[CANCER RESEARCH 54, 3088–3091, June 15, 1994]

*Advances in Brief*

# Reversal of Multidrug Resistance by RU 486[1]

**Donald J. Gruol,[2] Michele C. Zee, Joseph Trotter, and Suzanne Bourgeois**

*The Salk Institute for Biological Studies, Regulatory Biology Laboratory, San Diego, California 92186-5800*

## Abstract

**P-Glycoproteins represent a family of drug efflux proteins that convey multidrug resistance to cells in which they are expressed. This phenomenon can lower the efficacy of drugs used in chemotherapy. The steroid progesterone has been shown to bind P-glycoproteins and inhibit their drug efflux. We report that the antiprogestin RU 486 can reverse multidrug resistance in cells overexpressing the mouse *mdr*1 gene. Using flow cytometry to measure inhibition of P-glycoprotein-dependent efflux of rhodamine 123, RU 486 was found to be considerably more effective than progesterone and one-half as effective as verapamil. The results suggest a valuable new use for RU 486.**

## Introduction

The beneficial effects of chemotherapy can be compromised by cellular mechanisms that allow neoplastic tissue to evade the toxicity of drugs (1, 2). In some cases, pleiotropic resistance to a variety of unrelated drugs has been observed, and this phenomenon has been called multidrug resistance (3, 4). MDR[3] may be gained through several mechanisms. One such mechanism is a reduction in drug accumulation due to increased rates of drug efflux from cells expressing P-glycoproteins (5–8). P-glycoproteins represent a family of transport proteins capable of causing an ATP-dependent efflux of a wide variety of compounds across the plasma membrane. The drugs involved in this form of MDR share little structural similarity but are most often small and hydrophobic and thought to enter cells by diffusion across the cell membrane (9–14). This category includes such diverse compounds as *Vinca* alkaloids (vinblastine and vincristine), anthracyclins (daunomycin), colchicine, taxol, and puromycin. Resistance to hydrophobic peptides, such as gramacidin, has also been observed (15). A variety of hydrophobic compounds have also been identified that have a potent capacity to inhibit drug transport (16). Recently, we and others have found evidence that certain steroids are transported by P-glycoproteins (17–19). The mouse *mdr*1 P-glycoprotein causes a reduced accumulation of corticosteroids containing an hydroxyl group at the 11-position of the steroid molecule. An additional hydroxyl group at the 17-position greatly enhances this capacity. Thus, cortisol, dexamethasone, aldosterone, and other similar steroids appear to be substrates for transport. In contrast, steroids such as progesterone and cortexolone, which lack the 11-hydroxyl group, are not transported (19). However, progesterone has been shown to bind to the mouse and human P-glycoproteins and to inhibit their function, even though progesterone is not transported (20–22). RU38486 (RU 486) was developed as an antiprogestin with high affinity binding to both the progesterone and glucocorticoid receptors (23, 24). Its structure contains an 11$\beta$-(dimethyl amino phenyl) substitution which contributes to its antagonist properties. Interestingly, earlier studies of MDR inhibitors had indicated that a hydrophobic ring and an associated tertiary amine group could contribute to anti-MDR activity of phenothiazine compounds (16). Thus, the substitution at the 11-position of RU 486 also has the potential to enhance its capacity to inhibit P-glycoprotein function. (For a comparison of the structures of dexamethasone, RU 486, and progesterone, see Fig. 4*C*.)

## Materials and Methods

**Cell Culture.** WEHI-7 is a thymoma cell line obtained from a female BALB/c mouse after X-irradiation (25). W7TB is a derivative of this line which is resistant to bromodeoxyuridine, a marker that is unrelated to MDR. S7CD-5 is a derivative of W7TB that was selected through a series of steps for resistance to dexamethasone and colchicine (26). S7CD-5 overexpresses the mouse *mdr*1 gene (19). The cells were grown in suspension in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The incubator was maintained at 37°C and had a humidified atmosphere of 13% $CO_2$ and 87% air. The number of living cells in the cultures was determined by the trypan blue exclusion technique. The effects of drugs on cell proliferation was measured as described by Bourgeois and Newby (27). Briefly, cell cultures were set up ($5 \times 10^4$ cells/ml) in varied concentrations of drugs and incubated for 5 days. The amount of accumulated cellular material was assayed by measuring the turbidity of the cultures (660 nm) and by expressing the values as normalized to those from cultures grown in the absence of drug. The relative turbidity values reflect the amount of cellular material synthesized during the period of incubation and provide a sensitive measure of the capacity of the cells to proliferate even if a large portion are killed.

