STRUCTURE ACTIVITY RELATIONSHIPS IN THE COLCHICINE MOLECULE WITH RESPECT TO INTERACTION WITH THE MAMMALIAN MULTIDRUG TRANSPORTER, P-GLYCOPROTEIN

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Abstract -- Colchicine forms part of a group of structurally unrelated cytotoxic drugs to which P-glycoprotein overexpression confers resistance to, both in cultured cells in vitro and tumor cells in vivo. Modifications of the methoxy groups on the A and C rings modulated cellular toxicity but had no effect on P-gp interaction. 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. Examination of calculated molar refractivities (CMR) revealed that only compounds showing CMR values greater than 9.7 were P-gp substrates, suggesting a minimal size requirement for efficient interaction with P-gp.

Multidrug resistance (MDR) is a major limitation to the successful chemotherapeutic treatment of many types 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 many others.[1, 2] 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.[3, 4] In rodents, P-gp is encoded by three mdr genes, mdr1, mdr2, and mdr3,[5 - 8] while only two forms, MDR1 and MDR2 exist in humans.[9, 10] All P-gps 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 (Figure 1) 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.[11] In gene transfer experiments, both mdr1 and mdr3 can confer drug resistance, while mdr2 cannot.[6, 7, 12]

Although P-glycoprotein overexpression clearly results in a net decrease in cellular drug accumulation, the underlying mechanism of action is not well understood and a matter of controversy, P-glycoprotein being proposed to be either a drug efflux pump,[4] chloride channel,[13] ATP carrier,[14] pH regulator,[15] and lipid flippase.[16] 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. An improved understanding of the molecular requirements for drug/protein interactions by identifying putative common features and defining structural requirements for P-gp/drug interactions are essential prerequisites to the rational design of compounds capable of either by-passing or blocking the action of P-gp in drug resistant tumor cells.

Essential pharmacologic features of MDR substrates. The biochemical characterization[17 - 21] of P-gp and analyses of chimeric[22 - 24] and mutant P-gps[25 - 30] showing altered substrate specificities point to the membrane-associated segments as key functional determinants for drug-recognition and transport. Recently, the predicted transmembrane domain 11 has been identifed as a putative drug binding site in functional analyses of P-gp mutants.[23] However, the specific structural requirements and chemical groups of drug molecules required for

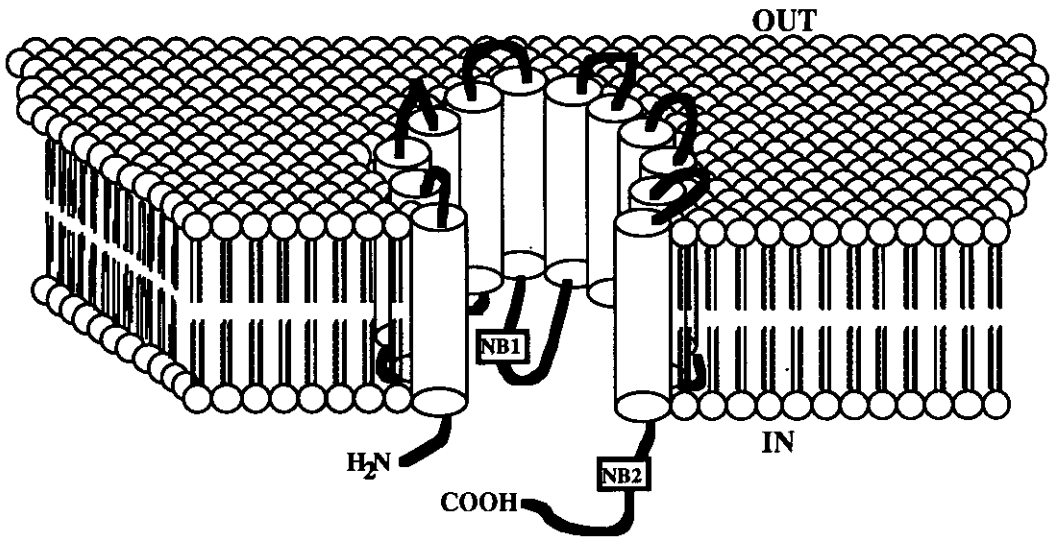


Figure 1: The putative structure of P-glycoprotein. (NB 1- nucleotide binding site 1; NB2- nucleotide binding site 2)

