The Nitrogen of the Acetamido Group of Colchicine Modulates P-Glycoprotein-Mediated Multidrug Resistance†

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ABSTRACT: The substituents of drug molecules and the specific amino acid residues of P-glycoprotein (P-gp) implicated in drug/protein interactions are largely unknown. We have used a series of colchicine analogs modified on the A, B, and C rings to identify the discrete chemical groups on the colchicine molecule that are required for recognition by P-gp. For this, the toxicity of these analogs was tested on independent cell clones expressing either of the two mouse *mdr* genes, *mdr1* and *mdr3*, known to confer multidrug resistance. Modifications of the methoxy groups on the A and C rings modulated cellular toxicity but had no effect on P-gp recognition; however, modifications at the C7 position of the B ring, in particular the removal of the nitrogen atom of the acetamido group, had a dramatic effect. Analogs bearing a hydrogen at that position were not substrates for P-gp. The importance of the nitrogen at C7 was independently verified in thiocolchicine and allocolchicine analogs similarly modified, although overall levels of resistance to these compounds were somewhat reduced compared to their colchicine counterparts. The study of allocolchicine congeners bearing a six-carbon C ring and of two other analogs completely lacking a B ring suggested that intact B and C rings were important for interaction with P-gp. These results suggest that the structural determinants for cytotoxicity (tubulin binding) and P-gp recognition map to nonoverlapping sites in the colchicine analogs analyzed. Examination of calculated molar refractivities (CMR) revealed that only compounds showing CMR values greater than 9.7 were P-gp substrates. Taken together, these results indicate that both a minimal size and a nitrogen atom at C7 of the B ring are important requirements in the colchicine molecule for efficient interaction with P-gp.

Multidrug resistance (MDR) is a major impediment to the successful chemotherapeutic treatment of human tumors. Multidrug-resistant tumor cells *in vivo* and cultured cells *in vitro* become simultaneously resistant to a large group of structurally and functionally unrelated cytotoxic drugs which include colchicine, Vinca alkaloids, adriamycin, actinomycin-D, and others (Gerlach et al., 1986; Moscow & Cowman, 1988). MDR is caused by the overexpression of a membrane phosphoglycoprotein, termed P-glycoprotein (P-gp), which is capable of binding drugs and ATP analogs and is believed to function as an ATP-dependent drug efflux pump in resistant cells (Endicott & Ling, 1989; Roninson, 1991). In rodents, P-gp is encoded by three *mdr* genes, *mdr1*, *mdr2*, and *mdr3* (Gros et al., 1986a, 1988; Devault & Gros, 1990, Hsu et al., 1990), while only two forms *MDR1* and *MDR2*, exist in humans (Chen et al., 1986; Van der Bliek et al., 1987). All P-gp's share a high degree of amino acid sequence homology and predicted structural features, which include 12 transmembrane domains, two nucleotide binding sites, and a symmetrical structure with each half showing homology to a number of bacterial transport proteins participating in the import or export of amino acids, carbohydrates, and large peptides (reviewed by Higgins et al. (1990)). In gene-transfer experiments, both *mdr1* and *mdr3* can confer drug resistance, while *mdr2* cannot (Gros et al., 1986b, 1988; Devault & Gros, 1990).

One unresolved aspect of P-gp function is the apparent capacity of a simple transporter to recognize structurally and functionally unrelated substrates. A better comprehension of the molecular requirements for drug/protein interactions is a necessary prerequisite to the rational design of new compounds capable of either bypassing or blocking the action of P-gp. The biochemical characterization (Bruggeman et al., 1989; Yoshimura et al., 1990; Greenberger et al., 1992; Raviv et al., 1990) of P-gp and analyses of chimeric (Bushman & Gros, 1991; Dhir & Gros, 1992) and mutant P-gps (Choi et al., 1988; Safa et al., 1990; Gros et al., 1991; Devine et al., 1992) showing altered substrate specificities point to the membrane-associated segments as key functional determinants for drug recognition and transport. However, the specific structural requirements and chemical groups of drug molecules required for P-gp recognition and transport remain poorly defined (Zamora et al., 1988). A comprehensive study of reserpine analogs as well as ellipticine and olivacine derivatives indicates that lipid solubility and the presence of a basic nitrogen atom and at least two aromatic rings are essential requirements for drug interaction with P-gp (Pearce et al., 1989; Chevallier-Multon et al., 1990).

Colchicine is a plant alkaloid composed of three rings, two of which are aromatic, that form the phenyltropolone backbone of the molecule with four methoxy groups and an acetamido group attached to the periphery. The drug can enter cells by passive diffusion across the lipid bilayer, and it exerts its cytotoxic effect by binding to tubulin, arresting cell division

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(Weisenberg et al., 1968). Colchicine is a known MDR drug, and cultured cells transfected with and overexpressing either mouse *mdr1* or *mdr3* show 30- and 50-fold increases in resistance to this drug, respectively (Devault & Gros, 1990; Gros et al., 1991). Transport studies in P-gp expressing cells show decreased intracellular accumulation of radiolabeled colchicine in these cells (Safa et al., 1990). Photoactivatable analogs of colchicine have been shown to bind to P-gp; this binding is specific and is competed for by nonradioactive colchicine and other MDR drugs (Safa et al., 1989).

