[CANCER RESEARCH 48, 2064–2067, April 15, 1988]

Reversal of Resistance to Vincristine in P388 Leukemia by Various Polycyclic Clinical Drugs, with a Special Emphasis on Quinacrine[1]

Makoto Inaba[2] and Eiji Maruyama

Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan

ABSTRACT

We investigated several lipophilic drugs with a polycyclic structure for their effect on the net uptake of vincristine in vincristine-resistant P388 leukemia cells. Fourteen of 23 agents promoted vincristine uptake in the resistant cells. The net increase in vincristine uptake was caused by prevention of its outward transport rather than by stimulation of inward transport. Some of these drugs, *e.g.*, quinacrine, dilazep, syrosingopine, simetride, etc., remarkably potentiated the cytotoxicity of vincristine against the resistant cells *in vitro*. Quinacrine, an antimalarial drug which had the greatest effect on vincristine uptake and relatively low host toxicity, exhibited potent therapeutic synergism in combination with vincristine in resistant leukemia-bearing mice.

INTRODUCTION

It is generally accepted that a tumor as an assembly of tumor cells is extremely heterogeneous in terms of drug sensitivity, and after repeated treatments the tumor often acquires definite resistance even to combination chemotherapy consisting of 3 or more drugs. Therefore, we need to have as many drugs with different chemical structures or modes of action as possible. In this regard, so-called "multidrug resistance," which means a broad cross-resistance among structurally unrelated drugs such as anthracycline antibiotics, *Vinca* alkaloids, etc., raises a critical problem for cancer chemotherapy: the tumor becomes refractory to several unrelated drugs simultaneously, which substantially reduces the number of available antitumor drugs. On the contrary, if we can find some regimen to potentiate activity of these antitumor agents against a resistant tumor, it may be possible to restore the lost toxicity of plural drugs to the resistant tumor at the same time.

In our previous studies (1, 2), we found that both anthracycline analogues and *Vinca* alkaloids with no or little antitumor activity can reverse multidrug resistance by strongly inhibiting the outward transport of these antitumor drugs, thus increasing their intracellular accumulation. These findings suggested the possibility that other polycyclic compounds, like other non-antitumor anthracyclines and *Vinca* alkaloids, might also prevent outward transport of antitumor agents. Based on this assumption, in the present study we screened clinically used lipophilic polycyclic drugs that may reverse multidrug resistance.

MATERIALS AND METHODS

Chemicals. Generic names, applications, and suppliers of various clinical drugs used as second agent in the present study are shown in Table 1. Vincristine sulfate (Oncovin) was purchased from Shionogi and Co., Ltd., Osaka, Japan; and Adriamycin hydrochloride was provided by Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. [*G*-^{3}H]vincristine sulfate (specific activity, 2.7 Ci/mmol) was purchased from Amersham International plc, Amersham, England; 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide, was obtained from Wako Pure Chemical Ind., Ltd., Tokyo, Japan.

Tumor Cells. P388 leukemia, P388/VCR[3], and P388/ADR were supplied by the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, MD. The parental line and these resistance sublines were passaged weekly through DBA/2 and BALB/c × DBA/2 ($CD2F_1$) mice (Charles River Japan, Inc., and Atsugi, Kanagawa, Japan), respectively.

Growth Inhibition Assay. RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (M. A. Bioproducts), 10 μM 2-hydroxyethyldisulfide (Aldrich Chemical Co., Inc., Milwaukee, WI), and 100 μg/ml Kanamycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan) were used as culture medium. Cells were harvested from tumor-bearing mice 5–6 days after transplantation and suspended in the culture medium with antitumor agent and/or various additives, plated at a final cell density of 5×10^4 cells/ml, and incubated in a CO_2 incubator at 37°C for 48 h. The number of cells was counted in a Model ZBI Coulter Counter after a 5-min incubation with 0.25% trypsin to dissociate the cells.

