Biochemical Pharmacology, Vol. 52, pp. 213–217, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00178-5

Study of P-Glycoprotein Functionality in Living Resistant K562 Cells After Photolabeling with a Verapamil Analogue

Samlee Mankhetkorn, Elisabetta Teodori,† Serena Scapecchi† and Arlette Garnier-Suillerot*‡*

*Laboratoire de Physicochimie Biomoléculaire et Cellulaire (URA 2056 CNRS), Université Paris Nord, 74 rue Marcel Cachin, Bobigny 93017 France, and †Dipartimento di Scienze Farmaceutiche, Universita di Firenze, via G. Capponi 9, 50121 Firenze, Italy

ABSTRACT. To our knowledge, this is the first study to investigate the modification of P-glycoprotein functionality in living resistant cells after photolabeling. For this purpose, four new photoactive verapamil analogues were synthesized. These compounds have the same efficacy as verapamil to increase pirarubicin (pira) incorporation into living multidrug resistant (MDR) K562 cells and to sensitize them to the cytotoxic effect of this anthracycline derivative, indicating that they act as typical MDR modifiers in MDR cells. These compounds were used to photolabel P-glycoprotein (P-gp) in living resistant cells. Irradiation did not result in photodamage to cells, and P-gp functionality was verified by the ability of living cells to incorporate pira. The irradiation of resistant cells, 10^6/mL, in the presence of a verapamil analogue at concentrations equal to or higher than 3 μM yielded 70% inhibition of P-gp functionality. Our data provide the first evidence that the binding of a verapamil analogue to P-gp is not sufficient to completely inhibit the efflux of this anthracycline. The cells were, subsequently, cultured for several days. Resistance was progressively recovered with time, with the treated cells being just as resistant as before photolabeling after 6 days. BIOCHEM PHARMACOL 52;2:213–217, 1996.

KEY WORDS. multidrug resistance; P-glycoprotein; photoaffinity labeling; living cells; verapamil analogue

MDR§ is frequently expressed by neoplastic cell types and results in a broad spectrum of resistance to drugs that show little structural similarity. Characteristic of MDR cells is overexpression of a 150–180 kDa membrane protein, P-gp, which is an energy-dependent drug efflux pump [1, 2]. Using photoaffinity analogues, it has been shown that drugs involved in the MDR phenotype, such as vinblastine [3], colchicine [4], daunorubicin [5, 6], as well as compounds able to enhance the toxicity of MDR-related drugs, such as calcium channel blockers [7, 8] and cyclosporin [9], bind specifically to P-gp.

Most of the studies performed using photoaffinity analogues have been designed to demonstrate the binding of these analogues to P-gp, but none has reported a study of P-gp functionality after photolabeling.

The overall goal of this work was to show that it is possible to photolabel P-gp in living cells and to study how its functionality is modified after this treatment. For this purpose, we synthesized 4 photoactive analogues of verapamil and, by using a spectrofluorometric method described previously [10–12], we studied the alteration of P-gp functionality directly in living cells after irradiation in the presence of these compounds. Sensitive and resistant K562 cells were used, and their ability to incorporate an anthracycline derivative, pirarubicin determined.

MATERIALS AND METHODS

Cell Culture and Cytotoxicity Assay

Doxorubicin-sensitive and -resistant erythroleukemia K562 cells were routinely cultured as described previously [11]. For the assays, culture was initiated at 5×10^5 cells/mL to have cells in the exponential growth phase; the cells were used 24 hr later, when the culture had grown to approximately 8×10^5 cells/mL. Cell viability was assessed by Trypan Blue exclusion. The number of cells was determined by Coulter counter analysis.

The IC_{50} of cells resistant to pirarubicin was equal to 40 nM and decreased to 16 nM in the presence of 2 μM verapamil or 2 μM of the verapamil analogues used in this study, indicating that these new derivatives, as well as verapamil, act as typical MDR modifiers in K562-resistant cells. The IC_{50} was determined as follows: cells, 10^5/mL, were incubated in the presence of various pirarubicin concentrations, and in the simultaneous presence or absence of verapamil analogues. The IC_{50} was determined by plotting

‡ Corresponding author. Tel. 33 (1) 48 38 77 48; FAX 33 (1) 48 38 77 77.