**Flow Cytometry.** Flow cytometric assays were performed on cells that had been pre-incubated with rhodamine 123 by using a BD FACStar Plus cell sorter; subsequent listmode data analysis was done on a Sun SPARCStation 2 with software facilitating the time-slicing of kinetics assays. Laser excitation was at 100 mW from an argon laser tuned to 488 nm. The flow rate was maintained between 100 to 200 cells per s, and rhodamine 123 fluorescence was measured over a 4-decade range through the standard BD FL1 fluorescein bandpass filter (520 nm). All listmode data files were collected on a Hewlett Packard Consort 32 System using LYSYS II (BD) configured to include time as a correlated listmode parameter over a period of 1024 s. Typically, files were time-sliced into 32 points, and rhodamine 123 fluorescence was averaged over a period of 4 s/point. The averaged values (geometric means) for each time-slice were then normalized to the first point in the series to yield a value representing the percentage of rhodamine 123 remaining within the cells for each time point.

One can define and calculate an average rate of efflux using the time that it takes for 50% of the rhodamine 123 to be transported out of the cells (50%/$t_{50}$). In order to compare results with different inhibitory drugs at varied concentrations, we also define a RFER as the average rate (with drug) divided by the average rate (without drug). Thus, the RFER can be calculated by dividing the $t_{50}$ (without drug) by the $t_{50}$ (with drug).

## Results and Discussion

If RU 486 can inhibit P-glycoprotein function, it should be able to reverse the drug-resistant phenotype of the murine thymoma cell line S7CD-5. The S7CD-5 cell line was derived from a steroid-sensitive mouse line, W7TB (26). It expresses the *mdr*1 gene and is resistant to a variety of drugs including colchicine, puromycin, daunomycin, and

Received 1/28/94; accepted 5/4/94.

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 work was supported by Core Grant CA-14195 from the National Cancer Institute and grants from the American Cancer Society and the Elsa U. Pardee Foundation (to S. B.).

[2] To whom requests for reprints should be addressed, at The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, CA 92186-5800.

[3] The abbreviations used are: MDR, multidrug resistance; BD, Becton Dickinson; RFER, retained fractional efflux rate.

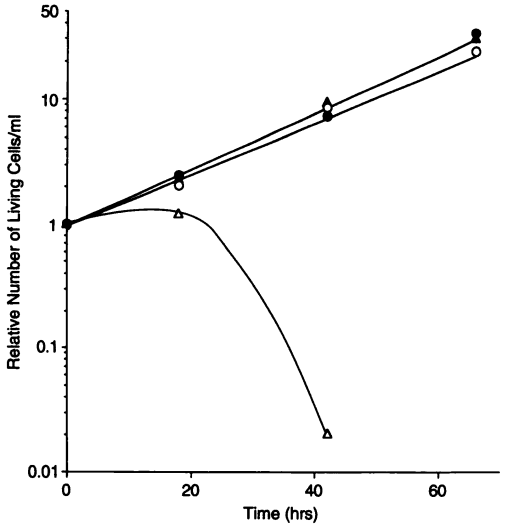


Fig. 1. Effects of RU 486 and puromycin on the viability of S7CD-5 cells. Separate cultures of S7CD-5 cells initially containing $4 \times 10^4$ cells/ml were incubated with: ●, no additions; ○, 5 μM RU 486; ▲, 20 μM puromycin; or △, 5 μM RU 486 plus 20 μM puromycin. At the times indicated, samples were removed, and the concentration of viable cells was evaluated using a trypan blue exclusion test. The values represent the mean of two determinations for each time point.