Colony-forming Inhibition Assay. One-fourth ml of 0.3% agarose (Sea Plaque; FMC Corporation, Rockland, ME) prepared by mixing 1 vol of melted 3% agarose with 9 vol of RPMI 1640 supplemented as mentioned above was added to each well of a 24-well plate (Corning Laboratory Sciences Co., NY) as an underlayer, and plates were refrigerated for 1 h at 4°C. On this layer, 0.25 ml of the mixture consisting of cell suspension (2000 cells), 10-fold higher than final concentration of drug solution and 3% agarose at the volume ratio of 8:1:1 were added, and the plates were again refrigerated for 1 h at 4°C. The plates were then transferred to a CO_2 incubator and the cells cultured for 6 days at 37°C, after which 40 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide solution (5 mg/ml of distilled water) were added to each well. After further incubation for 4 h at 37°C, the stained colonies were counted with a Model CA-7 Colony Analyzer (Oriental Instruments, Ltd., Tokyo, Japan).

***In Vivo* Treatment.** One million P388/VCR cells were inoculated i.p. into female $CD2F_1$ mice weighing 21–23 g. VCR or quinacrine dissolved in sterile physiological saline was administered i.p. on Days 1–5.

Uptake and Outward Transport. Cells were harvested 7 days after transplantation, washed 3 times by centrifugation, and suspended in Hanks' balanced salt solution to a final cell density of 2×10^6 cells/ml. Cells were preincubated for 10 min before the addition of [^{3}H]VCR with or without an additive such as quinacrine. After a 30-min incubation, triplicate 1-ml aliquots were withdrawn and added to centrifuge tubes containing 4 ml of cold 0.9% NaCl solution, followed by washing twice with cold 0.9% NaCl solution and centrifugation (600 × *g*, 5 min, 4°C). After the tubes were placed upside down on filter paper and left there for 2–3 h to remove the remaining washing solution, cells were dissolved in 0.5 ml of Protosol (New England Nuclear, Boston, MA). After addition of 15 ml of liquid scintillation cocktail (Econofluor; New England Nuclear) the radioactivity was determined in a Beckman Model LS-355 liquid scintillation counter having a 52% counting efficiency for tritium.

In outward transport experiments, cells were preloaded with [^{3}H]-VCR by incubation in a glucose-free and 1 mM 2,4-dinitrophenol-containing Hanks' balanced salt solution. After 20 min, a small quantity of glucose solution (final concentration of 1 mg/ml) with or without

Received 12/29/86; revised 10/21/87, 12/28/87; accepted 1/15/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, and Culture, Japan.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: VCR, vincristine; ADR, Adriamycin; P388/VCR, vincristine-resistant P388 leukemia; P388/ADR, Adriamycin-resistant P388 leukemia.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/48/8/2064/2434782/cr0480082064.pdf by guest on 06 July 2022

Table 1 *Generic name, application, and supplier of clinical drugs used in this study*

Generic name	Application	Supplier
Meclizine	Antitussive	Pfizer Taito Co., Ltd., Tokyo, Japan
Hydroxyzine	Minor tranquilizer	Pfizer Taito
Dilazep	Coronary vasodilator	Kowa Company Ltd., Tokyo, Japan
Eprazinone	Antitussive	Chugai Pharmaceutical Co., Ltd., Tokyo, Japan
Syrosingopine	Antihypertensive	Toyo Jozo Co., Ltd., Tokyo, Japan
Simetride	Analgesic	Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan
Carbinoxamine	Antihistamic	Taisho Pharmaceutical Co., Ltd., Tokyo, Japan
Pirenzepine	Antiulcerative	Nippon Boehringer Ingelheim Co., Ltd., Tokyo, Japan
Etomidoline	Antispasmodic	Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan
Quinacrine	Antimalarial	Sigma Chemical Co., St. Louis, MO
Triprolidine	Antihistamic	Sigma
Primaquine	Antimalarial	Sigma
Tilorone	Antiviral	Sigma
Dimethindene	Antihistamic	Sigma
Nafcillin	Antibacterial	Sigma
Trimethoprim	Antibacterial	Sigma
Prazosin	Antihypertensive	Banyu Pharmaceutical Co., Ltd., Tokyo, Japan
Oxolinic acid	Antibacterial	Sigma
Vancomycin	Antibacterial	Sigma
Piromidic acid	Antibacterial	Dainippon Pharmaceutical Co., Ltd., Osaka, Japan
Pipemidic acid	Antibacterial	Dainippon
Rolitetracycline	Antibacterial	Banyu
Zomepirac	Antiinflammatory	Sigma

Table 2 *Effects of various polycyclic clinical drugs on vincristine uptake by P388/VCR cells*

Cells were incubated with 3×10^{-5} mM [^{3}H]VCR in the presence or absence of 3 μg/ml of each drug as a second agent for 30 min at 37°C.