§ *Abbreviations:* MDR, multidrug resistance; pira, pirarubicin; P-gp, P-glycoprotein.

Received 22 November 1995; accepted 1 March 1996.

the percentage of cell growth inhibition vs the logarithm of the antitumor drug concentration: IC_{50} is the drug concentration that inhibits cell growth by 50% when measured at 72 hr. We verified that the verapamil analogues alone, at the concentrations used, have no effect on cell proliferation.

Total RNA was prepared from frozen cells according to the CsCl-guanidinium isothiocyanate method proposed by Maniatis *et al.* [13] and adapted by Ferrandis *et al.* [14]. The transcript level of the MDR1 gene was measured comparatively to that of the KB-8-5 cell line, which shows an arbitrary expression of 30 a.u. [15]. Our K562-resistant cells exhibited an MDR1 gene transcript level of 800 a.u. (Benard and Garnier-Suillerot, unpublished data).

Drugs and Chemicals

Purified pira (4′-*o*-tetrahydropyranyldoxorubicin) was kindly provided by Laboratoire Roger Bellon (France). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using $\epsilon 480 = 11500\ M^{-1}cm^{-1}$. Stock solutions were prepared just before use. Verapamil was obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents were of the highest quality available. Deionized double-distilled water was used throughout the experiments, which were performed in Hepes Na^+ buffer solutions containing 20 mM Hepes buffer plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.25, in the presence of 5 mM glucose.

Absorption spectra were recorded on a Cary 219 spectrophotometer and fluorescence spectra on a Perkin Elmer LS 50 B spectrofluorometer.

The synthetic pathway used to obtain verapamil analogues is reported in Scheme 1. The ^1H NMR and IR spectra were consistent with the chemical structure of the compounds. Microanalysis data were within ± 0.4 of the theoretical value derived from the formula reported.

2,2-DIPHENYL-5-[N-1-(O-AZIDOPHENYL)ETHYLAMINO]VALERONITRILE 1. 0.5 g (1.6 mmol) of 2,2-diphenyl-5-bromovaleronitrile [16], 0.25 g (1.6 mmol) of 1-(*o*-azidophenyl)ethylamine [17], and 1.1 mL of triethylamine were heated under reflux for 6 hr. The mixture was, then, treated with $CHCl_3$ and washed with water. After anhydrification and evaporation of the solvent, the residue was purified by column chromatography using $CHCl_3$/MeOH (9/1) as eluting system. 1 (320 mg, 51% yield) was obtained as an oil. The oxalate crystallized from EtOH, m.p. 166–168°C dec. Anal. ($C_{25}H_{25}N_5 \cdot C_2H_2O_4$). Compound 2 was obtained in the same way, using 1-(*p*-azidophenyl) ethylamine obtained with the method described in [17]. The oxalate crystallized from EtOH, m.p. 176–178°C dec. Anal. ($C_{25}H_{25}N_5 \cdot C_2H_2N_4$).

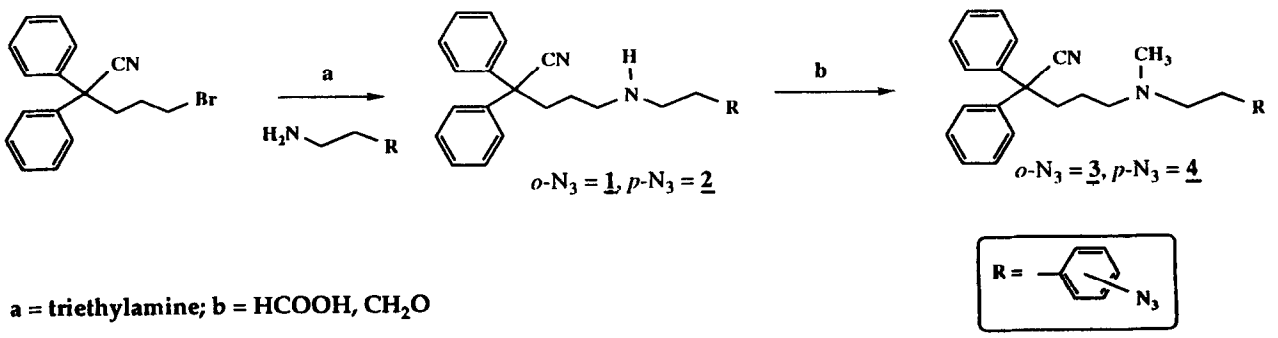
2,2-DIPHENYL-5-[N-METHYL-N-1-(O-AZIDOPHENYL)ETHYLAMINO]VALERONITRILE 3. A solution of 2,2-diphenyl-5-[N-1-(*o*-azidophenyl)ethylamino]valeronitrile (1) (150 mg, 0.38 mmol) and HCOOH (4 mL of 85% solution) in absolute ethanol (5 mL) was heated under reflux for 1 hr; then, formaline (4 mL of 40% solution) was added and the mixture heated under reflux for 1.5 hr. After removal of the solvent, the residue was dissolved in CH_2Cl_2 and washed with 10% NaOH. Anhydrification and evaporation of the solvent gave an oil (150 mg, 96% yield) that was suitably pure. The oxalate crystallized from EtOH, m.p. 172–174°C dec. Anal. ($C_{26}H_{27}N_5 \cdot C_2H_2O_4$). Compound 4 was obtained in the same way. The oxalate crystallized from EtOH, m.p. 176–178°C dec. Anal. ($C_{26}H_{27}N_5 \cdot C_2H_2O_4$).