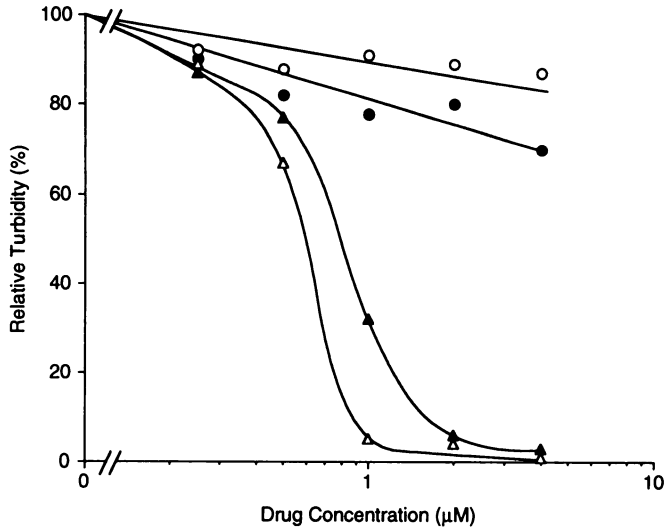


Fig. 3. Concentration dependence of drug resistance reversal by RU 486 and verapamil. Separate sets of S7CD-5 cultures, initially containing $5 \times 10^4$ cells/ml, were incubated in the indicated concentrations of either RU 486 (●, ▲) or verapamil (○, △). Two sets of cells were prepared for each drug, either with (▲, △) or without (●, ○) 20 μM puromycin. After 5 days, the cultures were analyzed as described for Fig. 2. These data are representative of results obtained in three separate experiments.

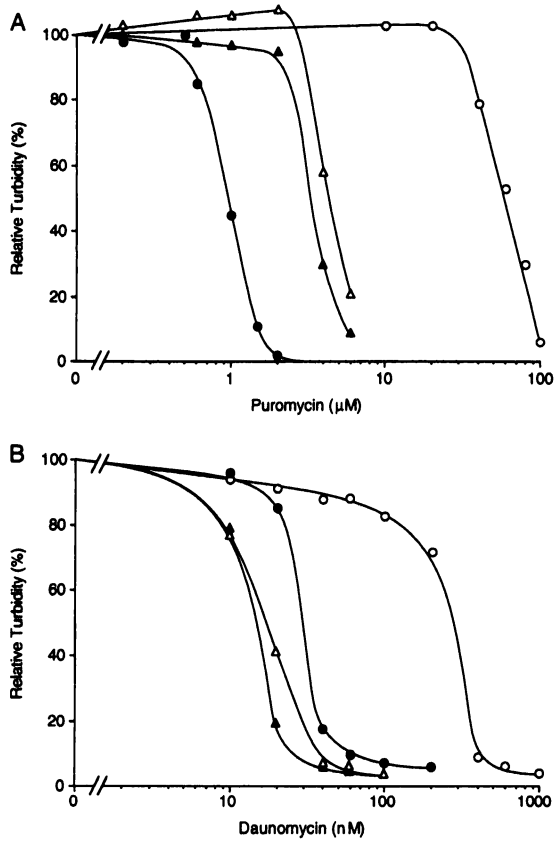


Fig. 2. *A*, reversal of puromycin resistance in S7CD-5 cells by RU 486 and verapamil. Separate sets of S7CD-5 cultures initially containing $5 \times 10^4$ cells/ml were incubated in the indicated concentrations of puromycin for 5 days. The cultures contained: ○, no additions; △, 5 μM RU 486; or ▲, 5 μM verapamil. Another set of cultures containing the parental W7TB line without additions (●) is shown for comparison. At the end of the incubation period, the turbidities of the cultures ($A_{660\,nm}$) were measured and normalized to values from the cultures without puromycin. Each value represents the mean of two determinations. These data are representative of three separate experiments. *B*, reversal of daunomycin resistance in S7CD-5 cells by RU 486 and verapamil. Separate sets of S7CD-5 cultures, containing RU 486 or verapamil, were set up and incubated in the indicated concentrations of daunomycin in a manner similar to the experiment shown in *A*. A set of cultures containing W7TB cells was again used for comparison. These data are representative of two separate experiments.