Drug	Vincristine uptake[a] (treated *versus* control %)
Quinacrine	357 ± 3.0[b]
Meclizine	255 ± 15
Dilazep	225 ± 13
Eprazinone	225 ± 14
Syrosingopine	224 ± 10
Simetride	217 ± 22
Triprolidine	214 ± 43
Primaquine	208 ± 43
Carbinoxamine	206 ± 78
Tilorone	162 ± 22
Dimethindine	158 ± 9.0
Pirenzepine	149 ± 24
Hydroxyzine	149 ± 6
Etomidoline	146 ± 22

[a] $\left(\frac{\text{VCR uptake with second agent}}{\text{VCR uptake without second agent}}\right) \times 100$.

[b] Mean ± SD of 3 different samples from the same experiments.

quinacrine (final concentration of 3 μg/ml) was added to the cell suspension, and incubation was continued for a further 30 min at 37°C. Triplicate 1-ml aliquots were withdrawn at appropriate time points and the radioactivities associated with the cells were measured as described above.

RESULTS

Various lipophilic clinical drugs with a polycyclic structure were examined for their ability to enhance VCR uptake by P388/VCR cells. As shown in Table 2, 14 of 23 drugs tested exhibited a significant VCR uptake-enhancing effect. Among them, quinacrine, an antimalarial agent, was the most active. While nafcillin, trimethoprim, and prazosine were minimally effective, oxolinic acid, vancomycin, piromidic acid, pipemidic acid, rolitetracycline, and zomepirac had no effect on VCR uptake at concentrations as high as 3 μg/ml. VCR uptake by P388/VCR cells was approximately one-half that of the sensitive cells; therefore, all of the drugs in Table 2 were expected to affect the VCR sensitivity of P388/VCR cells *in vitro*.

As a result, marked synergistic effects were observed in *in vitro* cell growth experiments, and several of the drugs potentiated VCR cytotoxicity against the resistant cells, causing complete or severe growth inhibition at subtoxic concentrations (Table 3).

Further studies were performed with quinacrine only, and Fig. 1 demonstrates its effect on active outward transport of VCR from P388/VCR cells. No change in the intracellular VCR level was seen in the presence of 3 μg/ml of quinacrine after outward transport had been induced by the addition of glucose. This strongly suggests that the observed increase in VCR uptake with quinacrine is due to inhibition of VCR outward transport.

We next investigated how much sensitivity to VCR or ADR was restored when the resistant cells were cultured for 48 h in the presence of 0.5 μg/ml of quinacrine. The approximately 15-fold resistance to VCR of P388/VCR cells was completely reversed by quinacrine. However, the 240-fold resistance to ADR of P388/ADR cells was only partially reversed, as shown in Fig. 2. Furthermore, the effect of quinacrine on VCR resist-

Table 3 *Combined effects of various polycyclic clinical drugs on growth inhibition by vincristine in P388/VCR cells*

Cells were cultured in a suspension form with a subcytotoxic concentration (5×10^{-6} mM) of VCR in the presence or absence of each drug as a second agent for 48 h at 37°C, and cell number was counted by a Coulter Counter. Each value is the mean of 3 determinations with coefficient of variation less than 10%.

Second agent	Concentration (μg/ml)	Growth rate (% of control): Second agent alone	Growth rate (% of control): VCR plus second agent
Quinacrine	0.5	82	0
Meclizine	2.0	106	8
Dilazep	2.5	78	4
Eprazinone	2.0	109	7
Syrosingopine	1.25	77	3
Simetride	3.0	87	0
Triprolidine	4.0	115	10
Primaquine	5.0	84	5
Carbinoxamine	10	115	10
Tilorone	0.25	108	17

