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Fluorescent Verapamil Derivative for Monitoring Activity of the Multidrug Transporter

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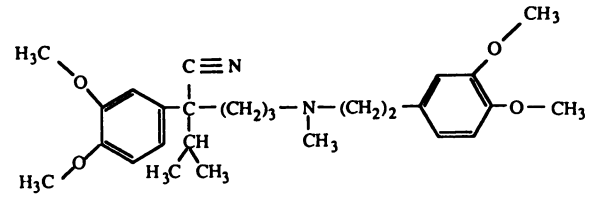
SUMMARY

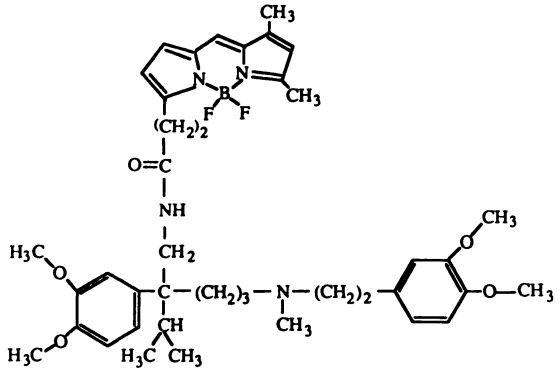
Multidrug resistance to amphipathic natural product chemotherapeutic drugs is conferred on cancer cells by expression of the *MDR*1 gene, which encodes the 170-kDa multidrug transporter known as P glycoprotein. The P glycoprotein-mediated efflux of toxic chemotherapeutic drugs can be reversed by agents such as verapamil, which is a substrate for the multidrug transporter and appears to be a competitive inhibitor of the efflux pump. In this study, Bodipy-verapamil, a fluorescent derivative of verapamil, has been shown to be a substrate for the efflux pump activity of P glycoprotein. Single-cell fluorescence analysis reveals that Bodipy-verapamil accumulates in lysosomes of drug-sensitive NIH3T3 and KB cells but is rapidly effluxed from multidrug-resistant derivatives of these cell lines. Although Bodipy-verapamil is a substrate for the multidrug transporter, it is not an efficient inhibitor of the pump and does not reverse resistance to vinblastine and colchicine as effectively as does verapamil. This new derivative may be a useful tool for imaging of lysosomes in drug-sensitive cells and for rapid screening for the multidrug-resistant phenotype in other cell types.

The outward translocation of many natural product chemotherapeutic drugs through the lipid bilayer is mediated by a plasma membrane transport glycoprotein referred to as P glycoprotein or the multidrug transporter (1–4). P glycoprotein is expressed at the surface of cells exhibiting the MDR phenotype (5). Drugs that have been shown to be expelled by the transporter are lipophilic compounds, which frequently have a positive charge at physiological pH, usually due to a tertiary amine group, and often possess aromatic moieties. By an unknown recognition mechanism, these common properties enable cross-resistance to certain classes of drugs, such as *Vinca* alkaloids, anthracyclines, colchicine, epipodophyllotoxins, and actinomycin D, in cultured MDR cells (1, 6–10). The physicochemical properties of some of these chemotherapeutic drugs allow direct spectrophotometric measurements of drug accumulation in cells (11, 12). In addition, specific fluorescent dyes, such as the mitochondrial dye rhodamine-123, which is also a substrate for transport by P glycoprotein (13–18), have also proven useful for study of the multidrug transport system.

P glycoprotein-mediated transport is inhibited by other unrelated molecules such as verapamil, reserpine, and quinidine. These drugs may act as inhibitors of the transport process by virtue of being substrates for the transporter that overwhelm its capacity to handle chemotherapeutic drugs. To develop tools to analyze the function of the multidrug transporter in living cells, we have studied a fluorescent derivative of verapamil. This derivative possesses the Bodipy fluorophore, which has



Verapamil



Bodipy-verapamil

Fig. 1. Structural formulas of verapamil and Bodipy-verapamil.

ABBREVIATIONS: MDR, multidrug resistance; PBS, phosphate-buffered saline.

spectral properties similar to those of fluorescein. In this study, we compare the effect of both Bodipy-verapamil and verapamil on MDR human KB carcinoma cells and MDR NIH3T3 mouse fibroblasts and their drug-sensitive parental cell lines. The results show that Bodipy-verapamil is a substrate for P glycoprotein in MDR cells and that this derivative is preferentially concentrated in lysosomes of drug-sensitive cells only. Accumulation of the derivative in MDR cells was increased by cotreatment of the cells with verapamil. Our results suggest that Bodipy-verapamil may be a useful tool for examining the MDR phenotype in other cell types.