Cellular Drug Accumulation

The uptake of anthracycline in cells was monitored by following the decrease in the fluorescence signal at 590 nm (λ_{ex} = 480 nm) as previously described [10–12]. Using this method, it is possible to accurately quantify the kinetics of uptake of the drug by the cells and the amount of anthracycline intercalated between the base pairs in the nucleus in the steady-state, because incubation of the cells with the drug proceeds without compromising cell viability. All experiments were conducted in 1-cm quartz cuvettes containing 2 mL of buffer at 37°C.

Irradiation

Ultraviolet irradiation was carried out using a 125-watt lamp (HPW 125 W-TS, Philips) as a light source. Cells, 2 × 10^6 in 2 mL were placed in a 25-mL cultured flask, and irradiation was carried out at a distance of 7 cm from the



SCHEME 1.

cells for 20 min at 25°C. The plastic flask allowed transmittance of UV light in the 300–400 nm range, and no additional filter was necessary. The intensity, I_0^i, of the light beam incident just within the flask was measured using the potassium ferrioxalate system as actinometer [18]. I_0^i was equal to 2.5 ± 0.5 × 10^{14} quanta/sec.

Cells were irradiated either in the presence or absence of verapamil analogues. To eliminate the unbound verapamil analogue, they were, then, washed twice in a 25 mL volume of buffer.

RESULTS

Determination of the Overall Concentration of Pirarubicin Bound to the Nucleus at the Steady-State in the Presence of Various Concentrations of Verapamil Derivatives

Cells, 10^6/mL, were incubated at 37°C and pH 7.2 with pira 1 μM in the presence of the four compounds 1, 2, 3, or 4 at concentrations ranging from 0 to 15 μM. Figure 1 shows a typical record of an experiment dealing with 5 μM compound 1. In each case, the steady-state was reached within ca. 30 min. C_n, the overall concentration of pira bound to the nucleus of drug-resistant cells, was plotted as a function of the verapamil analogue concentrations. In the 4 cases, C_n increased as verapamil analogue concentrations increased. This can be quantified using the following equation:

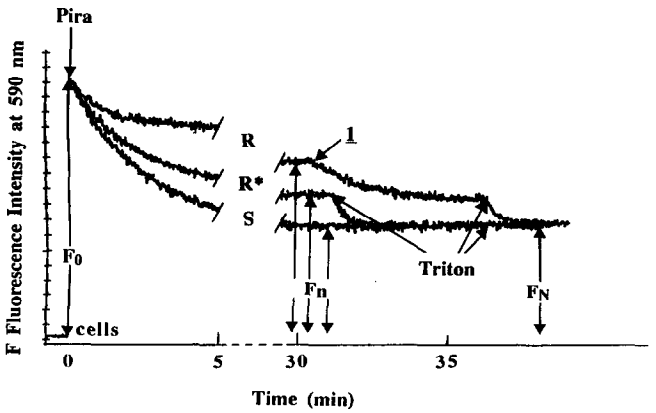


FIG. 1. Uptake of pirarubicin by drug-sensitive (S), drug-resistant (R), and drug-resistant K562 cells after photoirradiation in the presence of 5 μM verapamil analogue 1 (R*). Fluorescence intensity at 590 nm (λ_{ex} = 480 nm) was recorded as a function of time. Cells (2 × 10^6) were suspended in a cuvette filled with 2 mL buffer at pH_e = 7.25 under vigorous stirring. At t = 0, 20 μL of a 100-μM stock pirarubicin (pira) solution was added to the cells, yielding a C_T = 1 μM pirarubicin solution. The fluorescence intensity was then F_0. After the steady-state was reached, the fluorescence was F_n and the concentration of drug intercalated between the base pairs in the nucleus was $C_n = C_T(F_0 - F_n)/F_0$. At the steady-state, 5 μM compound 1 was added to the resistant cells (R). The addition of 0.05% Triton X-100 yielded the equilibrium state. The overall concentration (C_N) of drug intercalated between the base pairs in the nucleus was then $C_N = C_T \cdot (F_0 - F_N)/F_0$.

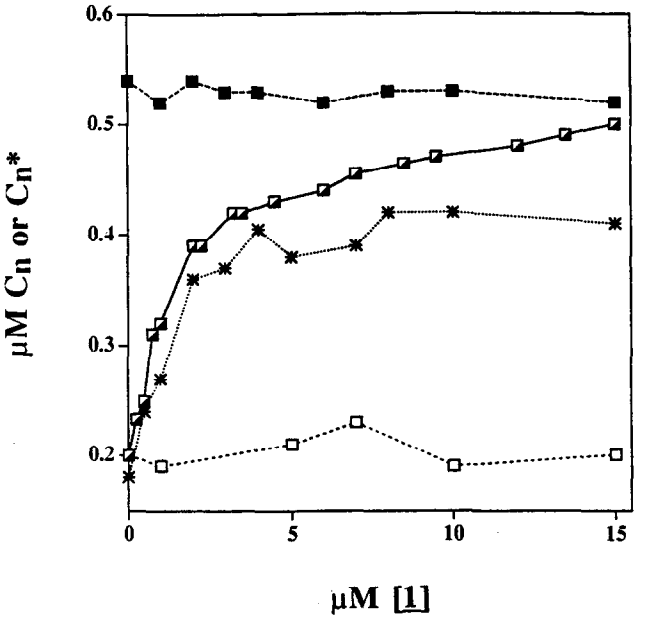


FIG. 2. Effect of verapamil analogue 1 on pirarubicin accumulation in living cells. Cn, the overall concentration of pirarubicin bound to the nucleus at the steady-state was plotted as a function of 1 concentration. The cells were sensitive (■) and resistant (◪) and the assay was performed in the presence of the indicated concentration of 1. The cells were resistant (*) and the measure was performed after irradiation in the presence of the indicated concentration of 1 and 2 washings to eliminate unbound 1. The cells were resistant, (□) incubated with the indicated 1 concentrations, and then washed twice. The experimental conditions are described in Materials and Methods. Each point represents the average of 3 determinations.

$$\alpha = [(C_n)_{Ri} - (C_n)_{R0}]/[(C_n)_S - (C_n)_{R0}] \quad (1)$$

where $(C_n)_S$ is the overall concentration of drug bound to the nucleus of sensitive cells and $(C_n)_{R0}$ and $(C_n)_{Ri}$ are the overall concentrations of drug bound to the nucleus of resistant cells in the absence and presence of a concentration [*i*] of inhibitor, respectively. α is the fold increase in the nuclear concentration of pira in the presence of reversing agent. α varies between 0 (in the absence of inhibitor) and 1 (when the amount of drug in resistant cells is the same as in sensitive cells) [19, 20]. The efficacy of the 4 verapamil analogues was the same within the limits of experimental error, and the concentration required to obtain α = 0.5 (i.e., to cause a half-maximal increase in cellular pira accumulation) was equal to 1.3 ± 0.2 μM. The same value was also obtained with verapamil [19]. The data obtained with compound 1 are shown in Fig. 2.

It was important to verify whether or not it was possible to eliminate the verapamil analogues from the cells. For this purpose, cells (2 × 10^6/2 mL) were incubated for 10 min with verapamil analogues at concentrations ranging from 0 to 15 μM. They were, then, washed twice in 25 mL buffer and their ability to incorporate pira measured. As can be seen in Fig. 2, the intranuclear amount of pira after washing was the same as in cells that had not been incubated with

a verapamil analogue, indicating that it had indeed been eliminated.