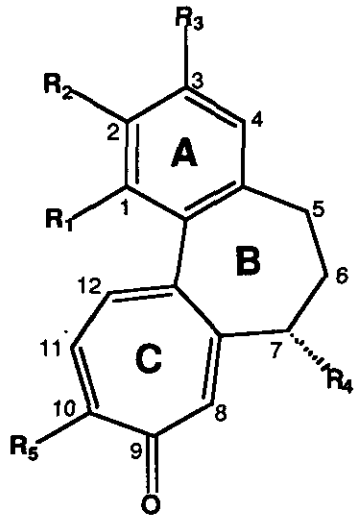
P-gp recognition and transport remain poorly defined.[31] Few studies aimed at identifying structure/activity relationships in MDR drug analogs have been published. A comprehensive study on the activity of derivatives of ellipticine and olivacine bearing modified side chains has been recently analyzed on human and mouse multidrug resistant cell lines overexpressing P-gp.[32] 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 important for defining the essential requirements for drug interaction with P-gp.

Structure/activity relationships amongst P-gp modulators, such as verapamil, quinacrine, and indole alkaloids have been analyzed[31]; 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 photolabelling 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 modulation of P-gp.[33] Similarly, reversal of MDR by phenothiazine analogs showed that in addition to the hydrophobic tricyclic aromatic ring structure, the spatial orientation of a charged tertiary amine side chain with respect to the aromatic skeleton was important for interaction with P-gp.[34] 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.[35]

Colchicine as model substrate for P-gp. Colchicine is a cytotoxic plant alkaloid and is part of the MDR group.of drugs, recognized and transported by P-glycoprotein (P-gp). The drug can enter cells by passive diffusion across the lipid bilayer and exerts its cytotoxic effect by binding to tubulin, arresting cell division.[36] The biologically active form of colchicine ((-)-(aS, 7S)-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 form the phenyl-tropolone backbone of the molecule with the 4 methoxy groups and an acetamido group attached to the periphery.[37] Cultured cells transfected with and overexpressing either mouse mdr1 or mdr3 show 30 and 50 fold increase in resistance to this drug, respectively.[7, 27] Transport studies in P-gp expressing cells show decreased intracellular accumulation of radiolabelled colchicine in these cells.[26] Photoactivatable analogs of colchicine have been shown to bind to P-gp, this binding being specific and competed by non-radioactive colchicine and other MDR drugs.[38]

Because of its relatively simple structure and the few peripheral chemical groups, we have used a series of colchicine analogs, provided by Dr. A. Brossi, that were 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. 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, 2, 3). For this, we measured the effect of these discrete chemical substitutions 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 pump, known to confer multidrug resistance. 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.



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

Figure 2: Structures of colchicine analogs.

Effect of lipophilicity on cytotoxicity. Cellular toxicity of these compounds (expressed as the D_{50} or dose required to reduce plating efficiency by 50%), was established for 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 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-gps encoded by mdr1 and mdr3 in two to four independent experiments, each done in duplicate. In these experiments, a minimal degree of three to four fold resistance was required to classify a compound as P-gp substrate.

The ability of these analogs to be recognized and transported by P-gp was established from the degree of resistance to these analogs, expressed by cell clones stably expressing either one of the two active mouse P-gp efflux pumps, mdr1 and mdr3. Cellular toxicity of colchicine analogs is dependent both on affinity for the intracellular target, tubulin, and accessibility of the target (entry into cells). Since colchicine enters the cell by passive diffusion across the lipid bilayer, lipophilicity of the analogs would be an important parameter of cytotoxicity. On the other hand, results from energy transfer experiments with photoactivatable membrane probes[21] together with the analysis of discrete P-gp mutants in the predicted membrane associated regions of the protein and showing altered substrate specificity,[25, 27, 29] 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 the study of ellipticine and olivacine analogs.[32] Therefore, it would appear that 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 have shown that there was no correlation, linear or quadratic, between (1) hydrophobicity and P-gp recognition nor (2) between hydrophobicity and toxicity. Therefore, it would appear that varying degrees of lipophilicity in colchicine analogs do not dramatically affect drug/P-gp interactions. Other factors, such as specific molecular groups and overall size of the molecule seemed to be of greater importance (see below).

