with CH_2Cl_2–EtOH 8:2 (2 × 400 mL) and filtered through glass wool. The solution was spin evaporated in vacuo to give 8.01 g (69%) of 5-amino-4-chloro-6-[[1-(3-hydroxyphenyl)ethyl]-amino]pyrimidine (**27**) as a foam that was a single spot on TLC (EtOAc–cyclohexane 1:1) and was used without further purification in the next step; UV (0.1 N HCl) λ_{max} 304 nm; UV (0.1 N NaOH) λ_{max} 292 nm.

A mixture of **27** (8.00 g, 30.2 mmol), ethanesulfonic acid (0.15 g, 1.3 mmol), and triethyl orthoformate (100 mL) was stirred at ambient temperature for 40 h. The dark solution was treated with charcoal, filtered through Celite, and spin evaporated in vacuo at 80 °C to give a quantitative yield of **29** as a glass, which was a single spot on TLC (EtOAc–cyclohexane 1:1) and was used without further purification in the next steps; UV (0.1 N HCl) λ_{max} 266.5 nm; UV (0.1 N NaOH) λ_{max} 267 nm; NMR (DMSO-d_6) δ 2.00 (d, 3 H, J = 7 Hz, CH_3), 5.95 (q, 1 H, J = 7 Hz, CH), 6.6–7.3 (complex m, 4 H, Ar), 8.77 (s, 1 H, purine H), 8.97 (s, 1 H, purine H), 9.42 (br s, 1 H, OH).

6-(Dimethylamino)-9-[1-(3-nitrophenyl)ethyl]-9*H*-purine (30). A solution of **28** (10.0 g, 33 mmol) and 10% dimethylamine in EtOH (100 mL) was stirred at ambient temperature for 24 h. The solution was spin evaporated in vacuo, and the residue was dissolved in EtOAc and washed with H_2O. The organic layer was dried ($MgSO_4$) and spin evaporated in vacuo to give a light yellow solid. Recrystallization from toluene gave 10.2 g (99%) of **30**, mp 136–138 °C. Another recrystallization from toluene gave the analytical sample: mp 137–138 °C; TLC (EtOAc); UV (pH 7 buffer + 9.5% EtOH) λ_{max} 274.5 nm (ε 25 800); NMR (DMSO-d_6) δ 2.02 (d, 3 H, J = 7.1 Hz, CH_3), 3.47 (s, 6 H, $N(CH_3)_2$), 6.03 (q, 1 H, J = 7.1 Hz, CH), 7.7 (complex m, 4 H, Ar), 8.21 (s, 1 H, purine H), 8.52 (s, 1 H, purine H). Anal. ($C_{15}H_{16}N_6O_2$) C, H, N.

8-Bromo-6-(dimethylamino)-9-[1-(3-nitrophenyl)ethyl]-9*H*-purine (31). This compound was prepared from **30** as described for preparation of 8-bromo-6-(dimethylamino)-9-(3-nitrobenzyl)-9*H*-purine.[8] The product was crystallized from EtOH to give 3.5 g (80%) of **31**, mp 168–169 °C; TLC (EtOAc–Hexane 1:1); NMR (DMSO-d_6) δ 2.12 (d, 3 H, J = 7.25 Hz, CH_3C), 3.4 (br s, 6 H, $N(CH_3)_2$), 6.11 (q, 1 H, J = 7.25 Hz, CH), 7.6–7.8 (m, 2 H, ArH), 8.19 (s, 1 H, purine H), 8.15–8.25 (m, 2 H, ArH). Anal. ($C_{15}H_{15}N_6BrO_2$) C, H, N.

Benzodiazepine-Binding Assay. The compounds in Table I were assayed for BZR-binding activity by the method described in ref 7. The IC_{50}s are the concentration at which specific binding of 1.5 nM [^{3}H]diazepam to rat brain receptors was decreased by 50%. Increased potency of the compound as an inhibitor of [^{3}H]diazepam binding was assumed to reflect increased affinity of the agent for the receptor.

Pharmacology. Conflict Responding Test. The compounds in Table I that were tested for conflict responding were tested as described in ref 7. This paradigm was a modification of a Geller–Seifter conflict schedule[9,10] in which chlordiazepoxide (CDP) produced significant dose-related increases in responding. At 10 and 20 mg/kg, CDP increased responding by 46 and 67%, respectively.

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Structure–Activity Relationships of Antineoplastic Agents in Multidrug Resistance

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Clinical resistance to many antineoplastic agents is a major cause of treatment failure. The well-documented phenomenon addressed as multidrug resistance (MDR) allows cells to withstand exposure to lethal doses of drugs with dissimilar chemical structures, modes of action, and physicochemical properties. In one of the earliest studies on MDR, Biedler and Riehm in an attempt to explain the cross-resistance profile of actinomycin D resistant Chinese hamster cells suggested that molecular weight was an important determinant. Our statistical analysis of their data validates their claim and indeed strongly demonstrates that cross resistance is enhanced by the increased size and hydrophobicity of the antitumor agent. Our preliminary studies with methotrexate-resistant L1210 cells indicates that cross resistance is increased in the case of moderate-sized, hydrophilic drugs. These two studies and others suggest that current chemotherapy regimens may be improved by treating resistant cells with antineoplastic agents displaying physicochemical characteristics opposite to that of the original inducing agent.

