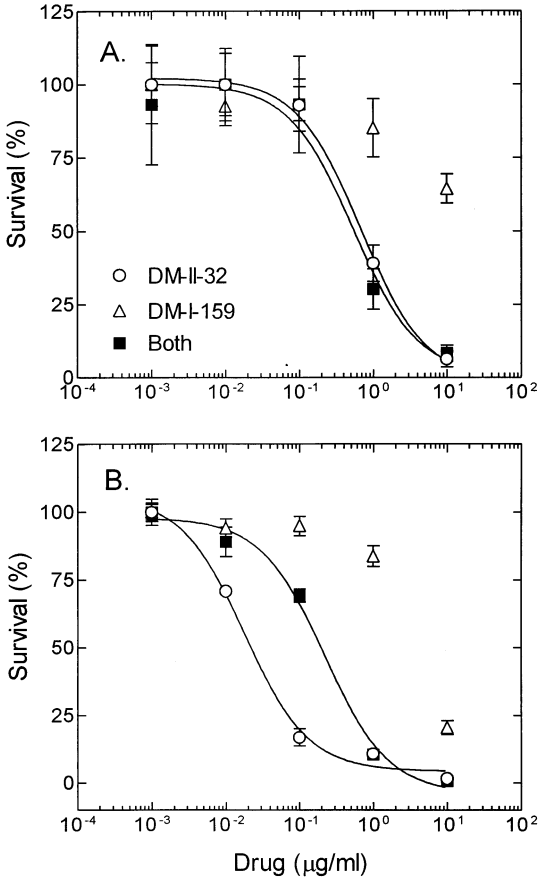


FIG. 5. Effects of palmatine (DM-I-159) on the cytotoxicity of DM-II-32 in RPMI 8402 compared with SF-268 cells. Cytotoxicity assays were performed as described in Materials and Methods using increasing concentrations of DM-II-32 alone (○), palmatine (DM-I-159) alone (△), or DM-II-32 in the presence of 10 μg/mL of palmatine (■). Data (± SEM) from triplicate samples from a single experiment are plotted as log drug concentration versus percent survival of the cells. The solid lines show fits of the DM-II-32 alone, and DM-II-32 plus 10 μg/mL of palmatine, data to the sigmoidal inhibitory effect model (Materials and Methods). Panel A, RPMI 8402 cells: Panel B, SF-268 cells. Comparable results were obtained in two independent experiments.

that express cDNAs coding for the dopamine, norepinephrine, or serotonin uptake$_1$ transporters were evaluated to determine whether any of these systems was important.

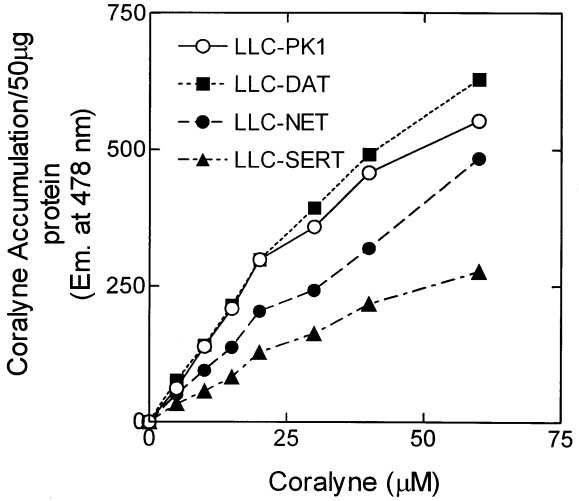


FIG. 6. Concentration dependence of coralyne accumulation in LLC-PK1 cells expressing individual biogenic amine transporters. The experiment was carried out as described in the legend to Fig. 3B and in Materials and Methods. Each point represents a mean of ≥3. The stably transformed cell lines were LLC-PK1 control (○), LLC-DAT expressing the dopamine transporter (■), LLC-NET expressing the norepinephrine transporter (●), and LLC-SERT expressing the serotonin transporter (▲).