dexamethasone (19). The resistance to dexamethasone is due to reduced intracellular accumulation of steroid, even though the glucocorticoid receptors in S7CD-5 are present at normal levels and are fully functional. The drug resistance in this cell line is effectively reversed by 5 μM verapamil, an established inhibitor of P-glycoprotein function (16, 28, 29). Fig. 1 illustrates the effect of growing S7CD-5 cells in the presence of RU 486 (5 μM), puromycin (20 μM), or a combination of both drugs. Neither drug alone has a significant effect on the viability or proliferation of the cells. The combination, on the other hand, causes a complete loss of viability at times greater than 42 h. The result with RU 486 alone is not unexpected since it normally has little or no agonist activity for the glucocorticoid receptor (30). Thus, the results shown in Fig. 1 are consistent with the possibility that RU 486 acts at another target and promotes the accumulation of puromycin in the cells.

Fig. 2*A* compares the relative abilities of verapamil and RU 486 to alter the resistance of S7CD-5 to puromycin. The two drugs have a very similar effect. Each lowers the puromycin resistance approximately 17-fold to a level nearly equal to that seen with the sensitive parental cell line. Fig. 2*B* demonstrates that RU 486 and verapamil also have the capacity to completely reverse the resistance to daunomycin seen in the S7CD-5 cells. RU 486 and verapamil have the additional capacity to reverse colchicine resistance in S7CD-5 cells and in cells expressing the *mdr*3 gene (data not shown). This similarity in behavior between RU 486 and verapamil is particularly significant since verapamil has been found to be a potent inhibitor of P-glycoprotein function.

To determine the relative concentrations at which RU 486 and verapamil begin to reverse drug resistance, the experiment shown in Fig. 3 was carried out. Fig. 3 shows the effects of growing S7CD-5 cells in increasing concentrations of either verapamil or RU 486 in the presence or absence of 20 μM puromycin. Without puromycin, verapamil and RU 486 have only modest effects on the proliferation of cells and no visible effect on viability. In the presence of puromycin, verapamil causes a sharp decrease in proliferation at concentrations above 0.5 μM. RU 486 exhibits a very similar profile, only slightly displaced to higher concentrations. Microscopic inspection of the cultures revealed that all of the cells were dead in the presence of 20 μM puromycin and verapamil or RU 486 at concentrations above 2 μM.