Resistance of tumor cells to multiple cytotoxic agents is one of the major causes of treatment failure in cancer chemotherapy. Malignancies that exhibit de novo resistance seem to be associated with previous exposure to carcinogens, e.g. lung cancer. Acquired resistance generally results from exposure of drug-sensitive malignant cells to various antineoplastic agents. Many experimental cell lines selected for resistance to actinomycin D, colchicine, vincristine, adriamycin, and trimetrexate have demonstrated multidrug resistance to a variety of antitumor agents with dissimilar chemical structures, modes of action, and physicochemical properties.[1–4] This general phenomenon of "pleiotropic drug resistance" is now addressed as multidrug resistance (MDR).

In a pioneering study, Beidler and Riehm were the first to describe the MDR phenomenon.[4] They found that exposure of several sublines of Chinese hamster cells to increasing concentrations of actinomycin D resulted in resistance to a broad range of structurally varied agents (Table I). Their results indicated that cross resistance was correlated with the molecular weights of the drugs.

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(1) Klohs, W. D.; Steinkampf, R. W.; Besserer, J. A.; Fry, D. W. *Cancer Lett.* 1986, *31*, 253–260.
(2) Bech-Hansen, N. T.; Till, J. E., Ling, V. *J. Cell Physiol.* 1976, *88*, 23–31.
(3) Conter, V.; Beck, W. T. *Cancer Treat. Rep.* 1984, *68*, 831–839.
(4) Biedler, J. L.; Reihm, H. *Cancer Res.* 1970, *30*, 1174–1184.

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Table I. Data on Cross Resistance of Chinese Hamster Cells Resistant to Actinomycin D

	log CR				
antineoplastic agent	obsd	pred[a]	deviation	log MW[f]	log P[b]
mithramycin	2.83	2.62	0.21	3.04	−0.25
vincristine	2.28	2.50	−0.22	2.97	2.57
puromycin	1.92	1.42	0.50	2.67	0.86
daunomycin	1.46	1.59	−0.13	2.72	0.66
demecolsine	1.26	1.07	0.19	2.57	1.37
mitomycin C	0.49	0.58	−0.09	2.52	−0.38
proflavine	0.46	0.75	−0.29	2.49	1.10
novobiocin[c]	0.28	1.95	−1.67	2.80	1.58
bromodeoxyuridine	0.08	0.49	−0.41	2.49	−0.29
nitroquinoline *N*-oxide	0.04	−0.06	0.10	2.28	1.02
amethopterin	0.04	0.01	0.03	2.66	−2.52
6-mercaptopurine	−0.30	−0.42	0.12	2.23	0.01
hydrocortisone[c]	−0.40	1.53	−1.93	2.69	1.20
nitrogen mustard[d]	−0.40	−0.50[e]	−0.17	2.28	−2.00[e]
actinomycin D	2.58	2.83	−0.25	3.10	3.21
vinblastine	2.38	2.12	0.26	2.96	3.69

[a] Predicted with eq 3. [b] Partition coefficient in octanol/phosphate buffer, pH 7.40 [c] Not included in the derivation of eq 3. [d] Not included in the analysis since the partition coefficient is not measurable. [e] Estimated. [f] r^2 (log MW vs log P) = 0.21.

This landmark study was followed by another intriguing study by Ling's group using CHO cells resistant to colchicine.[2] They were the first to suggest that there was a positive correlation between partitioning of the drugs into a hydrophobic phase and the cross resistance generated in the mutant cell line. The diverse range of drugs affected by the MDR phenomenon pinpointed the plasma membrane as the site of alteration. Juliano and Ling then identified the 170 000 Da glycoprotein (GP-170) associated with the plasma membrane in their MDR line.[5] In most cases, MDR has been shown to be associated with reduced drug accumulation, which has been attributed to two different phenomena. One involves drug influx via normal channels but drug extrusion by an energy-dependent efflux pump that is more effective in resistant cells.[6] The other mode of action suggests the existence of an energy-dependent permeability barrier which effectively restricts drug entry into the cells.[7] Hence different studies suggest that the determinants of intracellular drug concentration and the ensuing cross resistance profiles are indeed complex and at present not well delineated.

While Beidler and Reihm clearly saw the correlation between the effectiveness of drugs in resistant cells and molecular weight, they did not treat their data statistically. Statistical evaluation of their data in Table I has led to the derivation of the following equations (1–3).

$$\log CR = 3.65\ (\pm 1.1) \log MW - 8.54\ (\pm 2.9) \quad (1)$$

$$n = 13,\ r = 0.915,\ s = 0.470,\ F_{1,11} = 56.9$$

$$\log CR = 3.21\ (\pm 1.1) \log MW + 0.16\ (\pm 0.18) \log P - 7.50\ (\pm 2.8) \quad (2)$$

$$n = 13,\ r = 0.940,\ s = 0.417,\ F_{1,10} = 3.95$$

$$\log CR = 3.85\ (\pm 0.91) \log MW + 0.26\ (\pm 0.15) \log P - 0.09\ (\pm 0.06) (\log P)^2 - 9.00\ (\pm 2.3) \quad (3)$$

$$n = 13,\ r = 0.973,\ s = 0.300,\ \log P_o = 1.44,\ F_{1,9} = 10.3$$

(5) Juliano, R. L., Ling, V. *Biochim. Biophys. Acta* **1976**, *455*, 152–162.
(6) Dano, K. *Biochim. Biophys. Acta* **1974**, *323*, 466–482.
(7) Beidler, J. L.; Peterson, R. H. F. In *Molecular Actions and Targets for Cancer Chemotherapeutic Agents*; Academic Press: New York, 1981; pp 453–482.