Because of its relatively simple structure and few peripheral chemical groups, we have used a series of colchicine analogs as model substrates to determine the molecular requirements for substrate recognition by P-gp. For this, we measured the effect of discrete chemical substitutions on the A, B, and C rings of colchicine on (i) cellular toxicity, (ii) relative molar volume of the compound (calculated molar refractivity), and (iii) resistance levels expressed by cell clones expressing either *mdr1* or *mdr3* drug efflux pumps. Our results show that (i) the methoxy groups of the A and C rings previously shown to be important for tubulin binding are not required for P-gp recognition, while (ii) a minimal size and (iii) the presence of a nitrogen atom at the C7 position of the B ring are required for P-gp recognition and transport.

MATERIALS AND METHODS

Cytotoxic Drugs. Colchicine analogs were provided by Dr. A. Brossi (National Institutes of Health, Bethesda, MD) according to published procedures (Boyé & Brossi, 1992). Phenyltropone was a generous gift of Dr. T. Fitzgerald (Florida Agricultural and Mechanical University). Vinblastine, colchicine, and demecolcine were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of demecolcine and colchicine analogs were prepared in dimethyl sulfoxide (DMSO) at concentrations of 1.0–10 mg/mL, aliquoted, stored at –80 °C, and protected from light until use. A stock solution of vinblastine was prepared at 1.0 mg/mL in phosphate-buffered saline (PBS) and stored in the same conditions at –80 °C.

Cell Lines and Tissue Culture. Chinese hamster LR73 ovary cells and their drug-resistant derivatives transfected and overexpressing either *mdr1* (clone 1S) or *mdr3* (clone 3S) cDNAs were prepared and maintained as previously described (Gros et al., 1991). All cell lines were grown in α-minimal essential medium (MEM) supplemented with 10% fetal calf serum, 3 mM glutamine, penicillin (50 units/mL), and streptomycin (50 mg/mL). The drug-resistant transfectants were grown in the same medium supplemented with vinblastine at 50 (clone 1S) or 100 ng/mL (clone 3S).

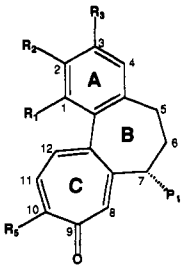
Detection of P-Glycoproteins. Membrane-enriched fractions of parental LR73 cells and drug-resistant transfectants were prepared by ultracentrifugation, as previously described (Devault & Gros, 1990). Protein concentrations in the extracts were measured using an amido black based commercial assay (Bio-Rad). Membrane proteins from control and *mdr*-transfected cells (40 μg) were separated by sodium dodecyl sulfate–polyacrylamide (final concentration 7.5%) gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane by electroblotting. The Western blot was incubated with the monoclonal mouse anti-P-gp antibody C219 (Centocor Corp., Philadelphia) at a dilution of 1:300 for 1 h at 20 °C, and specific immune complexes were revealed with goat anti-mouse IgG antiserum coupled to alkaline phosphatase used at a 1:3000 dilution, as described in Devault and Gros (1990).

Cytotoxicity Assay. A modification of a cell survival assay (Skehan et al., 1989) based on sulforhodamine B (SRB) staining of total cell protein was used. Briefly, 5×10^3 drug-sensitive LR73 control cells or *mdr*-transfected cells were plated in 96-well titer plates in complete medium containing increasing concentrations of a given colchicine analog and incubated for 72 h at 37 °C. Cells were then washed once in ice-cold PBS, fixed in 17% trichloroacetic acid in PBS for 1 h at 4 °C, and then washed extensively in tap water. Cellular proteins were stained with a solution of 0.4% SRB in 1% acetic acid for 15 min at room temperature, followed by four washes with 1% acetic acid to remove excess stain. After the plates were dried, the stain was dissolved in 10 mM Tris (pH 9.0), and quantitation was carried out using an automated ELISA plate reader (Bio-Rad Model 450) set at 490 nm. The relative plating efficiency of each clone was calculated by dividing the absorbance observed at a given drug concentration by the absorbance detected in the same clone in medium devoid of drug; it is expressed as a percentage. D_{50} is defined as the drug dose required to reduce the plating efficiency of each clone by 50%.

Molecular Calculations. The calculated molar refractivity (CMR) and lipophilicity (*c* log *P*) of the analogs were calculated using MedChem Software Release 3.54 (Daylight Chemical Information Systems Inc., Pomona College Medicinal Chemistry Project). CMR is a value that is proportional to the ratio of molecular weight to density, or molar volume, and is calculated by summation of the appropriate fragmental constants for each analog. The calculated lipophilicity (*c* log *P*) predicts the partition coefficient of neutral compounds in an octanol/water system. The calculated *c* log *P* value for colchicine was 1.03 and was identical to that measured experimentally in an octanol/phosphate buffer (pH 4.7) (Selassie et al., 1990). Since log *D* (where *D* represents the distribution at a particular pH) is considered to be a more relevant parameter than log *P*, calculated lipophilicities (*c* log *P*) were used to estimate the effective lipophilicities at pH 7.4 (reported as *c* log *D* values) according to the relationship $c \log D = c \log P - \log [1 + \text{antilog}(pK_a - pH)]$ (Leo et al., 1979; Scherrer & Howard, 1977). The pK_a of demecolcine was determined by microtitration in water and found to be 7.45 ± 0.02. (A complete list of *c* log *D* and CMR values for all but two colchicine analogs tested is presented in Table I of the supplementary material.)