Materials and Methods

Cell cultures. KB-3-1, a cloned, drug-sensitive, human KB carcinoma cell line (10), and its vinblastine-selected subline KB-V1 (19) were grown as monolayer cultures at 37° in 5% CO_2, using Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (GIBCO), 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. The drug-resistant cell line was maintained continuously in 1 μg/ml vinblastine.

NIH3T3-MDR cells were derived from drug-sensitive parental NIH3T3 by selection in 1 μg/ml colchicine after transfection of a cloned *MDR*1 cDNA, as previously described (20). NIH3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Cell survival measured by colony formation. Dose-response curves for KB-3-1 and its drug-resistant KB-V1 subline were determined by plating 300 cells and 600 cells, respectively, in 60-mm dishes, in the absence of any drug. NIH3T3 and NIH3T3-MDR cells were plated at a density of 300 cells/60-mm dish. Various drugs from dimethyl sulfoxide stock solutions were added 16 hr later. The final concentration of dimethyl sulfoxide did not exceed 0.1% in the culture media. In individual experiments, either colchicine or vinblastine was added at various concentrations in combination either with verapamil or with Bodipy-verapamil, as specified in the figure legends.

After incubation for 10 days at 37°, plates were stained with 0.5% methylene blue in 50% methanol and counted. The survival of control