Table II. Cross Resistance Profiles of Antitumor Drugs versus L1210/R71 Cells

	log CR[a]				
antineoplastic agent	obsd	pred[b]	deviation	log MW[f]	log P[c]
3-H[d]	2.18	1.91	0.27	2.40	−3.00
3-$CONH_2$[d]	1.85	2.20	−0.35	2.47	−4.49
3-$COCH_3$[d]	2.20	2.08	0.12	2.47	−3.55
3-OH[d]	1.86	2.04	−0.18	2.43	−3.67
3-Br[d]	2.06	1.94	0.12	2.52	−2.14
3-$C_{12}H_{25}$[d]	0.52	1.27	−0.75	2.62	3.41
3-$CH_2OC_6H_4$-3′-C_6H_5[d]	1.22	1.60	−0.38	2.64	0.69
hydroxyurea	0.30	−0.12	0.42	1.88	−1.27
guanazole	0.08	0.49	−0.41	2.00	−1.61
5-fluorouracil[e]	−0.04	0.84	−0.88	2.11	−0.89
6-mercaptopurine	0.60	1.14	−0.54	2.23	0.01
azacytidine	1.54	1.79	−0.25	2.39	−2.17
cytosine arabinoside	1.61	1.86	−0.25	2.44	−2.13
metoprine	1.70	1.26	0.44	2.43	2.56
etoprine	1.69	1.24	0.45	2.45	2.93
mitomycin C	1.89	1.72	0.17	2.52	−0.38
DAMP	1.63	1.37	0.26	2.57	2.64
piritrexim	1.89	1.47	0.42	2.64	1.77
methotrexate	2.55	2.00	0.55	2.66	−2.52
puromycin	1.82	1.57	0.25	2.67	0.86
trimetrexate	1.75	1.55	0.20	2.70	0.84
bakers antifol I	2.18	1.85	0.33	2.73	−1.84
bakers antifol II	1.12	1.23	−0.11	2.79	2.42
tamoxifen	0.59	1.09	−0.50	2.75	4.03
maytansine	1.23	1.19	0.04	2.84	1.99
mithramycin	0.66	0.91	−0.25	3.04	−0.25
valinomycin	0.59	0.44	0.15	3.05	3.24
actinomycin D	0.12	0.25	−0.13	3.10	3.21
bleomycin	0.24	0.75	−0.51	3.15	−2.38
liblomycin	0.29	−0.14	0.43	3.31	−1.11

[a] log CR = log [(ED_{50} of L12100R71)/(ED_{50} of L1210/0)]. [b] Predicted with eq 5. [c] Partition coefficient in octanol/phosphate buffer, pH 7.40. [d] 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3-X-phenyl)-*s*-triazines. [e] Not used in the derivation of eq 5. [f] r^2 (log MW vs log P) = 0.09.

In these equations, CR represents the cross resistance to actinomycin D ($ED_{50}R/ED_{50}S$), where $ED_{50}R$ and $ED_{50}S$ are the molar concentrations of drug inducing 50% inhibition of growth in resistant and sensitive cells, respectively. MW represents the molecular weight of the drug, P is the octanol/water partition coefficient, n represents the number of data points used to derive the equation, r is the correlation coefficient, s is the standard deviation, $\log P_o$ is the optimum log P, and F is the test value for the significance of each additional term. Equation 1 clearly shows a parallel between large molecular weight and diminished effectiveness versus resistant cells. In addition, as eqs 2 and 3 indicate, lipophilicity plays a minor role as Ling's group suspected; hydrophilic (negative log P) drugs are more cytotoxic to these actinomycin D resistant cells. The results of the above analysis and the dearth of systematic SAR studies in this area prompted us to extend Biedler and Riehm's approach to MDR. In doing so, we turned to an L1210 cell line with which we have had considerable experience.[8-10]

Cell Cytotoxicity Studies. The procedures that we employed with the L1210/0 and L1210/R71 cell lines have been previously published.[8] Puromycin, ara-C, mithramycin, bleomycin, and maytansine were generous gifts

(8) Selassie, C. D.; Strong, C. D.; Hansch, C.; Delcamp, T. J.; Freisheim, J. H.; Khwaja, T. A. *Cancer Res.* **1986**, *46*, 744–756.
(9) Selassie, C. D.; Hansch, C.; Zheng, Y. C.; Zhu, H.; Khwaja, T.; Freisheim, T.; Delcamp, T. The Structure Activity Relationships of Novel Triazine Antifolates. In *Chemistry and Biology of Pteridines*; Cooper, B. A., Whitehead, V. M., Eds.; Walter de Gruyter: New York, 1986; pp 959–962.
(10) Selassie, C. D.; Hansch, C.; Khwaja, T.; Freisheim, T. *Proc. Am. Assoc. Cancer Res.* **1986**, *27*, 259.