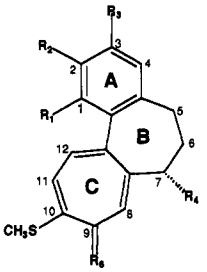
RESULTS

Colchicine is a cytotoxic plant alkaloid and is part of the MDR group of drugs recognized and transported by P-glycoprotein (P-gp). The biologically active form of colchicine ((–)-(7*S*)-colchicine) is formed by three rings: the A ring is a benzene ring having methoxy groups at positions C1, C2, and C3, the B ring is a seven-carbon ring with an acetamido group at the C7 position, while the C ring is a seven-carbon aromatic (tropolone) ring with a carbonyl group at C9 and a methoxy group at C10 (Figure 1). The A and C rings are rotated out of the plane of the molecule and arranged in a counterclockwise helicity (Boyé & Brossi, 1992). To identify, on the colchicine molecule, the specific structural requirements necessary for P-gp recognition, we analyzed a number of colchicine, thiocolchicine, and allocolchicine analogs bearing unique or combined substitutions at positions C1, C2, C3, C7, C9, and C10 (Figures 1–3). Cellular toxicity of these compounds (expressed as the D_{50} or dose required to reduce plating efficiency by 50%) was established for the drug-sensitive control LR73 hamster cells and their drug-resistant *mdr* transfectants overexpressing P-gp. The capacity of these analogs to be recognized by P-gp was deduced from the relative



		A-RING			B-RING	C-RING
		R_1	R_2	R_3	R_4	R_5
1	colchicine	OCH_3	OCH_3	OCH_3	$NHCOCH_3$	OCH_3
2	1-acetoxy-demethylcolchicine	CH_3COO	OCH_3	OCH_3	$NHCOCH_3$	OCH_3
3	2-demethylcolchicine	OCH_3	OH	OCH_3	$NHCOCH_3$	OCH_3
4	3-demethylcolchicine	OCH_3	OCH_3	OH	$NHCOCH_3$	OCH_3
5	cornigerine	OCH_3	-O-CH_2-O-		$NHCOCH_3$	OCH_3
6	deacetylcolchicine	OCH_3	OCH_3	OCH_3	NH_2	OCH_3
7	deacetamidocolchicine	OCH_3	OCH_3	OCH_3	H	OCH_3
8	5,6-dehydro-7-deacetamidocolchicine (5,6 -CH=CH-)	OCH_3	OCH_3	OCH_3	H	OCH_3
9	N-trifluoroacetyl-deacetylcolchicine	OCH_3	OCH_3	OCH_3	$NHCOCF_3$	OCH_3
10	N-ethoxycarbonyl-deacetylcolchicine	OCH_3	OCH_3	OCH_3	$NHCOOC_2H_5$	OCH_3
11	N-propoxycarbonyl-deacetylcolchicine	OCH_3	OCH_3	OCH_3	$NHCOOC_3H_7$	OCH_3
12	demecolcine	OCH_3	OCH_3	OCH_3	$NHCH_3$	OCH_3
13	N-formyl-demecolcine	OCH_3	OCH_3	OCH_3	$N(CH_3)CHO$	OCH_3
14	speciosine	OCH_3	OCH_3	OCH_3	$N(CH_3)CH_2C_6H_4(OH)$	OCH_3
15	deacetylcolchiceine	OCH_3	OCH_3	OCH_3	NH_2	OH
16	colchicide	OCH_3	OCH_3	OCH_3	$NHCOCH_3$	H
17	colchiceine	OCH_3	OCH_3	OCH_3	$NHCOCH_3$	OH
18	colchiceinamide	OCH_3	OCH_3	OCH_3	$NHCOCH_3$	NH_2

FIGURE 1: Structures of colchicine analogs.



		A-RING			B-RING	C-RING
		R_1	R_2	R_3	R_4	R_6
19	thiocolchicine	OCH_3	OCH_3	OCH_3	$NHCOCH_3$	O
20	3-demethylthiocolchicine	OCH_3	OCH_3	OH	$NHCOCH_3$	O
21	deacetylthiocolchicine	OCH_3	OCH_3	OCH_3	NH_2	O
22	3-demethyl-N-butyryl-deacetylthiocolchicine	OCH_3	OCH_3	OH	$NHCOC_3H_7$	O
23	thiodemecolcine	OCH_3	OCH_3	OCH_3	$NHCH_3$	O
24	N-formyl-thiocolchicine	OCH_3	OCH_3	OCH_3	NHCHO	O
25	N-ethoxycarbonyl-deacetylthiocolchicine	OCH_3	OCH_3	OCH_3	$NHCOOC_2H_5$	O
26	7-isothiocyanato-deacetamidothiocolchicine	OCH_3	OCH_3	OCH_3	NCS	O
27	9-thiodeoxo-thiocolchicine	OCH_3	OCH_3	OCH_3	$NHCOCH_3$	S

FIGURE 2: Structures of thiocolchicine analogs.

degree of cellular resistance detected in the P-gp expressing cells over background levels measured in LR73 cells (Table I). To unambiguously establish P-gp specific effects, all compounds were tested against individual cell clones expressing either one of the two biologically active mouse P-gp's encoded by *mdr1* and *mdr3* in 2–4 independent experiments, each performed in duplicate. In these experiments, a minimal degree of 3–4-fold resistance was required to classify a compound as P-gp substrate.