Colchicine analogs. We have previously shown that both mdr1 and mdr3 confer multidrug resistance in transfection experiments, with mdr3 confering higher levels of resistance than mdr1, possibly encoding a more efficient drug efflux pump.[27] A mutant of mdr3 which differs from wild-type by a single amino acid substitution confers a lower resistance to colchicine.[7, 27] Immunoblotting experiments with the mouse anti-P-glycoprotein monoclonal antibody C219 showed that cell clones mdr1S, mdr3S and mdr3F transfected with either wild type mdr1 (clone 1S) and mdr3 (clone 3S) or mutant mdr3 (clone 3F), respectively, express in membrane enriched fractions equivalent amounts of 180 and 160 kD proteins, the known molecular mass of these two mouse P-gps.[27] 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 (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-acetoxy-demethylcolchicine (#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-Acetoxy-demethylcolchicine (#2) was found to be non toxic for LR73 control cells, even at very high concentrations (10 μg/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; 3F, 5X), #4 (1S, 15X; 3S, 72X; 3F, 5X), and #5 (1S, 15X; 3S, 67X; 3F, 5X) showed that both mdr1S (1S) and mdr3S (3S), as well as the mutant mdr3 (3F) 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 recognition of colchicine by P-gp. The importance of the methoxy group at position C10 of the C ring was next analyzed in 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; 3F, 3X) and #18 (1S, 7X; 3S, 38X; 3F, 4X) show that removal or replacement of C10 methoxy has little effect on recognition of these analogs by P-gp; however, analog #17 appeared to be a P-gp substrate, although a poor one (1S, 2X; 3S, 4X; 3F, 2X). Overall, these results indicate that the four methoxy groups on the A and C rings are not key determinants for P-gp recognition; however, modifications of these groups did affect 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.[39]

Table I: The effect of colchicine analogs on survival characteristics of cell clones stably transfected with *mdr1* and *mdr3*