P-glycoprotein Functionality after Cell Irradiation in the Absence and in the Presence of Verapamil Analogue

Cells were irradiated in the absence of verapamil analogues for time intervals ranging from 1 min to 1 hr. Using Trypan Blue, we determined that the number of dead cells did not exceed 5%. We also ensured that pira incorporation was the same before and after irradiation.

Cells, 10^6/mL, were irradiated for 20 min in the presence of verapamil analogue at concentrations ranging from 1 to 15 μM and, to eliminate unbound molecules, were washed twice as described above. Pira incorporation was measured. $(C_n^*)_{Ri}$ is used to stand for the overall intranuclear concentration of pirarubicin and α* for the fold increase in the nuclear concentration of pirarubicin after irradiation in the presence of verapamil analogue at concentration [*i*]. C_n^* was plotted as a function of the verapamil analogue concentration added to the cells during irradiation. Figure 2 shows the data obtained with the compound 1. They are the mean of 3 independent experiments. Because strictly analogous data were obtained with the 4 compounds, only those obtained with 1 are reported below. We verified that 20-min irradiations were necessary to obtain the maximal effect and that no further modifications were observed with longer time irradiations.

Under these conditions, the P-gp-mediated efflux of pira could not be completely inhibited and the coefficient α* value never exceeded 0.7, even when the irradiation was performed in the presence of 1 15 μM. We, then, sought to determine whether or not a further addition of 1, which was still present in the solution, would be able to inhibit the remaining efflux. Pira incorporation was, thus, measured in cells, previously photolabeled, in the presence of additional increasing concentrations of compound 1. When this concentration was increased from 0 to 3 μM, no further increase in pira incorporation was observed. However, at concentrations higher than 3 μM, a further increase in pira incorporation, up to the value obtained in sensitive cells, was observed.

In summary, according to Eqn (1), α = 0.5 and α* = 0.5 are obtained for [1] = 1.3 ± 0.2 μM. However, the better α* value obtained with 1 15 μM after irradiation and elimination of the unbound molecules is 0.7, whereas at 1 15 μM, α is equal to 1.

Ability of MDR Cells to Recover Resistance after Photolabeling

Resistant and sensitive cells, 10^6/mL, were irradiated in the presence of 1, 2, 3, or 5 μM compound 1, respectively and washed twice to eliminate unbound molecules. They were, then, cultured for 3 days in the presence of various pira concentrations. The IC_{50} values thus obtained were plotted as a function of the verapamil analogue concentration used during the irradiation (Fig. 3a). The IC_{50} value of MDR cells photolabeled with 1, at concentrations equal to or higher than 3 μM, was twice that of sensitive cells.

In a second set of experiments, cells were irradiated in the presence of 2.5 μM compound 1. After two washings, cells, 5×10^5/mL, were cultured for 1 day and their ability to incorporate pira measured. The same cells, 5×10^5/mL, were again cultured for 1 day and their ability to incorporate pira measured. This operation was repeated every day for 5 days. α*, as defined by Eqn (1) was plotted as a function of time (Fig. 3b). As can be seen, their ability to