We have previously shown that both *mdr1* and *mdr3* confer multidrug resistance in transfection experiments, with *mdr3* conferring higher levels of resistance than *mdr1*, possibly encoding a more efficient drug efflux pump (Gros et al., 1991). Immunoblotting experiments with the mouse anti-P-glycoprotein monoclonal antibody C219 (Figure 4) show that cell clones *mdr1S* (1S) and *mdr3S* (3S) transfected with wild-type *mdr1* and *mdr3*, respectively, express in membrane-enriched fractions equivalent amounts of 180- and 160-kDa proteins, the known molecular mass of these two mouse P-gp's (Devault & Gros, 1990). The cytotoxicity of various colchicine analogs for LR73 control cells and *mdr* transfectants was then tested.

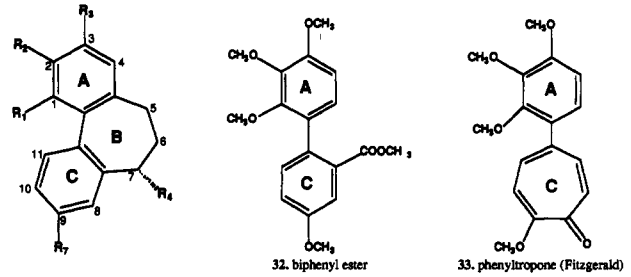
The effect of substituting the methoxy groups on the A ring was tested first (Table I). 2-Demethylcolchicine (3) and 3-demethylcolchicine (4) show replacement of the methoxy groups at either C2 or C3 by hydroxyl groups, respectively, while in 1-acetoxydemethylcolchicine (2) the C1 methoxy is replaced by an acetoxy group. Finally, in cornigerine (5) the C2 and C3 methoxy groups are bridged by a methylene group. 1-Acetyldemethylcolchicine (2) was found to be nontoxic for LR73 control cells, even at very high concentrations (10 000 ng/mL), and could not be tested as a possible substrate for P-gp. On the other hand, results with analogs 3 (1S, 13X; 3S, 30X), 4 (1S, 15X; 3S, 72X), and 5 (1S, 15X; 3S, 67X) showed that both *mdr1S* (1S) and *mdr3S* (3S) cell clones expressed significant levels of resistance to these compounds. These results indicate that modifications of the methoxy groups at C1, C2, and C3 do not affect the recognition of colchicine by P-gp. The importance of the methoxy group at position C10 of the C ring was next analyzed in the analogs colchicide (16), colchiceinamide (18), and colchiceine (17), where the methoxy at C10 has been either deleted or replaced by amino or hydroxyl groups, respectively. Results with compounds 16 (1S, 5X; 3S, 24X) and 18 (1S, 7X, 3S, 38X) show that removal or replacement of the C10 methoxy has little effect on recongition of these analogs by P-gp; however, analog 17 appeared to be a P-gp substrate, although a poor one (1S, 2X; 3S, 4X). Overall, our results indicate that the four methoxy groups on the A and C rings are not key determinants for P-gp recognition. The reduced toxicity detected in some of these anlogs (2, 3, 17) is in aggreement with the previous demonstration that these methoxy groups are essential for tubulin binding (Brossi, 1990).

The functional importance of the acetamido group at position C7 of the B ring for recognition by P-gp was then tested in 10 analogs (6–16) showing unique replacements at that position. Although most of these analogs were found to be substrates for P-gp, deletion of the acetamido group in deacetamidocolchicine (7; 1S, 1X; 3S, 1X) and 5,6-dehydro-7-deacetamidocolchicine (8; 1S, 0.4X; 3S, 0.4X) resulted in similar D_{50} values for drug-sensitive and *mdr* transfectants, suggesting that this group is critical for colchicine recognition by P-gp. This was not due to overall reduced toxicity of these compounds, as deacetamidocholchicine was indeed more toxic than colchicine. Deacetylcolchicine (6), in which the acetamido group at C7 has been replaced by an amino group, was found to be a substrate for P-gp (1S, 26X; 3S, 26X), strongly suggesting that the nitrogen rather than the acetyl group plays a key role at that position for P-gp recognition. Additional substitutions on the carbon atom of the carbonyl group at C7 were also tested. Replacement of the three hydrogens of the acetyl group by more electronegative fluorine atoms in *N*-(trifluoroacetyl)deacetylcolchicine (9) had little effect on recognition by P-gp (1S, 6X; 3S, 27X). Likewise, chain length extension, *N*-(ethoxycarbonyl)deacetylcolchicine (10; 1S, 10X; 3S, 24X) and *N*-(propoxycarbonyl)deacetylcolchicine (11; 1S, 11X; 3S, 46X), did not alter recognition by P-gp. The introduction of a *N*-methyl group instead of the *N*-acetyl group in demecolcine (12; 1S, 8X; 3S, 14X), thus changing the nitrogen-containing group from an amide to a secondary amine, was without consequences on recognition by P-gp, as was the introduction of either a formyl or a hydroxybenzyl group at that position in *N*-formyldemecolcine (13; 1S, 15X; 3S, 34X) and speciosine (14; 1S, 7X; 3S, 9X), respectively. Taken together, these results suggest an important role for the

Table I: Effect of Colchicine Analogs on Survival Characteristics of Cell Clones Stably Transfected with *mdr1* and *mdr3*