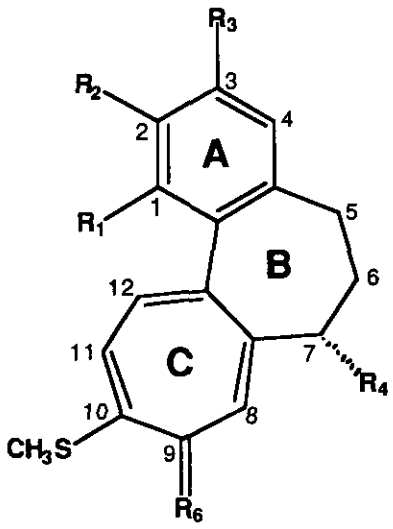
		LR73	*mdr1S*	*mdr3S*	*mdr3F*	n^c
1	colchicine	41[a] ± 6	1100 ± 100 (27X[b])	1900 ± 200 (46X)	200 ± 39 (5X)	6
2	1-acetoxydemethylcolchicine	NOT TOXIC				
3	2-demethylcolchicine	1100 ± 300	14000 ± 3000 (13X)	33000 ± 3000 (30X)[d]	5000 ± 2000 (5X)	3
4	3-demethylcolchicine	180 ± 10	2700 ± 800 (15X)	13000 ± 4000 (72X)	900 ± 400 (5X)	4
5	cornigerine	30 ± 10	440 ± 30 (15X)	2000 ± 1000 (67X)	160 ± 50 (5X)	3
6	deacetylcolchicine	14 ± 2	360 ± 50 (26X)	370 ± 30 (28X)	15 ± 8 (1X)	3
7	deacetamidocolchicine	8 ± 1	8 ± 1 (1X)	11 ± 4 (1X)	8 ± 1 (1X)	4
8	5,6-dehydro-7-deacetamidocolchicine	700 ± 100	290 ± 80 (0.4X)	260 ± 90 (0.4X)	240 ± 40 (0.3X)	3
9	*N*-trifluoroacetyl-deacetylcolchicine	7 ± 1	40 ± 10 (6X)	190 ± 50 (27X)	17 ± 5 (2X)	4
10	*N*-ethoxycarbonyl-deacetylcolchicine	11 ± 3	110 ± 30 (11X)	260 ± 30 (25X)	37 ± 7 (3X)	4
11	*N*-propoxycarbonyl-deacetylcolchicine	65 ± 9	700 ± 200 (11X)	3000 ± 300 (47X)	300 ± 100 (5X)	3
12	demecolcine	10 ± 1	78 ± 3 (8X)	140 ± 20 (15X)	19 ± 4 (2X)	3
13	*N*-formyldemecolcine	5 ± 1	73 ± 3 (15X)	170 ± 20 (34X)	25 ± 8 (5X)	4
14	speciosine	17 ± 3	120 ± 20 (7X)	150 ± 30 (9X)	30 ± 10 (2X)	4
15	deacetylcolchiceine	3500 ± 400	2300 ± 500 (0.7X)	2500 ± 800 (0.7X)	1600 ± 300 (0.5X)	3
16	colchicide	90 ± 10	410 ± 50 (5X)	2100 ± 600 (24X)	300 ± 100 (3X)	4
17	colchiceine	6000 ± 1000	15000 ± 2000 (2X)	25000 ± 1000 (4X)[d]	10000 ± 2000 (2X)	3
18	colchiceinamide	83 ± 6	550 ± 90 (7X)	3100 ± 400 (38X)	330 ± 70 (4X)	3
19	thiocolchicine	6 ± 2	50 ± 10 (9X)	260 ± 90 (42X)	16 ± 8 (3X)	4
20	3-demethylthiocolchicine	24 ± 5	210 ± 60 (9X)	1150 ± 70 (48X)[d]	120 ± 20 (5X)	3
21	deacetylthiocolchicine	7 ± 1	27 ± 5 (4X)	65 ± 5 (9X)	10 ± 2 (1X)	3
22	3-demethyl-*N*-butyryl-deacetylthiocolchicine	38 ± 8	300 ± 100 (7X)	2100 ± 700 (55X)	200 ± 100 (5X)	3
23	thiodemecolcine	6 ± 1	9 ± 2 (2X)	23 ± 4 (4X)	6 ± 1 (1X)	3
24	*N*-formylthiocolchicine	1.4 ± 0.1	17.3 ± 0.6 (12X)	50 ± 10 (36X)	5 ± 1 (4X)	3
25	*N*-ethoxycarbonyl-deacetylthiocolchicine	2.8 ± 0.8	8.1 ± 0.1 (3X)	40 ± 10 (17X)	6.5 ± 0.7 (2X)	2
26	7-isothiocyanato-deacetamidothiocolchicine	100 ± 30	120 ± 40 (1X)	190 ± 20 (2X)	150 ± 20 (2X)	3
27	9-thiodeoxothiocolchicine	9 ± 2	60 ± 20 (6X)	140 ± 20 (16X)	24 ± 4 (3X)	4
28	allocolchicine	16 ± 2	26 ± 5 (2X)	70 ± 20 (4X)	17 ± 4 (1X)	4
29	allocolchicine aldehyde	60 ± 20	120 ± 30 (2X)	310 ± 60 (5X)	80 ± 20 (1X)	4
30	*N*-acetylcolchinol methyl ether	13 ± 4	16 ± 1 (1X)	40 ± 10 (3X)	12 ± 5 (1X)	4
31	deaminocolchinol methyl ether	39 ± 2	33 ± 4 (1X)	38 ± 7 (1X)	40 ± 10 (1X)	4
32	biphenyl ester	2200 ± 200	2800 ± 400 (1X)	2000 ± 200 (1X)	2000 ± 200 (1X)	3
33	phenyltropone (Fitzgerald)	17[e]	16	17.5	NOT TESTED	

[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 D50 (*n*g/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 to 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.