Table 4 shows no change in cytotoxicity of coralyne or DM-II-32 for LLC-PK1 cells expressing any of the biogenic amine uptake$_1$ transporters compared with the LLC-PK1 control. To determine whether accumulation of coralyne differed between the parent LLC-PK1 cells and any of the biogenic amine transporter expressing cells, accumulation of coralyne was measured as a function of concentration. Figure 6 shows no significant changes in coralyne accumulation into cells expressing these transporters as compared with control. This experiment shows that none of the biogenic amine uptake$_1$ transport systems significantly changed the levels of coralyne accumulation into LLC-PK1 cells.

To determine whether any monoamine transporter was expressed in SF-268 cells, cDNAs encoding the three monoamine transporters [20] were labeled and used as probes for total RNA isolated from SF-268 and RPMI 8402 cells. No expression of any monoamine uptake$_1$ transporter was detected in any of the cell lines (data not shown).

TABLE 4. IC_{50} values of protoberberines and other topoisomerase poisons in cell lines expressing biogenic amine transporters.

	IC_{50} (μg/mL)			
Compound	LLC-PK1 (control)	LLC-DAT (dopamine)	LLC-NET (norepinephrine)	LLC-SERT (serotonin)
Camptothecin	0.008	0.01	0.01	0.023
Ho33342	2.2	ND*	2.0	3.0
VP-16	0.4	ND	0.35	0.6
DM-II-32	0.22	0.30	0.23	0.1
Coralyne	4.5	2.5	3.0	5.0

Data are representative of two independent experiments conducted with triplicate samples.
* ND = not determined.

DISCUSSION

Two previously characterized topoisomerase poisons, coralyne and nitidine, show a different spectrum of cytotoxicity against various tumor cell lines than the prototypic topoisomerase I poison camptothecin, and the topoisomerase II poisons VP-16 and doxorubicin (Table 1). DM-II-32, one of the most potent topoisomerase I poisons in the protoberberine series tested, showed the same spectrum of cytotoxicity as coralyne and nitidine. These protoberberine compounds were 3- to more than 10-fold more cytotoxic to cell lines derived from certain solid tumors (e.g. SF-268 glioblastoma cells) than to leukemias (e.g. RPMI 8402) (Table 2). The potential for understanding and exploiting this pattern of selective cytotoxicity is significant, since such selective cytotoxicity could form the basis of therapies directed toward specific malignancies.

The characteristics of protoberberine analog *selective cytotoxicity* show analogies to previous reports for ellipticinium derivatives [7, 8]. First, there is overlap in the group of sensitive cells. Second, the protoberberines showing selective cytotoxicity are quaternary (imminium) nitrogen-containing structures that carry a permanent positive charge. The ellipticinium derivatives reported previously to demonstrate selective cytotoxicity are also multi-ring, quaternary nitrogen-containing compounds [7]. In the case of these ellipticinium derivatives, the selective cytotoxicity toward nervous system-derived tumors was abolished in derivatives lacking the permanent positive charge [6, 7].

To evaluate the role of the various structural features of protoberberines in determining selective cytotoxicity (and cytotoxic potency), the IC_{50} value for each of a series of seventeen protoberberines was measured against RPMI 8402 lymphoblast cells and compared with that observed for SF-268 glioblastoma cells (Table 3). The data showed that the most important structure for determining selective cytotoxicity is the imminium nitrogen at the ring junction (position 7 in cores I–III). Simply moving the 7-nitrogen away from the ring junction position abolished selective cytotoxicity (Table 3). The data have also shown that substituents on the A ring (Table 3) affect consistency of selective cytotoxicity, with compounds possessing a 3,4-methylenedioxy substituent exhibiting selectivity consistently. In contrast, movement of the substituents on the D ring had little effect on selective cytotoxicity. Unpredictable and variable modulation of selective cytotoxicity was observed with saturation of the 5,6 double bond (5,6-dihydro derivatives) and with the presence of a methyl substituent at the 8-position (8-Me). Thus, the position of the imminium nitrogen on the ring junction, and A-ring substitution, are identified as determinants of consistency of potency and selective cytotoxicity.