Table III. Data on Cross Resistance of CCRF-CEM Cells Resistant to Vicristine

antineoplastic agent	log CR		deviation	log MW[d]	log P[b]
	obsd	pred[a]			
vindesine	3.00	2.76	0.24	2.93	0.67
maytansine	2.85	2.13	0.72	2.84	1.99
vincristine	2.76	3.03	−0.27	2.97	2.57
teniposide	1.56	2.00	−0.44	2.82	1.22
etoposide	1.51	1.65	−0.14	2.77	0.60
doxorubicin	1.46	1.51	−0.05	2.75	0.10
vinblastine[c]	1.28	2.96	−1.68	2.96	3.69
daunorobucin	1.20	1.31	−0.11	2.72	0.66
colchicine	1.08	0.48	0.60	2.60	1.03
podophyllotoxin	0.07	0.62	−0.55	2.62	2.01

[a] Predicted with eq 6. [b] Octanol/phosphate buffer, pH 7.40. [c] Not included in the derivation of eq 6. [d] r^2 (log MW vs log P) = 0.06.

Table IV. Collateral Sensitivity of CH^RC5 Cells to Local Anesthetics/Steroids

antineoplastic agent	log sensitivity[a]		deviation	log P[d]
	obsd[b]	pred[c]		
procaine	0.40	0.41	−0.01	0.30
tetracaine	0.70	0.69	0.01	1.00
xylocaine	1.00	0.89	0.11	1.65
propranolol	0.70	0.76	−0.06	1.18
3,20-pregnanedione	1.00	0.96	0.04	3.99[e]
deoxycorticosterone	1.00	1.05	−0.05	3.08
dehydrotestosterone	1.00	1.04	−0.04	3.14[e]
$N(bu)_4{}^+Br^-$	−0.84	−0.80	−0.04	−3.00[f]
TPMP[g]	−0.95	−0.48	−0.47	−2.00
cyclophosphamide	0.52	0.34	0.18	0.63

[a] Log sensitivity = log [ED_{50} of AUXB1/ED_{50} of CH^RC5]. [b] References 2 and 25. [c] Predicted with eq 8. [d] Measured in octanol/phosphate buffer at pH 7.40. [e] Calculated with MedChem Software 3.54. [f] Estimated values. [g] Triphenylmethylphosphonium bromide.

from N. Lomax at NCI. Liblomycin was obtained from Dr. T. Takita and Nippon Kayaku Co. Ltd. (Tokyo). Partition coefficients were obtained from the Pomona College MedChem Data Bank and when not available were measured by using established procedures.[11] In order to minimize the possibility of alterations of the mutant line (L1210/R71) while in culture, recently thawed aliquots of frozen stock samples were utilized. L1210/R71 is resistant to methotrexate (MTX) by virtue of a 100-fold elevation of dihydrofolate reductase levels. The absence or presence of GP-170 has not been determined in this line.

QSAR Studies. Multiple regression analyses were performed on all the data sets. In all data sets, both log P and log MW variables were examined; log MW was determined to be the most significant variable in Tables I, II, III, and V.

Results and Discussion

Using the results in Table II, we have formulated the following equations:

$$\log CR = 12.10\ (\pm 4.37) \log MW - 17.20\ (\pm 5.90) \log (\beta 10^{\log MW} + 1) - 19.51\ (\pm 7.77) \quad (4)$$

$$n = 29,\ r = 0.781,\ s = 0.491,\ F_{3,25} = 13.06,\ \log \beta = -2.13,\ \log MW_o = 2.50$$

$$\log CR = 7.44\ (\pm 2.10) \log MW - 14.97\ (\pm 3.94) \log (\beta 10^{\log MW} + 1) - 0.13\ (\pm 0.06) \log P - 13.13\ (\pm 4.22) \quad (5)$$

$$n = 29,\ r = 0.871,\ s = 0.394,\ F_{1,24} = 14.68,\ \log \beta = -2.60,\ \log MW_o = 2.60$$

(11) Leo, A. J.; Hansch, C.; Elkins, D. *Chem. Rev.* **1971**, *71*, 525–554.

Table V. Data on Cross Resistance of CHO Cells Resistant to Colchicine (CH^RC5)

antineoplastic agent	log CR		deviation	log MW[d]	log P[b]
	obsd	pred[a]			
colchicine	2.26	1.32	0.94	2.60	1.30
puromycin	2.02	1.47	0.55	2.67	0.86
daunomycin	1.88	1.55	0.33	2.72	0.66
emetine	1.46	1.34	0.12	2.74	3.24
ethidium bromide	1.04	1.33	−0.29	2.60	1.15
acriflavin	0.84	0.79	0.06	2.41	1.60
cytochalasin B	1.04	1.26	−0.22	2.68	3.37
erythromycin	0.70	1.35	−0.65	2.98	1.26
colcemid	1.20	1.25	−0.05	2.57	1.37
vinblastine	1.46	1.26	0.20	2.91	3.69
gramicidin D[c]	2.16	1.00	1.16	3.27	0.05
adriamycin	1.40	1.61	−0.21	2.75	0.10
proflavine	0.60	0.51	0.09	2.32	1.10
melphalan	1.18	1.25	−0.07	2.53	−0.52
mechlorethamine	0.48	0.38	0.10	2.28	0.91
chlorambucil	0.30	1.01	−0.71	2.48	1.47
ara-C	0.00	0.25	−0.25	2.44	−2.13
bleomycin	0.00	0.17	0.17	3.15	−2.38
5-flurouracil	0.00	0.30	0.30	2.11	−0.89
thiotepa	0.00	0.41	−0.41	2.28	0.53

[a] Calculated with eq 9. [b] Partition coefficient in octanol/phosphate buffer, pH 7.40. [c] Not used in derivation of eq 9. [d] r^2 (log MW vs log P) = 0.01.