The functional importance of the acetamido group at position C7 of the B ring for recognition by P-gp was tested in 10 analogs (#6 to #16) showing unique replacements at that position. Most of these analogs were found to be substrates for P-gp; however, deletion of the acetamido group in deacetamidocolchicine (#7; 1S, 1X; 3S, 1X; 3F, 1X) and 5,6-dehydro-7-deacetamidocolchicine (#8; 1S, 0.4X; 3S, 0.4X; 3F, 0.3X) resulted in D_{50} values similar for drug sensitive and *mdr* transfectants, suggesting that this group is critical for colchicine recognition by P-gp. The low level of resistance was not due to overall reduced toxicity of these compounds, as deacetylcolchicine 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, 28X; 3F, 1X), 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. Replacing the three hydrogens of the acetyl group by more electronegative fluorine atoms in *N*-trifluoroacetyldeacetylcolchicine (#9) had little effect on recognition by P-gp (1S, 6X; 3S, 27X; 3F, 2X). Likewise, extending the length of the chain, *N*-ethoxycarbonyldeacetylcolchicine (#10: 1S, 11X; 3S, 25X; 3F, 3X) and *N*-propoxycarbonyldeacetylcolchicine (#11: 1S, 11X; 3S, 47X; 3F, 5X), did not alter recognition by P-gp. The change of the nitrogen-containing group from an amide to a secondary amine by the introduction of a *N*-methyl group instead of the *N*-acetyl group in demecolcine (#12: 1S, 8X; 3S, 15X; 3F, 2X) 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; 3F, 5X) and speciosine (#14: 1S, 7X; 3S, 9X; 3F, 2X), respectively. Taken together, these results suggest an important role of the nitrogen atom of the acetamido group at C7 for recognition by P-gp. One notable exception was deacetylcolchiceine (#15) that 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. Although 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,[37, 39] 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.

Thicolchicine and allocolchicine analogs. Our conclusions on the relative importance of the nitrogen at C7 for P-gp interaction were verified in separate series of colchicine, thiocolchicine (#19 to #27), and allocolchicine (#28 to #31) analogs. Thiocolchicine (Figure 3) is similar to colchicine except that the C10 methoxy is replaced by an $-SCH_3$. Toxicity of thiocolchicine for drug sensitive LR73 cells is greater than colchicine, due to increased affinity for tubulin,[37] and drug resistant mdr transfectants show levels of resistance to thiocolchicine (1S, 9X; 3S, 43X; 3F, 3X) 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; 3F, 5X) and 3-demethyl-*N*-butyryldeacetylthiocolchicine (#22: 1S, 8X; 3S, 55X; 3F, 5X) did not reduce the degree of resistance expressed by mdr transfectants. mdr transfectants were also resistant to 9-thiodeoxothiocolchicine (#27: 1S, 6X; 3S, 16X; 3F, 3X), where the oxygen at C9 is replaced by sulfur. Replacement of the acetamido group at C7 with *N*-formamido (#24: 1S, 12X; 3S, 36X; 3F, 4X) and *N*-ethoxycarbamyl moieties (#25: 1S, 3X; 3S, 17X; 3F, 2X) had little effect on P-gp recognition. Removal of the acyl functionality from C7 in deacetylthiocolchicine (#21: 1S, 4X; 3S, 9X; 3F, 1X) and thiodemecolcine (#23: 1S, 2X; 3S, 4X; 3F, 1X) produced two compounds which 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-Isothiocyanatodeacetamidothiocolchicine (#26) which is an extremely poor substrate for P-gp (only 2X 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, although slightly lower, than to their colchicine counterparts. This was most obvious for the 6/21 and 12/23 pairs which varied by about 3 fold.

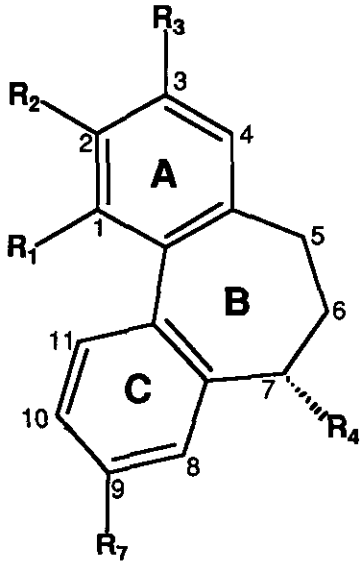
Studies of allocolchicine analogs (#28 to #31) (Figure 4) bearing a six carbon aromatic C ring showed that the integrity of the seven carbon C ring of the tropolone was also important for P-gp interaction. In these analogs, the seven carbon C ring is replaced by a six-membered aromatic ring. The four analogs showed D_{50} values for LR73 cells in the range of colchicine, and 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,