		LR73	*mdr1S*	*mdr3S*	*n*[c]
1	colchicine	41[a] ± 6	1100 ● 100 (27×[b])	1900 ● 200 (46×)	6
2	1-acetoxy-demethylcolchicine	not toxic			
3	2-demethylcolchicine	1100 ± 300	14000 ± 3000 (13×)	33000 ± 3000 (30×)[d]	3
4	3-demethylcolchicine	180 ± 10	2700 ● 800 (15×)	13000 ± 4000 (72×)	4
5	cornigerine	30 ± 10	440 ± 30 (15×)	2000 ± 1000 (67×)	3
6	deacetylcolchicine	14 ± 2	360 ± 50 (26×)	370 ± 30 (28×)	3
7	deacetamidocolchicine	8 ± 1	8 ± 1 (1×)	11 ● 4 (1×)	4
8	5,6-dehydro-7-deacetamidocolchicine	700 ± 100	290 ± 80 (0.4×)	260 ± 90 (0.4×)	3
9	*N*-(trifluoroacetyl)deacetylcolchicine	7 ± 1	40 ● 10 (6×)	190 ● 50 (27×)	4
10	*N*-(ethoxycarbonyl)deacetylcolchicine	11 ± 3	110 ± 30 (11×)	260 ± 30 (25×)	4
11	*N*-(propoxycarbonyl)deacetylcolchicine	65 ± 9	700 ± 200 (11×)	3000 ± 300 (47×)	3
12	demecolcine	10 ± 1	78 ± 3 (8×)	140 ± 20 (15×)	3
13	*N*-formyldemecolcine	5 ± 1	73 ● 3 (15×)	170 ± 20 (34×)	4
14	speciosine	17 ± 3	120 ± 20 (7×)	150 ± 30 (9×)	4
15	deacetylcolchiceine	3500 ● 400	2300 ± 500 (0.7×)	2500 ± 800 (0.7×)	3
16	colchicide	90 ± 10	410 ± 50 (5×)	2100 ± 600 (24×)	4
17	colchiceine	6000 ± 1000	15000 ± 2000 (2×)	25000 ± 1000 (4×)[d]	3
18	colchiceinamide	83 ± 6	550 ± 90 (7×)	3100 ± 400 (38×)	3
19	thiocolchicine	6 ± 2	50 ± 10 (9×)	260 ± 90 (42×)	4
20	3-demethylthiocolchicine	24 ± 5	210 ± 60 (9×)	1150 ± 70 (48×)[d]	3
21	deacetylthiocolchicine	7 ± 1	27 ± 5 (4×)	65 ± 5 (9×)	3
22	3-demethyl-*N*-butyryldeacetylthiocolchicine	38 ● 8	300 ± 100 (7×)	2100 ● 700 (55×)	3
23	thiodemecolcine	6 ± 1	9 ● 2 (2×)	23 ± 4 (4×)	3
24	*N*-formylthiocolchicine	1.4 ± 0.1	17.3 ± 0.6 (12×)	50 ± 10 (36×)	3
25	*N*-(ethoxycarbonyl)deacetylthiocolchicine	2.8 ± 0.8	8.1 ± 0.1 (3×)	40 ± 10 (17×)	2
26	7-(isothiocyanato)deacetamidothiocolchicine	100 ± 30	120 ● 40 (1×)	190 ● 20 (2×)	3
27	9-thiodeoxothiocolchicine	9 ± 2	60 ± 20 (6×)	140 ± 20 (16×)	4
28	allocolchicine	16 ± 2	26 ± 5 (2×)	70 ± 20 (4×)	4
29	allocolchicine aldehyde	60 ± 20	120 ± 30 (2×)	310 ± 60 (5×)	4
30	*N*-acetylcolchinol methyl ether	13 ± 4	16 ± 1 (1×)	40 ± 10 (3×)	4
31	deaminocolchinol methyl ether	39 ± 2	33 ± 4 (1×)	38 ± 7 (1×)	4
32	biphenyl ester	2200 ± 200	2800 ± 400 (1×)	2000 ± 200 (1×)	3
33	phenyltropone (Fitzgerald)	17[e]	16	17.5	

[a] The drug survival of LR73 drug-sensitive cells and multidrug-resistant clones transfected with either wild-type *mdr1* (1S) or *mdr3* (3S) is expressed as the D_{50} (ng/mL), or the dose necessary to reduce the plating efficiency of the control and transfected cell clones by 50%. [b] The number in parentheses represents the fold resistance expressed by the transfectants over the control, LR73, cells. [c] *n* represents the number of individual experiments. [d] Only two experiments were performed. A minimum degree of 3–4-fold resistance expressed by *mdr3S* was required to identify a compound as P-gp substrate. [e] Only one experiment in duplicate was carried out.



	A-RING R1	A-RING R2	A-RING R3	B-RING R4	C-RING R7
28 allocolchicine	OCH_3	OCH_3	OCH_3	$NHCOCH_3$	$COOCH_3$
29 allocolchicine aldehyde	OCH_3	OCH_3	OCH_3	$NHCOCH_3$	CHO
30 N-acetyl-colchinol methyl ether	OCH_3	OCH_3	OCH_3	$NHCOCH_3$	OCH_3
31 deamino colchinol methyl ether	OCH_3	OCH_3	OCH_3	H	OCH_3

FIGURE 3: Structures of allocolchicine analogs.

nitrogen atom of the acetamido group at C7 for recognition by P-gp. One notable exception was deacetylcholciceine (15), which bears an amine group at C7 but yet does not seem to be a substrate for P-gp. However, the additional hydroxyl group at C10 (C ring) in both colchiceine (17) and deacetylcolchiceine (15) greatly reduces toxicity, perhaps by affecting tubulin binding.