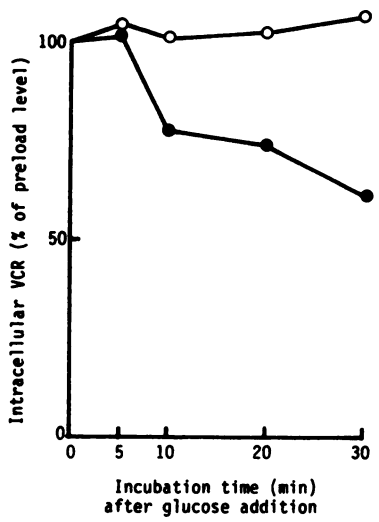

Fig. 1. Effects of quinacrine on active outward transport of vincristine in P388/VCR cells. Cells were incubated at 37°C in glucose-free Hanks' balanced salt solution containing 1 mM 2,4-dinitrophenol and 2.5×10^{-5} mM [^{3}H]VCR. After 20 min, a small volume of glucose solution with (○) or without (●) quinacrine at final concentrations of 1 mg/ml and 3 μg/ml for glucose and quinacrine, respectively, was added to induce outward transport, and the cells were incubated for an additional 30 min. The intracellular levels of VCR are presented as a percentage of the preload level.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/48/8/2064/2434782/cr0480082064.pdf by guest on 06 July 2022

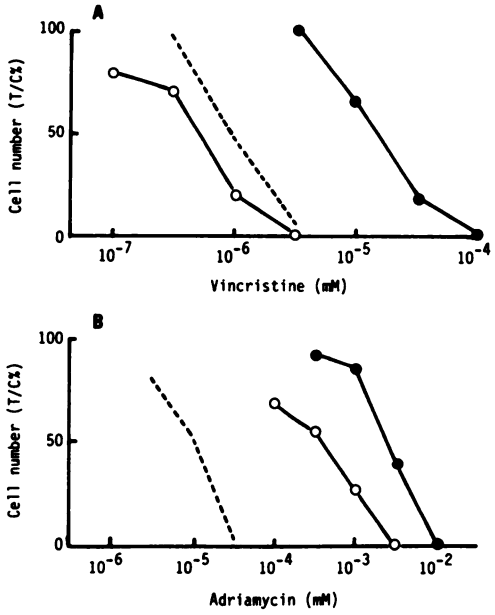

Fig. 2. Reversing effect of quinacrine on resistance to vincristine or Adriamycin in drug-resistant P388 leukemia cells *in vitro*. P388/VCR (*A*) or P388/ADR (*B*) cells were cultured with various concentrations of VCR (*A*) or ADR (*B*) in the presence (○) or absence (●) of 0.5 μg/ml of quinacrine for 48 h. Sensitive P388 cells (——) were cultured without quinacrine. *T/C*, treated *versus* control.

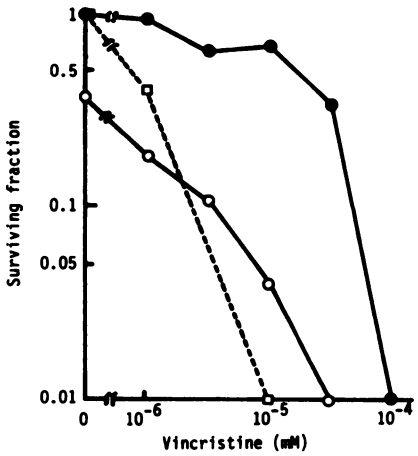

Fig. 3. Effects of quinacrine on resistance to VCR of P388/VCR cells in a colony-forming assay. P388/VCR cells were cultured on double layer of soft agarose containing various concentrations of VCR in the presence (○) or absence (●) of 0.5 μg/ml of quinacrine. After 6 days of culture, colonies formed were counted. Sensitive P388 cells (□) were cultured without quinacrine.

ance in P388/VCR cells was confirmed in the colony-forming assay (Fig. 3).

In vivo combined treatment with VCR and quinacrine was used in P388/VCR-bearing mice. Various treatment schedules for this combination therapy were attempted to find the optimal one and as a result, the protocol shown in Table 4 was selected as the best. VCR (0.1 mg/kg/day) alone exhibited a treated *versus* control value of 130%, and 40 and 80 mg/kg/day of quinacrine alone showed 100 and 129%, respectively. In combination therapy with VCR and quinacrine, treated *versus* control values of 157 and 185% were observed with the combination with low and high doses of quinacrine, respectively, although any long survivors were not observed. It is obvious that these combined effects are more than additive. In fact, they are approximately equivalent to the maximum effect observed with the sensitive P388 cells treated with VCR alone (data not shown).