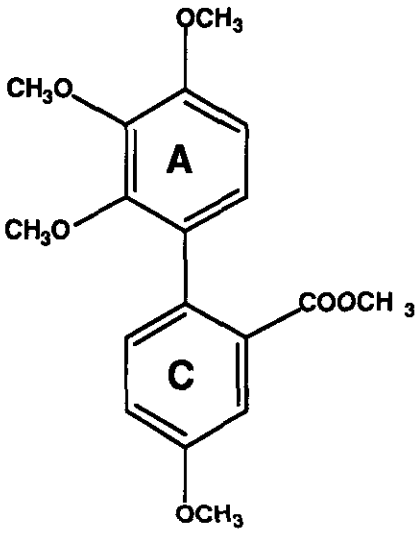


		A-RING			B-RING
		R_1	R_2	R_3	R_4
19	thiocolchicine	OCH_3	OCH_3	OCH_3	$NHCOCH_3$
20	3-demethylthiocolchicine	OCH_3	OCH_3	OH	$NHCOCH_3$
21	deacetylthiocolchicine	OCH_3	OCH_3	OCH_3	NH_2
22	3-demethyl-*N*-butyryldeacetylthiocolchicine	OCH_3	OCH_3	OH	$NHCOC_3H_7$
23	thiodemecolcine	OCH_3	OCH_3	OCH_3	$NHCH_3$
24	*N*-formylthiocolchicine	OCH_3	OCH_3	OCH_3	NHCHO
25	*N*-ethoxycarbonyldeacetylthiocolchicine	OCH_3	OCH_3	OCH_3	$NHCOOC_2H_5$
26	7-isothiocyanatodeacetamidothiocolchicine	OCH_3	OCH_3	OCH_3	NCS
27	9-thiodeoxothiocolchicine	OCH_3	OCH_3	OCH_3	$NHCOCH_3$

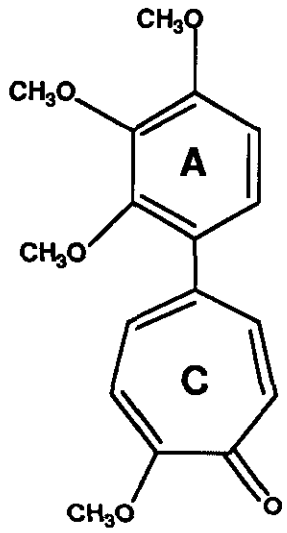
Figure 3: Structures of thiocolchicine analogs.

2X; 3S, 4X; 3F, 1X), allocolchicine aldehyde (#29: 1S, 2X; 3S, 5X; 3F, 1X) and *N*-acetylcolchicinol methyl ether (#30: 1S, 1X, 3S, 3X; 3F, 1X) 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 deamino-colchicinol 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.





32. biphenyl ester



33. phenyltropone (Fitzgerald)

		A-RING			B-RING	C-RING
		R_1	R_2	R_3	R_4	R_7
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*-acetylcolchinol methyl ether	OCH_3	OCH_3	OCH_3	$NHCOCH_3$	OCH_3
31	deaminocolchinol methyl ether	OCH_3	OCH_3	OCH_3	H	OCH_3

Figure 4: Structures of allocolchicine analogs.

Role of the C7 nitrogen. Previous studies of MDR modulators have shown that the spatial orientation of a tertiary amine with respect to an aromatic hydrophic core skeleton is important for P-gp/drug interaction.[31] A study among dipyridamole analogs showed that the electronegative atoms of the diethylamine group existing in a favorable conformation with respect to the carbon skeleton as well as the presence of at least two piperidine rings were important for reversal of adriamycin resistance.[40] Furthermore, they proposed that the core skeleton of pyrimidopyrimidines may serve to set the spatial relationship among substituents involved with activity. Phenoxazine and phenoxazine type compounds also showed that the presence of a highly electronegative atom

with one or more lone pairs of electrons opposite the NH group occupying position 10 in the tricyclic structure resulted in a superior MDR-modulator.[41]