These equations are both significant at the 0.99 level. It is important to note that the sign of the coefficient with the log P terms in eq 5 is opposite to that of eq 2 in dictating that the higher the hydrophobicity, the greater the toxicity to the methotrexate-resistant cells. In both equations above, the bilinear model of Kubinyl is used to describe the nonlinear structure pharmacokinetics relationship involved in permeability.[12] The corresponding parabolic equations of eqs 4 and 5 yield correlation coefficients of 0.767 and 0.865, respectively.

In eq 2, cross resistance was observed in a cell line made resistant to actinomycin D, a large, lipophilic drug (MW = 1300, log P = 3.21), while eq 5 arose from a leukemia cell line resistant to methotrexate, a drug of moderate size but highly hydrophilic (MW = 455, log P = −2.52). The great dependence on molecular weight in both these studies indicates that size constitutes a critical determinant in gaining access to the cytosolic compartment of the resistant cells. The bilinear dependence on the log MW term in eq 5 suggests that the greatest cross resistance will be experienced by a drug of moderate size (log MW = 2.60; MW = 398). Small drugs such as hydroxyurea and guanazole and large drugs like bleomycin show little or no cross resistance in the MTX-resistant cells. Hydroxyurea is the smallest, known antitumor agent; it is conceivable that no chemotherapeutic drug of the future will have a lower molecular weight. Thus, it represents the absolute lower limit of our scale. The behavior of the small drugs may be attributed to the inability of the resistant cells to sufficiently "tighten" their membranes in order to exclude small toxicants. 5-Fluorouracil was not included in the derivation of the equations as a result of its aberrant behavior. It retains collateral sensitivity in the MTX-resistant cells and this could be attributed to some complex interaction in the dihydrofolate reductase–thymidylate synthase cycle.

Bleomycin (Blenoxane) is moderately toxic to MTX-resistant cells. As observed, it is reasonably well predicted since its large size offsets its detrimental hydrophilic character. The MW term in eq 5 accounts for 61% of the

(12) Kubinyi, H. *Arzneim.-Forsch.* **1976**, *26*, 1991–1995.

variance in the data while the log P term only accounts for 15% of the variance. Our critical results with bleomycin, actinomycin D, and mithramycin intensified our search for an ultralarge antineoplastic agent.

Our quest ended with liblomycin, an interesting analogue of bleomycin. Positive preclinical attributes of liblomycin such as its absence of pulmonary toxicity and its low myelotoxicity have generated interest in its wide spectrum of activity against many human cell lines. Liblomycin (MW = 2026, log MW = 3.31) is well predicted and substantiates our findings that cross resistance is minimized in the case of high molecular weight drugs in resistant cells.

The results of eq 5 suggest alterations in microviscosity of the membrane and subsequent alterations in rigidity which eventually lead to a *depletion* of essential nutrients. This could result in an increase in endocytosis. Large drugs taken in via this process will be trapped within the confines of the cell while small drugs can easily traverse the membrane in either direction. Hence small drugs reveal the same toxicity to sensitive and resistant cells. Some resistant cell lines do show an alteration in the fluidity of the cell surface membrane which subsequently leads to increased/decreased structural order.[13,14] This "tightening" of the membrane may contribute to lower intracellular drug accumulation by effectively diminishing the influx of large molecules.

Studies on plasma membrane structural order have prompted other studies on membrane endocytosis in resistant cell lines.[15] Endocytosis is a specific process where the rates of uptake vary depending on the agent and the cell type.[16] Our results indicate that this process may be enhanced in the MTX-resistant cells. In fact, it has been demonstrated in anthracycline and vinca alkaloid resistant Ehrlich ascites cells that nonspecific adsorptive endocytosis is increased.[15]

Our results may be explained on the basis of the following model: two modes of entry into the resistant cells are operative. One involves normal diffusion governed by k_1, while the other includes entry via endocytosis controlled by k_3. Efflux out of the cell (the simple reverse of entry) would be directed by k_2. We assume that exocytosis is negligible compared to endocytosis. Three situations can arise based on the size—small, moderate and large—of the cytotoxic probes.

In the case of small molecules, $k_1 \gg k_3$ and hence the compounds enter via passive diffusion. As size of the drug increases, entry via the path controlled by k_1 slows until, at the critical size (MW $\approx$ 400), a change in mechanism begins and the endocytosis route prevails for drugs of increasing size. This is the only avenue open to resistant cells whereby they can obtain necessary nutrients of large size. Since $k_3 \gg k_2$ and $k_2 \cong k_1$, the large drugs become more tightly entrapped in the cytosol where they subsequently exert their cytotoxic actions. The break in linearity at log MW = 2.60 establishes the crossover point where concentration in the cell is governed by k_3 and k_2. Thus, the largest drugs in Table II have log CR values near 0, similar to that of the small drugs. That signifies that they are equipotent against sensitive and resistant cells. Hence their net concentrations within the confines of the cell in sensitive and resistant cells must be approximately the same. For large drugs, the net result of diffusion plus endocytosis for both sensitive and resistant cells is similar.

An alternative mechanism for the minimal cross resistance evoked by large drugs could be that they do not enter either sensitive or resistant cells but exert their cytotoxic actions at the membrane level outside the cell. This seems highly unlikely in view of their widely differing structural moieties and their well-established different modes of action at the molecular level.