Table 4 *Combined treatment in vivo of P388/VCR-bearing mice with vincristine and quinacrine*

P388/VCR cells (10^6) were inoculated i.p. and the animals were given injections i.p. of VCR and/or quinacrine on Days 1–5. Quinacrine was administered twice a day at intervals of approximately 8 h.

mg/kg/day		Median survival days (range)	Treated *versus* control (%)
Vincristine	Quinacrine		
		9.2 (9–11)	100
0.1		12.0 (11–13)	130
	25 × 2	9.2 (9–10)	100
	40 × 2	11.9 (10–12)	129
0.1	25 × 2	14.4 (14–15)	157
0.1	40 × 2	17.0 (16–18)	185

DISCUSSION

We have found that a number of clinical drugs selected solely on the basis of their polycyclic structure possess potent activity to enhance the reduced uptake of VCR by P388/VCR cells, thus potentiating VCR cytotoxicity against these cells (Tables 2 and 3). In addition to these 14 drugs, nafcillin, trimethoprim, and prazosine also showed weak but definite activity. The effect on VCR uptake of all drugs was examined at the same concentration, *i.e.*, 3 μg/ml. Therefore, some drugs found ineffective in the present test might show some activity if examined at concentrations higher than 3 μg/ml. At any rate, these results have clearly proved that some lipophilic compounds with a polycyclic structure, irrespective of their particular pharmacological actions, can enhance uptake of VCR by multidrug-resistant cells.

Quinacrine effectively prevented the active outward transport of VCR from these cells (Fig. 1). Therefore, with active drugs other than quinacrine, this inhibitory action on the outward transport also seems to be a direct mechanism for the increased net uptake of VCR. However, it is still unknown how these drugs can prevent active outward transport of VCR in the resistant cells. Recently, we reported kinetic data showing that active outward transport of VCR in P388 leukemia cells is a saturable process, suggesting the existence of a carrier system for outward transport in the membrane (3). Thus we speculated that this carrier protein possesses a broad affinity for lipophilic polycyclic compounds to varying extents. Since most drugs are generally lipophilic, the existence of this carrier system has an important pharmacological implication. If our speculation is true, these lipophilic drugs as well as non-antitumor anthracyclines and *Vinca* alkaloids, which can be retained at a much greater intracellular concentration than VCR because of their application at a much higher concentration than VCR to the cells, may be able to competitively inhibit a carrier-mediated outward transport of VCR through the cell membrane. Kinetic studies along this line are currently in progress in our laboratory.

It has been extensively reported that calcium antagonists such as calcium channel blockers and calmodulin inhibitors reverse multidrug resistance by increasing the net uptake of *Vinca* alkaloids and anthracyclines (4, 5). However, such pharmacological action seems to be not associated with their calcium-antagonizing activity (6–8). On the other hand, recently some other compounds with various physiological activities, namely, triparanol (9), tamoxifen (9), isoprenoids (10), chloroquine (11), etc., have been reported to possess the same activity toward these resistant cells. Based on the observation by Ramu *et al.* (6) that P388/ADR cells possess a higher degree of structural order of the membrane lipid than the parent line, they suggested the unique idea that amphipathic cationic compounds such as tamoxifen, triparanol, etc. affect the drug sen-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/48/8/2064/2434782/cr0480082064.pdf by guest on 06 July 2022

sitivity of the resistant cells by their specific interaction with the cell membrane lipid domain. Although that may be one possible mode of action, we speculate that the mechanism discussed above (*i.e.*, inhibition of carrier-mediated outward transport) might be also applicable to some of those agents. In this respect, Kessel and Willberding (12) have suggested the possibility that verapamil is a substrate for the outward transport system.

In considering the results of the present study, we place special emphasis on the *in vivo* experiment showing reversal of resistance, because agents effective *in vivo* in this respect are very few in number. In the present stage of experimentation, it is particularly important to find compounds which can overcome multidrug resistance *in vivo*. In this regard, quinacrine was selected as an additive to VCR for therapeutic experiments, owing to its potent activity *in vitro* (Fig. 2), its relatively low host toxicity, and its high solubility in water. It seems also important to find an optimal treatment regimen for this kind of combined treatment *in vivo*. According to the date on *in vitro* reversal of resistance (Fig. 2), it is expected that continuous exposure of cells to VCR and quinacrine should have a synergistic effect *in vivo*. Therefore, we initially attempted a 48-h continuous infusion of the mixed solution of VCR and quinacrine into the i.p. cavity of mice into which P388/VCR cells had been inoculated. However, no significant effect was observed with this combined treatment. It is possible that the i.p. concentration of quinacrine around the cells was not kept at its effective one, *i.e.*, 0.5 μg/ml, probably due to the extremely slow infusion rate (0.25 ml/h), in spite of the fact that its concentration in the infusion solution was quite high (180–275 μg/ml).