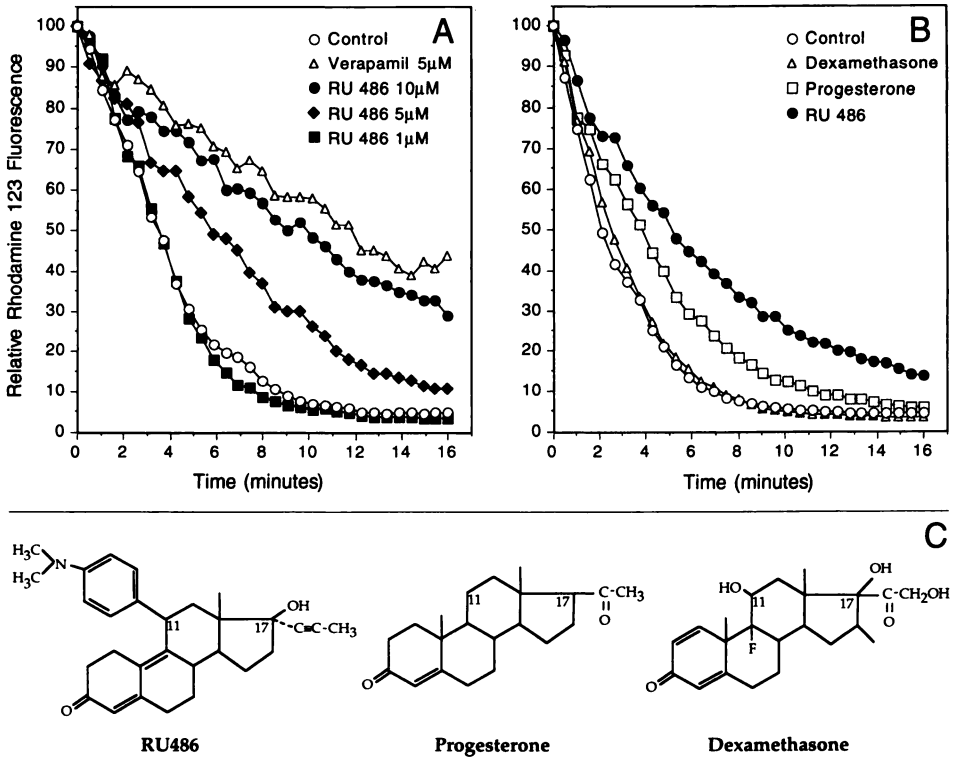


Fig. 4. *A* and *B*, efflux of rhodamine 123 from S7CD-5 cells. S7CD-5 cells were incubated for 30 min in 500 ng/ml of rhodamine 123. At the end of the incubation, the cells were placed into fresh medium without rhodamine and maintained at 0°C until analysis. Samples of cells were isolated by centrifugation and resuspended in phosphate-buffered saline at 37°C containing the drugs indicated in the figures. The controls contained no drug. Analysis of rhodamine efflux was carried out as described in "Materials and Methods." *C*, structures of RU 486, progesterone, and dexamethasone.

If RU 486 and verapamil act in a similar fashion, then the two drugs should have comparable capacities to inhibit the rate of drug efflux from S7CD-5 cells. This possibility can be tested directly since previous studies have shown that P-glycoproteins transport the fluorescent drug rhodamine 123 (31, 32). Rhodamine fluorescence can be used to measure its relative intracellular concentration. Fig. 4*A* presents a comparison by flow cytometry of the capacities of RU 486 and verapamil to inhibit the efflux of rhodamine 123. In the absence of inhibitory drugs, 50% of the rhodamine 123 was transported out of the cells within 3.4 min, and RU 486, at 1 μM, had no effect upon this rate. In the presence of 5 and 10 μM RU 486, the rates were significantly slower; 50% of the rhodamine 123 was transported in 6 and 10.3 min, respectively. The RFER can be calculated (see "Materials and Methods") by dividing the time needed to transport 50% of the rhodamine 123 (without drug) by the comparable time with drug. Thus, at 5 μM RU 486, the RFER was 57% of the untreated cells, and the value for 10 μM RU 486 was 33% of untreated cells. At 5 μM verapamil, the RFER was 29%. Using this comparison, 5 μM RU 486 appears to be one-half as effective as 5 μM verapamil (57 *versus* 29%) at inhibiting rhodamine 123 efflux from S7CD-5 cells. Fig. 4*B* compares the ability of RU 486 to inhibit rhodamine efflux with that of dexamethasone and progesterone. In this experiment, 10 μM dexamethasone had no effect on the efflux rate. As expected from previous reports (20–22), 10 μM progesterone had a measurable effect, but it was considerably smaller than the inhibition caused by 5 μM RU 486. In this instance, the RFER was 61% for progesterone and 45% for RU 486. In another similar experiment comparing 10 μM RU 486 and 10 μM progesterone, these inhibitors gave RFERs of 40 and 80%, respectively (data not shown). Thus, RU 486 was at least two times as effective as progesterone at inhibiting rhodamine 123 efflux.

These studies demonstrate that RU 486 has the capacity to efficiently inhibit drug efflux promoted by the mouse *mdr*1 P-glycoprotein and to reverse the multidrug-resistant phenotype conveyed by expression of this protein. RU 486 can achieve this effect at relatively low doses, just above 1 μM (Fig. 3). Studies carried out in humans have demonstrated that RU 486 serum concentrations above 1 μM are readily achievable (33). Thus, given the similarity between the mouse and human P-glycoproteins, the results indicate that RU 486 could potentially be used as a chemosensitizing agent. This is particularly true since high concentrations of RU 486 have been found not to have serious side effects. Recently, a series of hydrophobic compounds, including quinoline and cyclosporin derivatives and dihydropyridine analogues, were reported to be potent inhibitors of MDR function which act at submicromolar concentrations (34–36). The results with RU 486 suggest that there may be steroid derivatives with a similar capacity to inhibit drug transport by P-glycoproteins.