Equations 1–3 from Biedler and Riehm's study show that three data points are not included in their derivation. Novobiocin has almost the same toxicity to both resistant and sensitive cells, but it is predicted to be much less effective because of its rather high molecular weight and hydrophobicity. The fact that it penetrates a membrane that is resistant to many other drugs suggests a special means of transport or implies it does not need to cross the cell membrane to induce its cytotoxicity. Nitrogen mustard is in fact well predicted, assuming a log P of −2.00. Due to its instability in aqueous solution, its partition coefficient cannot be determined. The figure of −2.00 could be in considerable error without affecting its predicted activity substantially because of the small coefficient with the log P term in eq 3. Hydrocortisone is more toxic to resistant cells than expected, but reasons for its aberrant behavior are not apparent. In the case of both novobiocin and hydrocortisone, nonspecific membrane perturbations may be implicated particularly if high concentrations of drugs are involved (see eq 8).

Another interesting MDR study was done by Conter and Beck in which CCRF-CEM cells resistant to vincristine (MW = 930, log P = 2.57) were treated with 10 antitumor agents (Table III).[17] From the data, eq 6 has been formulated.

$$\log \mathrm{CR} = 6.89\ (\pm 3.1) \log \mathrm{MW} - 17.4\ (\pm 8.7)$$

$$n = 9,\ r = 0.893,\ s = 0.468,\ F_{1,17} = 27.5 \qquad (6)$$

Equation 6 is highly significant ($F_{1,17}, \alpha 0.01 = 12.3$). In the derivation of eq 6 one data point, vinblastine, has been omitted. The shortcomings of the above mentioned data set include a small range in the molecular weights of the drugs and clustering of the data points with respect to hydrophobicity. We believe that this is the reason behind the absence of the hydrophobicity term in eq 6. Equation 6 is of interest since the same type of drug (high MW, high log P) was used for eq 3, and in each case the slopes of eq 3 and 6 are positive. However, addition of a parabolic term in log P allows for inclusion of vinblastine with a correlation coefficient r = 0.900 for 10 compounds. In this case, vinblastine is very well predicted.

Equation 7 is very similar to eq 3 in demonstrating that cross resistance is dependent on size and hydrophobicity. However, the equation is not very robust statistically because of the small sample size (n = 10).

(13) Ramu, A.; Glaubiger, I.; Magrath, I. T.; Joshi, A. *Cancer Res.* **1983**, *43*, 5533–5537.
(14) Rintoul, D. A.; Center, M. S. *Cancer Res.* **1984**, *44*, 4978–4980.
(15) Schested, M.; Skorsgaard, P.; Van Deurs, B.; Winther-Nielson, H. *JNCI, J. Natl. Cancer Inst.* **1987**, *78*, 171–179.
(16) Duve, C.; Barsy, T.; Poole, B.; Trouet, A.; Tulkens, P.; Van Hoof, F. *Biochem. Pharmacol.* **1974**, *23*, 2495–2531.
(17) Conter, V.; Beck, W. T. *Cancer Treat. Rep.* **1984**, *68*, 831–839.
(18) Levin, V. J. *Med. Chem.* **1980**, *23*, 682–684.
(19) Hansch, C.; Bjorkroth, J. P.; Leo, A. *J. Pharm. Sci.* **1987**, *76*, 663–687.
(20) Beck, W. T.; Mueller, T. J.; Tanzer, L. R. *Cancer Res.* **1979**, *39*, 2070–2076.
(21) Kartner, N.; Riordan, J. R., Ling, V. *Science* **1983**, *222*, 1285–1288.
(22) McGrath, T.; Center, M. S. *Biochem. Biophys. Res. Commun.* **1987**, *145*, 1171–1175.
(23) Arsenault, A. L.; Ling, V.; Kartner, N. *Biochem. Biophys. Acta* **1988**, *938*, 315–321.

$$\log CR = 7.48\ (\pm 3.64) \log MW + 0.74\ (\pm 1.33) \log P - 0.30\ (\pm 0.35) (\log P)^2 - 19.35\ (\pm 10.18) \quad (7)$$

$$n = 10, r = 0.900, s = 0.494, \log P_0 = 1.20, F_{3,6} = 8.55$$

Two sets of data from Ling's laboratory are outlined in Tables IV and V. Using the Chinese hamster ovary line CH^RC5, they demonstrated variation in cross resistance and collateral sensitivity to a set of drugs that included local anesthetics, steroids, and detergents.[2,24,25] A number of the compounds tested by Ling are not typically cytotoxic (Table IV) and were therefore treated separately. Cyclophosphamide is include in this group although it is a well-known antitumor agent. However, it has been shown to be ineffective unless it undergoes microsomal activation, which was not done in Ling's studies. The SAR of these compounds is contained in eq 8.

$$\log \text{sensitivity} = 0.31\ (\pm 0.10) \log P + 0.14\ (\pm 0.23) \quad (8)$$

$$n = 10, r = 0.930, s = 0.290, F_{1,18} = 51.3$$

These results are significant at the 0.99 level ($F_{1,18},\alpha 0.01 = 11.3$) and constitute a typical Meyer and Overton type response at high drug concentrations to nonspecific membrane perturbations by compounds that are generally not cytotoxic. As expected, no role could be found for molecular weight.