On the other hand, when bolus injections of VCR once and quinacrine twice a day were given for 5 successive days, a remarkable effect was observed (Table 4). It is pharmacologically unclear why this regimen is so effective, because a very long exposure of the cells to quinacrine is not to be expected by this treatment. Accordingly, we speculate that an extremely high intracellular quinacrine concentration results temporarily from the bolus injection and is effective in preventing outward transport of VCR from the cells *in vivo*. Since quinacrine itself has marginal antitumor activity, it might be true that a synergistic effect *in vivo* between VCR and quinacrine cannot be clearly demonstrated. However, such antitumor activity of quinacrine itself will be advantageous for the overall therapeutic effectiveness of such combined treatment against resistant tumors.

ACKNOWLEDGMENTS

We thank Dr. Hirotaka Shinoda and Junko Yamabe for their technical cooperation.

REFERENCES

1. Inaba, M., Nagashima, K., Sakurai, Y., Fukui, M., and Yanagi, Y. Reversal of multidrug resistance by non-antitumor anthracycline analogs. Jpm. J. Cancer Res. (Gann), *75:* 1049–1052, 1984.
2. Inaba, M., and Nagashima, K.: Non-antitumor *Vinca* alkaloids reverse multidrug resistance in P388 leukemia cells *in vitro*. Jpm. J. Cancer Res. (Gann), *77:* 197–204, 1986.
3. Inaba, M., Watanabe, T., and Sugiyama, Y. Kinetic analysis of active efflux of vincristine from multidrug-resistant P388 leukemia cells. Jpm. J. Cancer Res. (Gann), *78:* 397–404, 1987.
4. Tsuruo, T., Iida, H., Tsukagoshi, S., and Sakurai, Y. Increased accumulation of vincristine and Adriamycin in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Res., *42:* 4730–4733, 1982.
5. Tsuruo, T., Iida, H., Nojiri, M., Tsukagoshi, S., and Sakurai, Y. Circumvention of vincristine and Adriamycin resistance *in vitro* and *in vivo* by calcium influx blockers. Cancer Res., *43:* 2906–2910, 1985.
6. Ramu, A., Spanier, R. H., Rahamimoff, H., and Fuks, Z. Restoration of doxorubicin responsiveness in doxorubicin-resistant P388 murine leukemia cells. Br. J. Cancer, *50:* 501–507, 1984.
7. Kessel, D., and Wilberding, C. Anthracycline resistance in P388 murine leukemia and its circumvention by calcium antagonists. Cancer Res., *45:* 1687–1691, 1985.
8. Nair, S., Samy, T. S. A., and Krishan, A. Calcium, calmodulin, and protein content of Adriamycin-resistant and -sensitive murine leukemic cells. Cancer Res., *46:* 229–232, 1986.
9. Ramu, A., Glaubiger, D., and Fuks, Z. Reversal of acquired resistance to doxorubicin in P388 murine leukemia cells by tamoxifen and other tripranol analogues. Cancer Res., *44:* 4392–4395, 1984.
10. Yamaguchi, T., Nakagawa, M., Shiraishi, N., Yoshida, T., Kiyosue, T., Arita, M., Akiyama, S., and Kuwano, M. Overcoming drug resistance in cancer cells with synthetic isoprenoids. J. Natl. Cancer Inst., *76:* 947–953, 1986.
11. Shiraishi, N., Akiyama, S., Kobayashi, M., and Kuwano, M. Lysosomotropic agents reverse multiple drug resistance in human cancer cells. Cancer Lett., *30:* 251–259, 1986.
12. Kessel, D., and Wilberding, C. Mode of action of calcium antagonists which alter anthracycline resistance. Biochem. Pharmacol., *33:* 1157–1160, 1984.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/48/8/2064/2434782/cr0480082064.pdf by guest on 06 July 2022