## Acknowledgments

RU 486 was obtained as a gift from Roussel Uclaf (Romainville, France). We thank Dr. Gail Baughman for critical reading of the manuscript.

## References

1. Warr, J. R., and Atkinson, G. F. Genetic aspects of resistance to anticancer drugs. Physiol. Rev., *68:* 1–26, 1988.
2. Hayes, J. D., and Wolf, C. R. Molecular mechanisms of drug resistance. Biochem. J., *272:* 281–295, 1990.
3. Moscow, J. A., and Cowan, K. H. Multidrug resistance. J. Natl. Cancer Inst., *80:* 14–20, 1988.
4. Arceci, R. J. Clinical significance of P-glycoprotein in multidrug resistance malignancies. Blood, *81:* 2215–2222, 1993.
5. Riehm, H., and Biedler, J. L. Cellular resistance to daunomycin in Chinese hamster cells *in vitro*. Cancer Res., *31:* 409–412, 1971.
6. Ling, V., and Thompson, L. H. Reduced permeability in CHO cells as a mechanism of resistance to colchicine. J. Cell. Physiol., *83:* 103–116, 1973.
7. Dano, K. Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. Biochim. Biophys. Acta, *323:* 466–483, 1973.
8. Carlsen, S. A., Till, J. E., and Ling, V. Modulation of membrane drug permeability in Chinese hamster ovary cells. Biochim. Biophys. Acta, *455:* 900–912, 1976.
9. Endicott, J. A., and Ling, V. The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu. Rev. Biochem., *58:* 137–171, 1989.
10. Biedler, J. L. Genetic aspects of multidrug resistance. Cancer (Phila.), *70:* 1799–1809, 1992.
11. Roninson, I. B. The role of the *mdr*1 (P-glycoprotein) gene in multidrug resistance *in vitro* and *in vivo*. Biochem. Pharmacol., *43:* 95–102, 1992.
12. Greenberger, L. M., Hsu, S. I-H., Yang, C-P. H., Cohen, D., Lothstein, L., Han, E. K-H., Kirschner, L. S., Piekarz, R. L., Yu, L., and Horwitz, S. B. A comparison of

the structure, function and expression of P-glycoproteins encoded by *mdr1a* and *mdr1b* in mouse. *In:* T. Tsuruo and M. Ogawa (eds.), Drug Resistance as a Biochemical Target in Cancer Chemotherapy, pp. 63–95. San Diego: Academic Press, Inc., 1992.