A possible mechanism for the selective cytotoxicity could be differences in drug uptake between the cell lines. Quantitation of coralyne fluorescence as a measure of accumulation supports this mechanism, as significant differences in the time course of accumulation in RPMI 8402 and SF-268 cells were observed (Fig. 3A). At the coralyne concentration used for the time–course experiment, we cannot conclude that accumulation was saturable; however, at higher concentrations, accumulation in the SF-268 cells did approach saturation (Fig. 3B). These data cannot be explained by *simple* unregulated carrier-mediated uptake, and apparently reflect the presence of kinetically complex and/or multiple accumulation systems. Therefore, we have investigated the ability of compounds structurally related to coralyne to inhibit accumulation of the drug.

Of the several protoberberines tested for inhibition of coralyne accumulation into SF-268 cells, palmatine and DM-II-24 were the most effective (Fig. 4). Two compounds structurally analogous to either half of the protoberberine backbone, DMI and its *N*-methyl derivative, MDMI, did not affect coralyne accumulation (Fig. 4). This contributes to the important conclusion that the minimal essential structure for inhibition of accumulation (i.e. the pharmacophore for accumulation) is *larger* than either the A plus B or the C plus D rings of the isoquinoline backbone. It suggests that the network of rings surrounding the imminium ion is required for the interaction of the protoberberine analogs with the cell surface components responsible for drug uptake. The inhibition studies documented in Fig. 4, in addition to tests of a large number of other weakly basic compounds (data not shown), lead to the conclusion that the uptake system does have structural specificity for protoberberine-like compounds. The concentration dependence of accumulation data in Fig. 3B suggests that the binding affinity of coralyne for the uptake system is in the micromolar range.

Palmatine substantially inhibits the accumulation of coralyne in SF-268 cells, but is two orders of magnitude less potent than DM-II-32 as a cytotoxic agent. We examined whether palmatine could shift the concentration–response curve for DM-II-32 or nitidine in the cytotoxicity assay. The fact that palmatine did shift the cytotoxicity curve in SF-268, but not in RPMI 8402 (Fig. 5), indicates that the difference in the IC_{50} values between the two cell lines is due to the inhibition of coralyne accumulation by palmatine. This finding supports the conclusion that the selective cytotoxicity observed for protoberberines in SF-268 compared with RPMI 8402 cells is due to selective uptake of protoberberines into SF-268 cells mediated by a palmatine-inhibitable system. This system is absent, or expressed at much lower levels, in RPMI 8402 cells.

The structural specificity of protoberberine uptake into SF-268 cells was demonstrated by a palmatine-mediated decrease of coralyne accumulation and a reversal of DM-II-32 cytotoxicity. Passive membrane diffusion is thus ruled out as a mechanism of coralyne accumulation, consistent with the fact that the cytotoxic protoberberines carry a permanently charged quaternary nitrogen. Several possible mechanisms of protoberberine accumulation were suggested by the biosynthetic derivation of protoberberines from the biogenic amine dopamine [5, 28]. These include: (1) the high affinity neuronal biogenic amine transport systems

(also known as uptake$_1$) for dopamine, norepinephrine, and serotonin (see, for example, Refs. [20] and [29], (2) the extraneuronal transport system, uptake$_2$, which contributes to glial cell uptake of the antitumor agent SarCNU [15, 30], and (3) the vesicular monoamine transport system known as VMAT, which mediates concentration of diverse cations into synaptic vesicles [reviewed in Refs. 31 and 32]. An uptake$_1$-mediated system was ruled out by inhibitor studies with dopamine and desipramine (Fig. 4), and by coralyne accumulation measurements and protoberberine cytotoxicity assays in LLC-PK1 cell lines individually expressing the norepinephrine, dopamine, or serotonin carriers (Fig. 6, Table 4) [20]. In addition, expression of mRNAs encoding the biogenic amine carrier proteins could not be detected in SF-268 cells (data not shown). Uptake$_2$ and the VMAT system were similarly ruled out using well-known blockers [15, 30–32] (data not shown). Thus, we conclude that biogenic amine-specific transport systems do not play a role in determining selective cytotoxicity of protoberberines.