Our conclusions on the relative importance of A, B, and C ring substitutions in the recognition of colchicine by P-gp were tested in a second series of analogs (19–27) of thiocolchicine (19) (Figure 3). Thiocolchicine is similar to colchicine except that the C10 methoxy is replaced by an SCH_3 group. Toxicity of thiocolchicine for drug-sensitive LR73 cells is greater than colchicine due to increased affinity for tubulin (Boyé & Brossi, 1992), and drug-resistant *mdr* transfectants show levels of resistance to thiocolchicine (1S, 9X; 3S, 43X) comparable to those measured for colchicine. Overall, the study of thiocolchicine analogs produced results very similar to those obtained with colchicine analogs. Modifications of the C3 methoxy in 3-demethylthiocolchicine (20; 1S, 9X; 3S, 48X) and 3-demethyl-*N*-butyryldeacetylthiocolchicine (22; 1S, 8X; 3S, 55X) did not reduce the degree of resistance expressed by *mdr* transfectants. *mdr* transfectants were also resistant to 9-thiodeoxothiocolchicine (27; 1S, 7X; 3S, 16X), where the oxygen at C9 is replaced by sulfur. Replacement of the acetamido group at C7 with *N*-formamido (24; 1S, 12X; 3S, 36X) and *N*-ethoxycarbamyl moieties (25; 1S, 3X; 3S, 13X) had little effect on P-gp recognition. Removal of the acyl functionality from C7 in deacetylthiocolchicine (21; 1S, 4X; 3S, 9X) and thiodemecolcine (23; 1S, 1.5X; 3S, 4X) produced two compounds that were still P-gp substrates, although poor ones. These results are compatible with the conclusion from our analysis of colchicine analogs indicating that the nitrogen at C7 is required for P-gp recognition. 7-(Isothiocyanato)deacetamidothiocolchicine (26), which is an extremely poor substrate for P-gp (only 2× resistance detected in 3S), is an interesting exception since it still retains a nitrogen at C7 but lacks the NH unit found in all other P-gp substrates. *mdr* transfectants generally expressed levels of resistance to the thiocolchicine analogs that were similar to, although slightly lower than, their colchicine counterparts. This was most obvious for the 6/21 and 12/23 pairs, which varied by about 3-fold.

To determine whether our conclusions would be verified in yet another set of analogs constructed on a different molecular

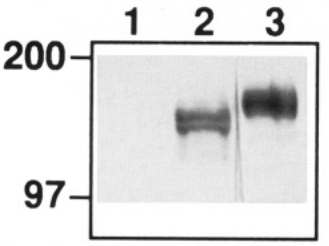


FIGURE 4: Identification of mouse *mdr1* and *mdr3* proteins. Crude membrane fractions (40 μg) of drug-sensitive LR73 hamster cells (lane 1) and cell clones stably transfected with wild-type *mdr3* (lane 2) and *mdr1* (lane 3) were analyzed by Western blotting using anti-P-glycoprotein monoclonal antibody C219. Molecular mass markers are myosin (200 kDa) and phosphorylase *b* (97 kDa).

backbone, analogs of allocolchicine (28–31) were tested (Figure 4). In these analogs, the seven-carbon C ring is replaced by a six-membered aromatic ring. The four analogs showed D_{50} values for LR73 cells in the range of colchicine, varying from 13 to 60 ng/mL, and all were poor substrates for P-gp. In fact, only *mdr3*-expressing cells (3S) showed low but significant levels of resistance to any of these compounds. These results suggest that the tropolone ring is important for efficient P-gp recognition. Nevertheless, the removal of the nitrogen at position C7 of the B ring still produced a notable effect: while allocolchicine (28; 1S, 2X; 3S, 4X), allocolchicine aldehyde (29; 1S, 2X; 3S, 5X), and *N*-acetylcolchicinol methyl ether (30; 1S, 1X, 3S, 3X) showing substitutions at C9 of the C ring were poor P-gp substrates, the deletion of the amino group at C7 of the B ring in deaminocolchicinol methyl ether (31) completely abrogated recognition by P-gp. Removal of the B ring altogether, in the biphenyl ester (32) and in the Fitzgerald phenyltropone (33), produced compounds that were not substrates for P-gp. Taken together, studies of these colchicine analogs suggest that the presence of intact B and C rings is required for optimal interaction with P-gp and also provide confirmation of the key role of the nitrogen atom at C7.

Molecular calculations on the colchicine, thiocolchicine, and allocolchicine analogs showed that there was no apparent direct or multifactorial correlation between lipophilicity and toxicity (data not shown) nor between lipophilicity and the degree of resistance expressed by *mdr* transfectants (data not shown), indicating that resistance and toxicity are not dependent on the lipophilicity of the analogs but rather are linked to other factors. The calculated molar refractivities (CMR) reflecting the size or molar volume of the compound were calculated for the three colchicine analog series. Examination of the molar refractivity values indicates that compounds with a CMR value below 9.7 are not substrates for P-gp, while those compounds with a CMR value greater than the 9.7 threshold are substrates for P-gp (Figure 5). These results suggest that there is a minimal size requirement for the efficient recognition and transport of colchicine analogs by P-gp. Of all of the congeners, only 7-(isothiocyanato)-deacetamidothiocolchicine is not a substrate for P-gp but has a CMR greater than 9.7. Finally, in compounds showing CMR values between 9.7 and 13.0, there was no correlation between a further increase in molar volume and the degree of resistance expressed by either *mdr1S* or *mdr3S* clones.