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 show that the C7 nitrogen lone-pair of electrons participates in the hyperconjugation (donation/sharing) of the π-aromatic electrons of the C-ring;[42 - 44] this interaction may account for the unexpectedly low pKa we observed for demecolcine (pKa = 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-isothiocyanatodeacetamidothiocolchicine, another non-P-gp substrate in our study, may not be available for hyperconjugation due to the intermediate sp^2-sp hybridization of nitrogen[45] as expected from its mesomeric involvement in the isothiocyanato group. Alternatively, the 7-isothiocyanto derviative (#26) is distinct from all of the other congeners bearing a nitrogen at C7 since it does not possess a polarized N-H moiety capable of hydrogen bond donation. The importance of this N-H functionality for P-gp interaction may be easily probed by comparisons with the corresponding *N*-methylacetamido or carbamyl analogs of colchicine. One additional 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 (see below). 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.

Calculated molar refractivity (CMR) analysis. Molecular calculations on the colchicine, thiocolchicine, and allocolchicine analogs show that there was no apparent direct or multifactorial correlation neither 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

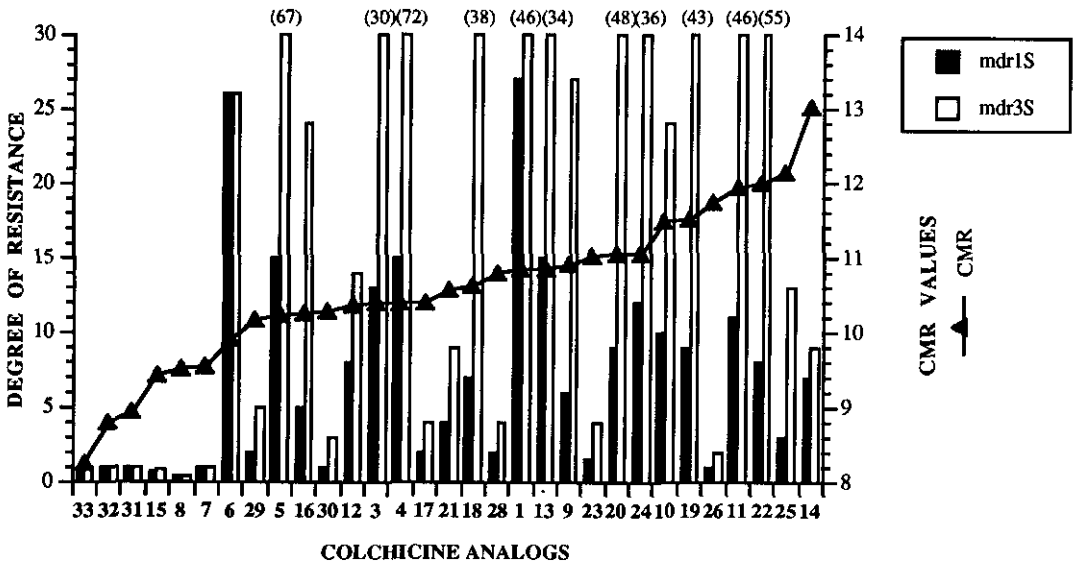


Figure 5: CMR (calculated molar refractivity) values of the various colchicine analogs are plotted against the degree of cellular resistance expressed by *mdr1S* and *mdr3S* cell clones for each of these compounds. The degree of resistance expressed by *mdr3S* for some of the compounds is indicated in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

molar volume of the compound, were calculated for the three colchicine analogs series (Table II). Examination of the molar refactivity 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 exists a minimal size requirement for the efficient recognition and transport of colchicine analogs by P-gp. Zamora *et al.*[31] had also suggested that a nitrogen atom and two aromatic domains share a common volume element. Interestingly, four of these five compounds (#7, #8, #31, #32) did not have a nitrogen at C7 of the B ring, raising the formal possibility that the importance of the C7 nitrogen in 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 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-isothiocyanatodeacetamidothiocolchicine (#26) which has a CMR value of 11.73 and a chemically distinct nitrogen at C7 is not a P-gp substrate. Finally, in compounds showing CMR values between 9.7 and 13.0, there was no correlation between further increase in molar volume and degree

Table II: Listing of CMR, clogP, and clogD values of colchicine analogs.