13. Gottesman, M. M., and Pastan, I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu. Rev. Biochem., *62:* 385–427, 1993.
14. Gros, P., and Buschman, E. The mouse multidrug resistance gene family: structural and functional analysis. Int. Rev. Cytol., *137C:* 169–197, 1993.
15. van der Bliek, A. M., and Borst, P. Multidrug resistance. Adv. Cancer Res., *52:* 165–203, 1989.
16. Ford, J. M., and Hait, W. N. Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol. Rev., *42:3:* 155–199, 1990.
17. Nelson, E. J., and Hinkle, P. A. Characterization of multidrug-resistant pituitary tumor cells. Endocrinology, *130:* 3246–3256, 1992.
18. Ueda, K., Okamura, N., Hira, M., Tanigawara, Y., Saeki, T., Kioka, N., Komano, T., and Hori, R. Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. J. Biol. Chem., *267:* 24248–24252, 1992.
19. Bourgeois, S., Gruol, D. J., Newby, R. F., and Rajah, F. M. Expression of an *mdr* gene is associated with a new form of resistance to dexamethasone-induced apoptosis. Mol. Endocrinol., *7:* 840–851, 1993.
20. Yang, C-P. H., Goei DePinho, S., Greenberger, L. M., Arceci, R. J., and Horwitz, S. B. Progesterone interacts with P-glycoprotein in multidrug-resistant cells and in the endometrium of gravid uterus. J. Biol. Chem., *264:* 782–788, 1989.
21. Qian, X-d., and Beck, W. T. Progesterone photoaffinity labels P-glycoprotein in multidrug-resistant human leukemic lymphoblasts. J. Biol. Chem., *265:* 18753–18756, 1990.
22. Yang, C-P. H., Cohen, D., Greenberger, L. M., Hsu, S. I-H., and Horwitz, S. B. Differential transport properties of two *mdr* gene products are distinguished by progesterone. J. Biol. Chem., *265:* 10282–10288, 1990.
23. Gravanis, A., Schaison, G., George, M., de Brux, J., Satyasworoop, P. G., Baulieu, E. E., and Robel, P. Endometrial and pituitary responses to the steroidal antiprogestin RU 486 in postmenopausal women. J. Clin. Endocrinol. Metab., *60:* 156–163, 1985.
24. Teutsch, G., Ojasoo, T., and Raynaud, J. P. 11β-Substituted steroids, an original pathway to antihormones. J. Steroid Biochem., *31:* 549–565, 1988.
25. Harris, A. W., Bankhurst, A. D., Mason, S., and Warner, N. L. Differentiated functions expressed by cultured mouse lymphoma cells. II. Theta antigen, surface immunoglobulin and a receptor on cells of a thymoma cell line. J. Immunol., *110:* 431–438, 1973.
26. Johnson, D. M., Newby, R. F., and Bourgeois, S. Membrane permeability as a determinant of dexamethasone resistance in murine thymoma cells. Cancer Res., *44:* 2435–2440, 1984.
27. Bourgeois, S., and Newby, R. F. Diploid and haploid states of the glucocorticoid receptor gene of mouse lymphoid cell lines. Cell, *11:* 423–430, 1977.
28. Willingham, M. C., Cornwell, M. M., Cardarelli, C. O., Gottesman, M. M., and Pastan, I. Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and -sensitive KB cells: effects of verapamil and other drugs. Cancer Res., *46:* 5941–5946, 1986.
29. Safa, A. R., Glover, C. J., Sewell, J. L., Meyers, M. B., Biedler, J. L., and Felsted, R. L. Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. J. Biol. Chem., *262:* 7884–7888, 1987.
30. Bourgeois, S., Pfahl, M., and Baulieu, E-E. DNA binding properties of glucocorticosteroid receptors bound to the steroid antagonist RU 486. EMBO J., *3:* 751–755, 1984.
31. Neyfakh, A. A. Use of fluorescent dyes as molecular probes for the study of multidrug resistance. Exp. Cell Res., *174:* 168–176, 1988.
32. Chaudhary, P. M., and Roninson, I. B. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell, *66:* 85–94, 1991.
33. Heikinheimo, O., and Kekkonen, R. Dose-response relationships of RU 486. Annals Med., *25:* 71–76, 1993.
34. Shudo, N., Mizoguchi, T., Kiyosue, T., Arita, M., Yoshimura, A., Seto, K., Sakoda, R., and Akiyama, S. Two pyridine analogues with more effective ability to reverse multidrug resistance and with lower calcium channel blocking activity than their dihydropyridine counterparts. Cancer Res., *50:* 3055–3061, 1990.
35. Sato, W., Fukazawa, N., Suzuki, T., Yusa, K., and Tsuruo, T. Circumvention of multidrug resistance by a newly synthesized quinoline derivative, MS-073. Cancer Res., *51:* 2420–2424, 1991.
36. Loor, F., Boesch, D., Gavériaux, C., Jachez, B., Pourtier-Manzanedo, A., and Emmer, G. SDZ 280–446, a novel semi-synthetic cyclopeptolide: *in vitro* and *in vivo* circumvention of the P-glycoprotein-mediated tumour cell multidrug resistance. Br. J. Cancer, *65:* 11–18, 1992.