DISCUSSION

One of the most puzzling aspects of P-glycoprotein-mediated multidrug resistance (MDR) is that cytotoxic drugs recognized as substrates by this transporter have few common structural and functional characteristics. However, the identification of putative common features and the definition of structural requirements for P-gp/drug interactions are essential prerequisites to the rational design of compounds capable of bypassing or blocking the action of P-gp in drug-resistant tumor cells. Few studies aimed at identifying structure–activity relationships in MDR drug analogs have been published. The activity of derivatives of ellipticine and olivacine bearing modified side chains has recently been analyzed on human and mouse multidrug-resistant cell lines overexpressing P-gp (Chevallier-Multon et al., 1990). Although compounds lacking either of the two nitrogen atoms on these molecules were not analyzed in this study, the quaternization of one of the two nitrogens and the degree of hydrophobicity of the side-chain substitutions appeared to be important for defining the MDR type of resistance in these compounds. Structure–activity relationships among P-gp modulators, such as verapamil, quinacrine, and indole alkaloids, have been analyzed (Zamora et al., 1988); lipid solubility at physiological pH, cationic charge, and molar refractivity were proposed as important determinants of these compounds for MDR reversal. Related studies on the capacity of reserpine and yohimbine analogs to compete with photolabeling of P-gp by an analog of vinblastine indicated that the relative disposition and conformation of the two aromatic rings and the basic nitrogen were important for the modulation of P-gp (Pearce et al., 1989). Finally, we have shown that simple lipophilic cations such as tetraphenylphosphonium, tetraphenylarsonium, and derivatives of these compounds where one or two phenyl groups had been substituted by methyl groups are substrates for P-gp (Gros et al., 1992).

In the present study, we used a series of colchicine analogs to establish structure–activity relationships for this known P-gp substrate and identify structural determinants important for P-gp recognition. The ability of these analogs to be recognized and transported by P-gp was established from the degree of resistance to these analogs, shown by cell clones stably expressing either one of the two active mouse P-gp efflux pumps, *mdr1* and *mdr3*. The cellular toxicity of colchicine analogs is dependent on both the affinity for the intracellular target, tubulin, and the accessibility of the target (entry into cells). Since colchicine enters the cell by passive diffusion across the lipid bilayer, the lipophilicity of the analogs would be an important parameter of cytotoxicity. On the other hand, results from energy-transfer experiments with photoactivatable membrane probes (Raviv et al., 1990), together with the analysis of discrete P-gp mutants in the predicted membrane-associated regions of the protein and showing altered substrate specificity (Choi et al., 1988; Gros et al., 1991; Devine et al., 1992), suggest that MDR drugs may be recognized by P-gp in association with the lipid bilayer. This double effect of relative hydrophobicity, i.e., increased toxicity for drug-sensitive cells and increased degree of resistance of the same cells overexpressing P-gp, was noticed in a study of ellipticine and olivacine analogs (Chevallier-Multon et al., 1990). Therefore, it would appear that the lipophilicity of the analogs may also influence their availability and capacity to be effluxed by P-gp in the type of assay system used here. However, calculations of effective lipophilicity in the colchicine analog series (with *c* log *D* values spanning 0.2 to 3.9; see Table I in the supplementary material) have shown that there was no correlation (linear or quadratic) between (1) hydrophobicity and P-gp recognition or (2) between hydrophobicity and toxicity (data not shown). Therefore, it would appear that varying degrees of lipophilicity in colchicine analogs do not dramatically affect drug/P-gp interactions.

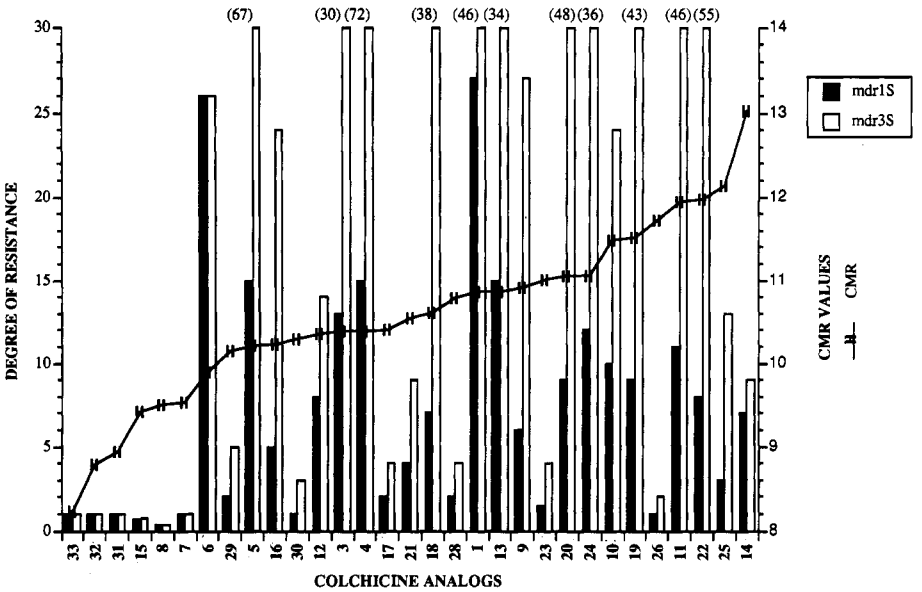


FIGURE 5: CMR (calculated molar refractivity) values of the various colchicine analogs plotted against the degree of cellular resistance expressed by *mdr1S* and *mdr3S* cell clones for each of the compounds. The degree of resistance expressed by *mdr3S* for some of the compounds is indicated in parentheses, when greater than 30-fold.