Further analysis of the compounds in CH^RC5 cells was undertaken because of the great number of cytotoxic drugs that were assayed in this particular line.[2] However, there is excellent correspondence between the two colchicine-resistant mutant lines CH^RC5 and CH^RC4 ($n = 14$, $r = 0.94$). The following bilinear equation was obtained for the cross-resistance profile of the drugs in Table V. The corresponding parabolic equation yielded a correlation coefficient of 0.790 and a standard deviation of 0.494.

$$\log CR = 1.03\ (\pm 1.31) \log P + 5.67\ (\pm 3.55) \log MW - 1.12\ (\pm 1.48) \log (\beta_1 10^{\log P} + 1) - 52.05\ (\pm 42.39) \log (\beta_2{}^{\log MW} + 1) - 10.17\ (\pm 8.25) \quad (9)$$

$$n = 19, r = 0.828, s = 0.486, F_{6,12} = 4.36, \log \beta_1 = 1.327, \log \beta_2 = -3.722, \log P_o = -0.30, \log MW_o = 2.81$$

These results are significant at the 0.975 level ($F_{6,12},\alpha 0.025 = 3.73$). One compound, gramicidin D, was not utilized in the derivation of the equation. Although the CH^RC5 line was selected for resistance with colchicine, it is much more resistant to gramicidin D, a cyclic peptide whose cytotoxicity is attributed to its probable insertion into the plasma membrane.[26]

A comparison of eq 9 with eq 5 reveals a great similarity between the two equations, particularly in the size and magnitude of the coefficients with log MW. The bilinear dependence on size in eq 9 denotes a cut-off point in size around a molecular weight of 645. In our analysis of Ling's work, the greatest cross resistance in these colchicine-resistant cells would be manifest by a moderate-sized, slightly hydrophilic drug. The hypotheses of our model can be extended to these results. Small drugs like melphalan traverse the membrane with ease and so do large drugs like bleomycin, albeit by a different mechanism. It is postulated that large drugs such as actinomycin D and liblomycin should demonstrate minimal cross resistance versus the CH^RC5 cells. A double bilinear plot of log MW and log P has been utilized in eq 9. It must be cautioned that, for the number of data points ($n = 19$) and the large number of parameters, the correlation equation is not very robust. For QSAR analysis at least 25 data points should be analyzed.

What is unusual about the above examples is not that a few points do not fit the equations or that eq 9 does not have a higher correlation coefficient, but that there is *any* correlation at all between molecular weight and drug resistance! Equations 3, 5, 6, and 9 are all highly significant and all point to a role for molecular weight in MDR. While it would be overly simplistic to expect only molecular weight to explain the SAR for MDR it would also be a mistake to perfunctorily dismiss it. When viewed alone one might well have doubts about any one of the above four data sets; however, considered together they create a strong case for the significant role of molecular weight in resistant cell cytotoxicity.

It is of interest to compare the role of molecular weight in the penetration of antineoplastic drugs across rat brain capillaries.[18] The data upon which eq 10 is based were

$$\log P_c = -1.43 \log MW + 0.050 \log P - 1.84 \quad (10)$$

$$n = 25, r = 0.927, s = 0.461$$

obtained by Levin. However, we have modified his results in a form suitable for comparison with the above studies.[19]

Equation 10 indicates that increase in molecular weight results in poorer (P_c) penetration through the capillaries. However, in the analysis of the data, if water and compounds with molecular weight above 500 are omitted, the remaining data can be correlated by log P alone. Although cell membranes and capillaries are not strictly comparable, the results do show that under certain conditions the size of drugs does affect their ability to penetrate biological entities.

Much attention has been accorded glycoprotein GP-170 which often[20,21] but not always[22] seems to be overproduced by resistant cells. There has been speculation that it acts as a pump to extrude toxic drugs from resistant cells, but recent evidence from freeze–fracture studies, indicates there is sufficient GP-170 embedded in membranes so that membrane structure must surely be distorted.[23] Hence we believe that GP-170 functions to constrict channels in membranes thus blocking the entrance of moderate-sized drugs, which is indicated by eqs, 3, 5, 6, and 9. When membranes become so tight as to withhold vital nutrients, an increase in endocytosis occurs, opening the cells to large toxic compounds, as shown in eqs 5 and 9.

An aspect of this study which merits attention is the use of numerical values to allow for statistical validation of a hypothesis and a clearer delineation of a multifaceted problem. Because of its structure, liblomycin is often referred to as a lipophilic drug! However, it is obvious from its negative log P value that it is quite hydrophilic. Log P values as ascertained from the octanol/water system have been carefully studied for the last 25 years and over 15 000 values have now been reported for a wide range of drugs and simple, organic compounds. Thus it is now a very widely used standard of hydrophobicity.

The treatment of cross-resistance data in terms of QSAR using physicochemical parameters is advantageous in that a statistically based working model focuses attention on compounds which may be behaving in an anomalous fashion. What is it about novobiocin in Table I and gramicidin D in Table V that distinguishes them from compounds of similar molecular weight and hydrophobicity?

A salient finding is that both small drugs (such as hydroxyurea, guanazole, 5-fluorouracil, and 6-mercaptopurine) and large drugs (like mithramycin, valinomycin,

(24) Eliott, E. M.; Ling, V. *Cancer Res.* **1981**, *41*, 393–400.
(25) Ling, V. *Chemotherapy* **1978**, *23*, 191–199.
(26) Gerlach, J. H.; Kartner, N.; Bell, D. R.; Ling, V. *Cancer Surv.* **1988**, *5*, 25–46.

actinomycin D, bleomycin, and liblomycin) show minimal cross resistance (log CR ≈ 0). This warrants further consideration. Although numerous papers on MDR appear almost daily, none of the work which has appeared so far, except that in Table II, has included a great enough range of drugs to address this most unusual finding. While our rationalization (eq 5) of the mechanism is probably not the last word on this complex and extremely important problem, it is a starting point which must be considered in the design of drugs against resistant neoplasms.