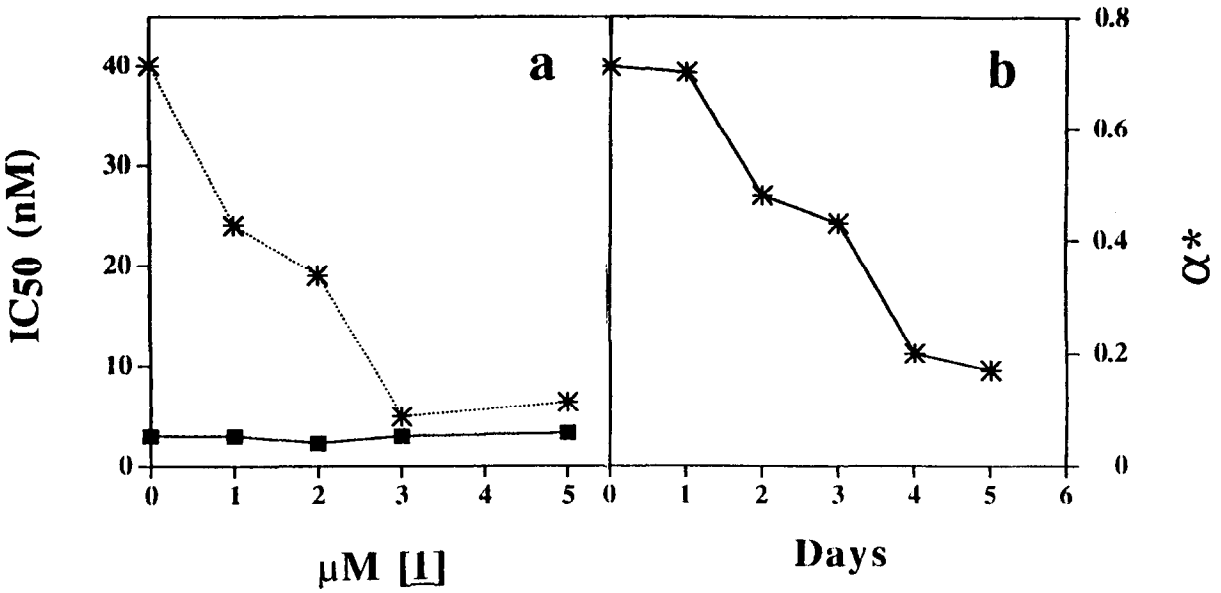


FIG. 3. Ability of MDR cells to recover resistance after photolabeling. (a) The IC_{50} of resistant (*) and sensitive cells (■) to pirarubicin was plotted as a function of 1 concentration used during their irradiation. (b) Resistant cells were photolabeled with 2.5 μM 1 and their ability to incorporate pirarubicin was verified every day for 5 days. α*, the fold increase in nuclear pirarubicin concentration (as compared to sensitive cells) was plotted as a function of the time elapsed since irradiation.

incorporate pira decreased as time elapsed and cell multiplication occurred.

A comparison of these 2 sets of experiments shows that, 3 days after photolabeling with 2.5 μM 1, 65 ± 5% of the reversal of resistance is maintained, determined either by the IC_{50} value (Fig. 3a) or by the capacities of drug extrusion (Fig. 3b). This finding can be interpreted as a dilution of the irradiated P-gp among newly generated P-gp, as cell division occurs.

DISCUSSION

Photoaffinity labeling has become one of the key approaches for identifying and characterising mediators of biochemical, physiological, and pharmacological phenomena. It has been used primarily to try to identify the drug-binding sites on P-gp, and it has been shown that drugs involved in the MDR phenotype bind specifically to P-gp [21, 22]. For this purpose, plasma membrane preparations of resistant MDR cells were photoaffinity labeled with various compounds. The photoaffinity labeling of P-gp in living cells has been performed by Raviv *et al.* [23]. However, these authors did not subsequently study P-gp functionality.

Our aim was to perform photoaffinity labeling in intact living cells and to study the modifications of P-gp functionality. Two essential points emerge from our data. The first is the demonstration that it is possible to photolabel P-gp in living cells without compromising their viability. Although we used verapamil analogues, any type of photoaffinity analogue might be employed. Second, the binding of the verapamil analogues does not completely reverse cell resistance to pira, and the amount of pira in these treated cells reaches the level observed in sensitive cells. However, further addition of this inhibitor, which, under this condition, should not interact specifically with P-gp, yields the same level of pira as in sensitive cells. This suggests that these verapamil analogues could have two effects: a direct one by binding to P-gp, and an indirect one by acting as a surfactant. This would be in agreement with observations showing that surfactants are able to reverse MDR phenotype through modification of membrane lipid fluidity [24].

This investigation was supported by the Université Paris Nord, the CNRS (Centre National de la Recherche Scientifique), and the ARC (Association pour la Recherche sur le Cancer).