Other factors, such as specific molecular groups and overall size of the molecule, seemed to be of greater importance (see below).

Our analysis of colchicine analogs clearly showed that substitutions of the methoxy groups of the A and C rings had little effect on the degree of resistance expressed by *mdr* transfectants, suggesting that these groups are not important for P-gp recognition. However, modifications of these groups did affect the overall toxicity. This is in agreement with results of tubulin binding studies on these analogs, showing that the methoxy groups at C1, C2, C3, and C10 are key determinants for binding (Brossi, 1990). On the other hand, we observed that the nitrogen atom of the acetamido group at C7 was critical for P-gp recognition, as *mdr*-transfected cells showed no resistance to colchicine analogs where this nitrogen had been replaced by hydrogen. The acetamido group at C7 is not required for tubulin binding, and tubulin binding is even enhanced by removal of the entire B ring of the molecule (Brossi, 1990; Boyé & Brossi, 1992). Our conclusions on the relative importance of the nitrogen at C7 for P-gp interaction were verified in separate series of colchicine, thiocolchicine, and allocolchicine analogs. Studies of allocolchicine analogs bearing a six-carbon aromatic C ring showed that the integrity of the seven-carbon C ring of the tropolone was also important for P-gp interaction. The various effects and our conclusions on discrete substitutions on colchicine analogs were verified for two P-gps encoded by mouse *mdr1* and *mdr3*. Moreover, the effects of these substitutions on cytotoxicity for *mdr* transfectants were not due to the mere presence of a large integral membrane protein in the cell membranes of these clones: cells transfected and expressing mutant forms of *mdr1* and *mdr3* (Gros et al., 1991) showing decreased activity and altered substrate specificity (in particular for colchicine) did not express resistance to the analogs tested herein (data not shown). One exception was deacetylcolchiceine, which has an amino group at C7 but yet is not a P-gp substrate. This compound has a CMR value smaller than 9.7, and its lack of recognition by P-gp may be due to a reduced molar volume. In addition, the very reduced toxicity of this compound and that of the parent compound colchiceine, which may be due to the replacement of the methoxy group by a hydroxyl at the C10 position (reduced hydrophobicity, tautomerization of the carbonyl at C9), may reduce the structure–activity value of these analogs. Taken together, our results suggest that different domains within colchicine are important for tubulin binding (methoxy groups on the A and C rings), while others are important for P-gp interaction (nitrogen at C7).

The previous physicochemical analysis of colchicine and some of the analogs tested in this study provides some interesting clues as to the possible role of the nitrogen atom at C7 in P-gp recognition. A comparison of the ultraviolet absorption spectra and circular dichroism spectroscopic measurements of colchicine, demecolcine, and deacetamidocolchicine shows that the C7 nitrogen lone pair of electrons participates in the hyperconjugation (donation/sharing) of the π-aromatic electrons of the C ring (Bhattacharyya et al., 1986; Bane-Hastie & Rava, 1989; Pyles et al., 1992); this interaction may account for the unexpectedly low pK_a value we observed for demecolcine (pK_a = 7.45 in water). Deacetamidocolchicine, which is not a substrate for P-gp, lacks this nitrogen and cannot participate in this hyperconjugation. The lone pair of the nitrogen of 7-(isothiocyanato)deacetamidothiocolchicine, another non-P-gp substrate in our study, may not be available for hyperconjugation due to the intermediate sp^2–sp hybridization of nitrogen (Beard & Dailey, 1949) as expected from its mesomeric involvement in the isothiocyanato group. Alternatively, the 7-isothiocyanto derivative (26) is distinct from all of the other congeners bearing a nitrogen at C7 since it does not possess a polarized NH moiety capable of hydrogen bond donation. The importance of this NH functionality for P-gp interaction may be easily probed by comparisons with the corresponding *N*-methylacetamido or carbamyl analogs of colchicine.

Examination of the calculated molar refractivities (CMR) indicated that a minimal size requirement exists in colchicine analogs for efficient P-gp recognition, since compounds with CMR values lower than 9.7 were not substrates for P-gp (Figure 5). Interestingly, five of these six compounds (7, 8, 31, and 32) did not have a nitrogen at C7 of the B ring, raising the formal possibility that the importance of the C7 nitrogen in the colchicine interaction with P-gp may not reflect specific chemical interactions, but rather may suggest a simple minimal size requirement at that position. This proposal can only be tested by analyzing additional novel compounds which are

roughly isosteric and isoelectronic at the C7 nitrogen position (such as hydroxyl or methyl ether derivatives). However, we feel that this hypothesis is unlikely since 7-(isothiocyanato)-deacetamidothiocolchicine (26), which has a CMR value of 11.73 and a chemically distinct nitrogen at C7, is not a P-gp substrate. Therefore, it appears that the minimum chemical structural requirements for effective colchicine/P-gp interaction include an intact phenyltropolone backbone, an overall size greater than the CMR 9.7 threshold, and the C7 nitrogen with either an available lone pair of electrons or an NH function capable of H-bond donation.

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SUPPLEMENTARY MATERIAL AVAILABLE

A table (Table I) containing the calculated molar refractivity values (CMR), log *P* (*c* log *P*), and log *D* (*c* log *D*) values for the colchicine analogs used in this study (1 page). Ordering information is given on any current masthead page.

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