Additional findings that corroborate this conclusion, and also direct attention to consideration of other accumulation systems, include data showing that coralyne accumulation was independent of sodium ion concentration and not energy dependent (results not shown). Viewed under the fluorescence microscope, SF-268 cells treated with coralyne have bright fluorescence associated with cytoplasmic vesicles (data not shown). These observations, in addition to the low affinity–high capacity accumulation implied by the data in Fig. 3B, are consistent with uptake via ligand translocation processes associated with the specialized membrane invaginations known as caveolae [reviewed in Ref. 33]. Caveolae are associated with receptor and non-receptor-mediated uptake of both negatively and positively charged drugs and peptides, including folate derivatives and analogs [33, 34], as well as the homeodomain of the *Drosophila* homeoprotein Antennapaedia [35, 36]. Other known systems that are not ruled out completely at this time include various carrier-mediated organic amine uptake systems (excluding uptake$_2$) [reviewed in Refs. 30 and 37]. Experiments are continuing to positively identify the cellular uptake system responsible for selective cytotoxicity of protoberberines in SF-268 and other susceptible cell lines.

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References

1. Makhey D, Gatto B, Yu C, Liu A, Liu LF and LaVoie EJ, Coralyne and related compounds as inhibitors of mammalian topoisomerase I and topoisoimerase II. *Bioorg Med Chem* **4:** 781–791, 1996.
2. Gatto B, Sanders MM, Yu C, Wu H-Y, Makhey D, LaVoie EJ and Liu LF, Identification of topoisomerase I as the cytotoxic target of the protoberberine alkaloid coralyne. *Cancer Res* **56:** 2795–2800, 1996.
3. Makhey D, Gatto B, Yu C, Liu A, Liu LF and LaVoie EJ, Protoberberine alkaloids and related compounds as dual inhibitors of mammalian topoisomerase I and II. *Med Chem Res* **5:** 1–12, 1995.
4. Suffness M and Cordell GA, Antitumor alkaloids, In: *The Alkaloids* (Ed. Brossi A), Vol. XXV, pp. 178–197. Academic Press, New York, 1985.
5. Bhakuni DS and Jain S, Protoberberine alkaloids. In: *The Alkaloids* (Ed. Brossi A), Vol. 28, pp. 95–181. Academic Press, Orlando, FL, 1986.
6. Jurayj J, Haugwitz RD, Varma RK, Paull KD, Barrett JF and Cushman M, Design and synthesis of ellipticinium salts and 1,2-dihydroellipticines with high selectivities against human CNS cancers *in vitro*. *J Med Chem* **37:** 2190–2197, 1994.
7. Acton EM, Narayanan VL, Risbood PA, Shoemaker RH, Vistica DT and Boyd MR, Anticancer specificity of some ellipticinium salts against human brain tumors *in vitro*. *J Med Chem* **37:** 2185–2189, 1994.
8. Kenney S, Vistica DT, Linden H and Boyd MR, Uptake and cytotoxicity of 9-methoxy-N^2-methylellipticinium acetate in human brain and non-brain tumor cell lines. *Biochem Pharmacol* **49:** 23–32, 1995.
9. Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J and Boyd MR, Feasibility of a high flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* **83:** 757–766, 1991.
10. Noe AJ, Malapetsa A and Panasci LC, Transport of (2-chloroethyl)-3-sarcosinamide-1-nitrosourea in the human glioma cell line SK-MG-1 is mediated by an epinephrine-sensitive carrier system. *Mol Pharmacol* **44:** 204–209, 1993.
11. Schimke RT, Gene amplification in cultured cells. *J Biol Chem* **263:** 5989–5992, 1988.
12. Schimke RT, Gene amplification in cultured animal cells. *Cell* **37:** 705–713, 1984.
13. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385–427, 1993.
14. Breuninger LM, Paul S, Gaughan K, Miki T, Chan A, Aaronson SA and Kruh GD, Expression of multidrug resistance-associated protein in NIH/3T3 cells confers multidrug resistance associated with increased drug efflux and altered intracellular drug distribution. *Cancer Res* **55:** 5342–5347, 1995.
15. Noë AJ, Marcantonio D, Barton J, Malapetsa A and Panasci LC, Characterization of the catecholamine extraneuronal uptake$_2$ carrier in human glioma cell lines SK-MG-1 and SKI-1 in relation to (2-chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU) selective cytotoxicity. *Biochem Pharmacol* **51:** 1639–1648, 1996.
16. Vistica DT, Kenney S, Hursey ML and Boyd MR, Cellular uptake as a determinant of cytotoxicity of quaternized ellipticines to human brain tumor cells. *Biochem Biophys Res Commun* **200:** 1762–1768, 1994.
17. Vistica DT, Kenney S, Hursey M and Boyd MR, Role of membrane potential in the accumulation of quaternized ellipticines by human tumor cell lines. *J Pharmacol Exp Ther* **279:** 1018–1025, 1997.
18. Shero JH, Bordwell B, Rothfield NF and Earnshaw WC, High titers of autoantibodies to topoisomerase I (Scl-70) in sera from scleroderma patients. *Science* **231:** 737–740, 1986.
19. Zee-Cheng K-Y and Cheng CC, Synthesis of 5,6-dihydro-6-methoxynitidine and a practical preparation of nitidine chloride. *Heterocyclics* **10:** 85–88, 1973.