References

1. Bradley G, Juranka PF and Ling V, Mechanism of multidrug resistance. *Biochim Biophys Acta* **948:** 87–128, 1988.
2. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385–427, 1993.
3. Safa AR, Glover CM, Meyers MB, Biedler JL and Felsted RL, Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. *J Biol Chem* **261:** 6137–6140, 1986.
4. Safa AR, Mehta ND and Agresti M, Photoaffinity labeling of P-glycoprotein in multidrug resistant cells with photoactive analogs of colchicine. *Biochem Biophys Res Commun* **162:** 1402–1408, 1989.
5. Busche R, Tummler B, Cano-Gauci DF and Riordan JR, Equilibrium, kinetic and photoaffinity labeling studies of daunomycin binding to P-glycoprotein-containing membranes of multidrug-resistant Chinese hamster ovary cells. *Eur J Biochem* **183:** 189–197, 1989.
6. Beck WT and Qian X-D, Photoaffinity substrates for P-glycoprotein. *Biochem Pharmacol* **43:** 89–93, 1992.
7. Safa AR, Photoaffinity labeling of the multi-drug-resistance-related P-glycoprotein with photoactive analogs of verapamil. *Proc Natl Acad Sci USA* **85:** 7187–7191, 1988.
8. Qian X-D and Beck WT, Progesterone photoaffinity labels P-glycoprotein in multidrug resistant human leukemic lymphoblasts. *J Biol Chem* **265:** 18753–18756, 1990.
9. Foxwell BMJ, Mackie A, Ling V and Ryffel B, Identification of the multidrug resistance-related P-glycoprotein as a cyclosporine binding protein. *Mol Pharmacol* **36:** 543–546, 1989.
10. Tarasiuk J, Frézard F, Gattegno L and Garnier-Suillerot A, Anthracycline incorporation in human lymphocytes. Kinetics of uptake and nuclear concentration. *Biochim Biophys Acta* **1013:** 109–117, 1989.
11. Frezard F and Garnier-Suillerot A, Comparison of the membrane transport of anthracycline derivatives in drug-resistant and drug-sensitive K562 cells. *Eur J Biochem* **196:** 483–491, 1991.
12. Frezard F and Garnier-Suillerot A, Determination of the osmotic active drug concentration in the cytoplasm of anthracycline-resistant and -sensitive K562 cells. *Biochim Biophys Acta* **1091:** 29–35, 1991.
13. Maniatis T, Fristch E and Sambrook J, *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
14. Ferrandis E, Da Silva J, Riou E and Bernard J, Coactivation of the MDR1 and MYCN genes in human neuroblastoma cells during the metastatic process in the nude mouse. *Cancer Res* **54:** 2256–2261, 1994.
15. Golstein LJ, Fojo AT, Crist W, Green A, Brodeur G, Pastan I and Gottesman MM, Expression of the multidrug resistance, MDR1, gene in neuroblastomas. *J Clin Oncol* **8:** 128–136, 1990.
16. Gualtieri F, Teodori E, Bellucci D, Pesce E and Piacenza G, SAR studies in the field of calcium(II) antagonists. Effect of modifications at the tetrasubstituted carbon of verapamil-like compounds. *J Med Chem* **28:** 1621–1625, 1985.
17. Molina P, Alajarin M and Vidal A, Synthetic applications of bis(iminophosphoranes). One-pot preparation of rigid bicyclic guanidines. *J Org Chem* **58:** 1687–1690, 1993.
18. Calvert JC and Pitts JN, *Photochemistry*, pp. 783–786. John Wiley and Sons, Inc., New York, 1966.
19. Pereira E and Garnier-Suillerot A, Correlation between the short-term measurements of drug accumulation in living cells and the long-term growth inhibition. *Biochem Pharmacol* **47:** 1851–1857, 1994.
20. Pereira E, Teodori E, Dei S, Gualtieri F and Garnier-Suillerot A, Reversal of multidrug resistance by verapamil analogues. *Biochem Pharmacol* **50:** 451–457, 1995.
21. Safa AR, Photoaffinity labeling of P-glycoprotein in multidrug-resistant cells. *Cancer Invest* **10:** 295–305, 1992.
22. Beck WT and Qian X-D, Photoaffinity substrates for P-glycoprotein. *Biochem Pharmacol* **43:** 89–93, 1992.
23. Raviv Y, Pollard H, Bruggeman EP, Pastan I and Gottesman MM, Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells. *J Biol Chem* **265:** 3975–3980, 1990.
24. Borrel MN, Fiallo M, Veress I and Garnier-Suillerot A, The effect of crown ethers, tetraalkylammonium salts and polyoxyethylene amphiphiles on pirarubicin incorporation in K562 resistant cells. *Biochem Pharmacol* **50:** 2069–2076, 1995.