Finally there are implications for multidrug chemotherapy. Although one does not expect a panacea since there are many ways by which cells acquire resistance, it might be worthwhile to study the simultaneous use of drugs at the extreme ends of the molecular weight and hydrophobicity scales. The study of MDR with a well-designed set of cytotoxic probes could yield new insights into membrane alterations and could eventually lead to improvements in current cancer chemotherapy regimens.

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2-(Arylalkylamino)adenosin-5′-uronamides: A New Class of Highly Selective Adenosine A_2 Receptor Ligands

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The synthesis and receptor-binding profiles at adenosine receptor subtypes for a series of 2-(arylalkylamino)-adenosin-5′-uronamides is described. Halogenated 2-phenethylamino analogues such as **3e** show greater than 200-fold selectivity for the A_2 receptor subtype on the basis of rat brain receptor binding. The general structure–activity relationship of this series of compounds is discussed both in terms of potency at A_2 receptors as well as receptor subtype selectivity. It is possible to introduce a hydrophilic carboxyalkyl substituent to this series such as in CGS 21680A (**3h**) and still retain good potency and selectivity for A_2 receptors. In addition, functional data in a perfused working rat heart model shows that these compounds possess full agonist properties at A_2 receptors with **3h** having a greater than 1500-fold separation between A_2 (coronary vosadilatory) and A_1 (negative chronotropic) receptor mediated events.

The purine nucleoside adenosine has been extensively studied as a modulator of cardiovascular function since it was shown to have potent hypotensive and bradycardic activity some 60 years ago by Drury and Szent-Gyorgyi.[1] The hypotensive actions of adenosine occur via several mechanisms among which are direct regulation of blood flow via vasodilation of the peripheral vasculature, including the coronary arteries.[2] Adenosine also produces sinus bradycardia and prolongation of impulse conduction in the atrioventricular node (AVN).[3] In addition, adenosine has the ability to inhibit neutrotransmitter release[4] and possesses potent central nervous system depressant and anticonvulsant activity.[5]

The vasodilator and conduction effects of adenosine are mediated through different receptor subtypes. In the heart, A_1 receptors present on nodal cells and cardiac myocytes are responsible for the negative dromo-, chrono-, and inotropic actions of adenosine.[3] Activation of A_2 receptors located on coronary smooth muscle results in vasodilation.[2] The potential use of an adenosine agonist as an antihypertensive agent has been limited by this spectrum of actions, nonselective agonists producing vasodilation that can be associated with cardiac depression as well as marked angina.[6] Selective A_2 receptor agonists may provide more viable agents as potential therapeutic candidates possessing effective vasodilatory hypotensive actions without the detrimental effects on cardiac conduction and renal function observed with currently available agonists. Whereas many highly selective agonists for the A_1 receptor have been described,[7] the prototypical A_2 agonist NECA (1) (*N*-ethyladenosine-5′-uronamide)[8] show little or no A_2 selectivity (see Table I). Until recently, the most selective A_2 agonist described was CV 1808 (2) ((2-phenylamino)adenosine), being approximately 5–10-fold selective for the A_2 receptor.[8] More recently, several N-6 substituted purine ribosides and NECA analogues with about 40-fold selectivity for the A_2 receptor have been described.[9] However, most of these analogues still possess reasonably potent A_1 receptor affinity (K_i ~ 200 nM). In the present paper, the synthesis and receptor-binding profiles at adenosine receptor subtypes for a series of 2-(arylalkylamino)adenosin-5′-uronamides are described. Some of the analogues described possess as much as 200-fold selectivity for the A_2 receptor on the basis

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(1) Drury, A. N.; Szent-Gyorgyi, A. *J. Physiol. (London)* **1929**, *68*, 214.
(2) Berne, R. M.; Winn, H. R.; Knabb, R. M.; Ely, S. W.; Rubio, R. In *Regulatory Function of Adenosine*; Berne, R. M., Rall, T. W., Rubio, R., Eds.; Nihoff: Boston, 1983; p 293.
(3) Bellardinelli, L.; West, A.; Crampton, R.; Berne, R. M. In *Regulatory Function of Adenosine*; Berne, R. M., Rall, T. W., Rubio, R., Eds.; Nihoff: Boston, 1983; p 378.
(4) Fredholm, B.; Dunwiddie, T. V. *Trends Pharmacol. Sci.* **1988**, *9*, 130.
(5) Dunwiddie, T. V. *Int. Rev. Neurobiol.* **1985**, *27*, 63.
(6) Sylven, C.; Beermann, B.; Jonzon, B.; Brandt, R. *Br. Med. J.* **1986**, *293*, 227.
(7) Daly, J. W. *J. Med. Chem.* **1982**, *25*, 197.
(8) Bruns, R. F.; Lu, G. H.; Pugsley, T. A. *Mol. Pharmacol.* **1986**, *29*, 331.
(9) Bridges, A. J.; Bruns, R. F.; Ortwine, D. F.; Priebe, S. R.; Szotek, D. L.; Trivedi, B. K. *J. Med. Chem.* **1988**, *31*, 1282.

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