20. Gu H, Wall SC and Rudnick G, Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics, and ion dependence. *J Biol Chem* **269:** 7124–7130, 1994.
21. Akiyama S, Fojo A, Hanover JA, Pastan I and Gottesman MM, Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. *Somat Cell Mol Genet* **11:** 117–126, 1985.
22. Beck WT, Cirtain MC, Danks MK, Felsted RL, Safa AR, Wolverton JS, Suttle DP and Tren JM, Pharmacological, molecular and cytogenetic analysis of "atypical" multidrug-resistant human leukemic cells. *Cancer Res* **47:** 5455–5460, 1987.
23. Andoh T, Ishii K, Suzuki Y, Ikegami Y, Kusunoki Y, Takemoto Y and Okada K, Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. *Proc Natl Acad Sci USA* **84:** 5565–5569, 1987.
24. Mosmann T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. *J Immunol Methods* **65:** 55–63, 1983.
25. Denizot F and Lang R, Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* **89:** 271–277, 1986.
26. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski JM, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH and Boyd MR, Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* **45:** 589–601, 1988.
27. Su L, Smythe AM, Stinson SF, Mullendore LA, Monks A, Scudiero DA, Paull KD, Koutsoukos AD, Rubinstein LV, Boyd MR and Shoemaker RH, Multidrug-resistant phenotype of disease-oriented panels of human tumor cell lines used for anticancer drug screening. *Cancer Res* **52:** 3029–3034, 1992.
28. Shamma M, *The Isoquinoline Alkaloids: Chemistry and Pharmacology*, p. 300. Academic Press, New York, 1972.
29. Rudnick G and Clark J, From synapse to vesicle: The reuptake and storage of biogenic amine transmitters. *Biochim Biophys Acta* **1144:** 249–263, 1993.
30. Trandelenburg U, The extraneuronal uptake and metabolism of catecholamines. In: *Catecholamines I* (Eds. Trandelenburg U and Weiner N), pp. 279–319. Springer, Berlin, 1988.
31. Schuldiner SA, Molecular glimpse of vesicular monoamine transporters. *J Neurochem* **62:** 2067–2078, 1994.
32. Yelin R and Schuldiner S, The pharmacological profile of the vesicular monoamine transporter resembles that of multidrug transporters. *FEBS Lett* **377:** 201–207, 1995.
33. Anderson RGW, Caveolae: Where incoming and outgoing messengers meet. *Proc Natl Acad Sci USA* **90:** 10909–10913, 1993.
34. Smart EJ, Ying Y-s and Anderson RGW, Hormonal regulation of caveolae internalization. *J Cell Biol* **131:** 929–938, 1995.
35. Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G and Prochiantz A, Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J Biol Chem* **271:** 18188–18193, 1996.
36. Joliot A, Trembleau A, Raposo G, Calvet S, Volovitch M and Prochiantz A, Association of Engrailed homeoproteins with vesicles presenting caveolae-like properties. *Development* **124:** 1865–1875, 1997.
37. Grundemann D, Gorboulev V, Gambaryan S, Veyhl M and Koepsell H, Drug excretion mediated by a new prototype of polyspecific transporter. *Nature* **372:** 549–552, 1994.