	COMPOUND	clogP	clogD	CMR
1	colchicine	1.03	1.03	10.86
2	1-acetoxydemethylcolchicine	NA	NA	NA
3	2-demethylcolchicine	0.83	0.83	10.39
4	3-demethylcolchicine	0.55	0.55	10.39
5	cornigerine	1.24	1.24	10.22
6	deacetylcolchicine	1.16	1.15	9.90
7	deacetamidocolchicine	2.81	2.81	9.53
8	5,6-dehydro-7-deacetamidocolchicine	2.51	2.51	9.50
9	*N*-trifluoroacetyldeacetylcolchicine	1.88	1.88	10.91
10	*N*-ethoxycarbonyldeacetylcolchicine	2.21	2.21	11.48
11	*N*-propoxycarbonyldeacetylcolchicine	2.74	2.74	11.94
12	demecolcine	1.58	1.25	10.36
13	*N*-formyldemecolcine	0.80	0.80	10.86
14	speciosine	2.20	2.20	13.02
15	deacetylcolchiceine	1.14	1.10	9.43
16	colchicide	0.43	0.43	10.24
17	colchiceine	0.74	0.74	10.40
18	colchiceinamide	0.21	0.20	10.61
19	thiocolchicine	1.20	1.20	11.51
20	3-demethylthiocolchicine	0.99	0.98	11.05
21	deacetylthiocolchicine	1.60	1.60	10.55
22	3-demethyl-*N*-butyryldeacetylthiocolchicine	2.04	2.04	11.98
23	thiodemecolcine	2.01	1.70	11.01
24	*N*-formyl-thiocolchicine	1.65	1.65	11.05
25	*N*-ethoxycarbonyl-deacetylthiocolchicine	2.65	2.65	12.13
26	7-isothiocyanato-deacetylcolchicine	3.71	3.71	11.73
27	9-thiodeoxo-thiocolchicine	NA	NA	NA
28	allocolchicine	1.93	1.93	10.79
29	allocolchicine aldehyde	1.32	1.32	10.16
30	*N*-acetylcolchinol methyl ether	1.87	1.87	10.29
31	deaminocolchinol methyl ether	3.91	3.91	8.95
32	biphenyl ester	2.61	2.61	8.78
33	phenyltropone (Fitzgerald)	1.24	1.24	8.24

The calculated molar refractivity (CMR) and lipophilicity (c logP) 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 (clogP) predicts the partition coefficient of neutral compounds in an octanol-water system. The calculated clogP value for colchicine was 1.03 and was identical to that measured experimentally in an octanol/phosphate buffer (pH 7.4).[48] Since logD (where D represents the distribution at a particular pH) is considered to be a more relevant parameter than logP, calculated lipophilicities (c logP) were used to estimate the effective lipophilicites at pH 7.4 (reported as c logD values) according to the relationship: c logD = c logP - log[1 + antilog (pKa-pH)].[49, 50] The pKa of demecolcine (#12) was determined by microtitration in water and found to be 7.45 ± 0.02.

of resistance expressed by either the 1S or the 3S clones. 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 N-H function capable of H-bond donation. 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 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 these substitutions on cytotoxicity for mdr transfectants were not due to the mere presence of a large integral membrane protein in the cell membrane of these clones because the cells transfected and expressing the mutant forms mdr3 showing decreased activity and altered substrate specificity, in particular for colchicine, did not express resistance to the analogs tested herein.

Recently, a theoretical model for the interaction of drug molecules and reversal agents with P-gp within the membrane has been proposed.[46] This model based on the predicted interaction of calmodulin with phenothiazines and thioxanthenes,[47] proposes that MDR drugs have a amphiphilic nature, with both a hydrophobic and a hydrophilic domain required for recognition by homologous domains on P-gp. The hydrophobic domain contributed by the phenyl groups would interact with overlapping aromatic and hydrophobic amino acid residues within the transmembrane domain of P-gp. Hydrophilic amino acid residues in proximity would interact with the charged/polar amino aicd side chain of the drug.[46] Results presented in this report on the indentification of structural determinants of colchicine essential for P-gp recognition, together with the above mentioned theoretical models may help design novel drug analogs capable of blocking P-gp mediated drug resistance.

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