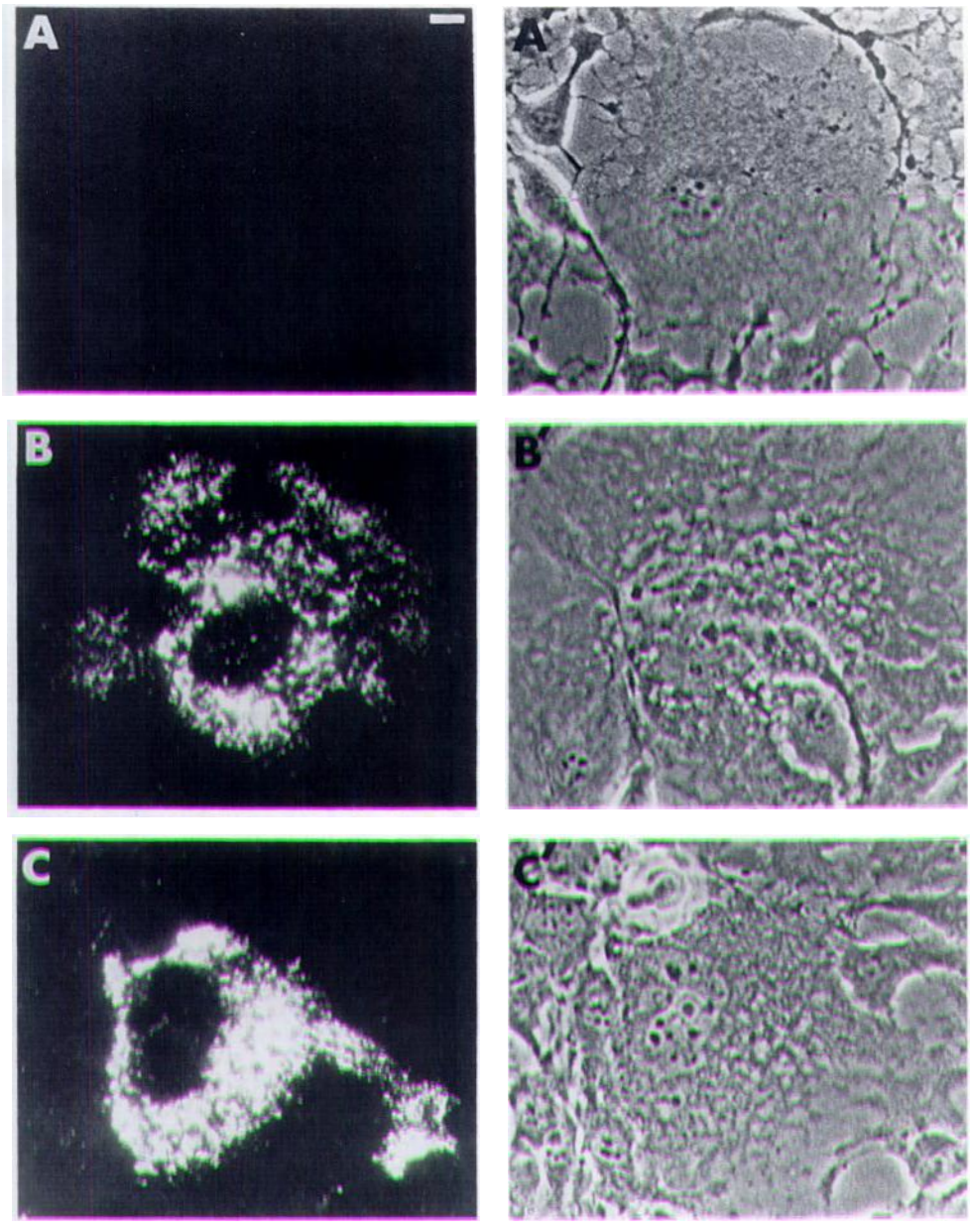


Fig. 2. Accumulation of Bodipy-verapamil in cultured multidrug-resistant NIH3T3 cells. NIH3T3-MDR cells were incubated with 1 μM Bodipy-verapamil for 20 min at 37°, in regular medium (A) or with the addition of 10 μM verapamil (B) or 10 μM vinblastine (C). The cells were then washed in PBS and examined by water immersion optics while still alive, using epifluorescence (A, B, and C) or phase contrast (A', B', and C') microscopy. Note the accumulation of Bodipy-verapamil in intracellular organelles (B and C) in the presence of either verapamil or vinblastine and the absence of accumulation in intracellular organelles when the transporter is allowed to function (A). (×380; *bar*, 10 μm).

cells after 10 days in culture in the absence of either verapamil or Bodipy-verapamil is considered to be 100% survival, and percentage of survival in the presence of drugs is given relative to this figure. The ID_{50} is the concentration of drug that reduced the cloning efficiency of the cells to 50% of the control without drug.

Drugs and chemicals. Vinblastine, colchicine, and racemic verapamil were from Sigma. Bodipy-verapamil, a fluorescent derivative of verapamil, was synthesized by Molecular Probes Inc., starting with an amino analog of verapamil originally described by Theodore *et al.* (21). This derivative possesses the Bodipy fluorophore, which has spectral properties similar to those of fluorescein (Fig. 1).

Accumulation studies. NIH3T3 sensitive cells and MDR cells were incubated with 1 μM Bodipy-verapamil at 37°, with or without 10 μM verapamil or 10 μM vinblastine, in regular medium. After 20 min of incubation, cells were washed in PBS and examined by water immersion optics, using epifluorescence or phase contrast microscopy.

Lysosomal accumulation studies. NIH3T3-MDR cells (on 35-mm plates) were incubated with 10 μM verapamil and 1 μM Bodipy-verapamil at 37°, in regular culture medium. After 15 min, cells on the plates were washed with PBS containing 10 μM verapamil, checked for fluorescence pattern, and divided into two batches. One batch was then incubated with PBS containing 10 μM verapamil (control), while the second batch was incubated with PBS containing 10 μM verapamil and 100 μM chloroquine. After 15 min of incubation at 37°, cells were examined alive, using epifluorescence microscopy.

Results and Discussion

Some tissue culture cells acquire resistance to a variety of unrelated drugs, such as vinblastine, colchicine, Adriamycin, and actinomycin D, by expressing an energy-dependent multidrug transporter (1, 6–10, 19). Selection for resistance to one of these agents results in high level resistance to other lipophilic compounds. These cell lines become fully drug sensitive when cultured in the presence of other pharmacological agents that may also be substrates for the multidrug transporter (22–25). Among these reversing agents, verapamil is a particularly potent competitive inhibitor of the drug transport. In this study, a Bodipy derivative of verapamil has been used as a fluorescent probe to study drug transport.

Single-cell fluorescence studies using Bodipy-verapamil. Epifluorescence and phase contrast microscopy studies performed on NIH3T3 cells incubated in the presence of 1 μM Bodipy-verapamil for 20 min at 37° showed that this compound concentrates in lysosomes. No other sites of concentration of the derivative could be demonstrated. The same incubation performed with the MDR cell line showed that Bodipy-verapamil failed to accumulate in lysosomes at the same level as seen in the sensitive cell line (Fig. 2, A and A′). When incubated

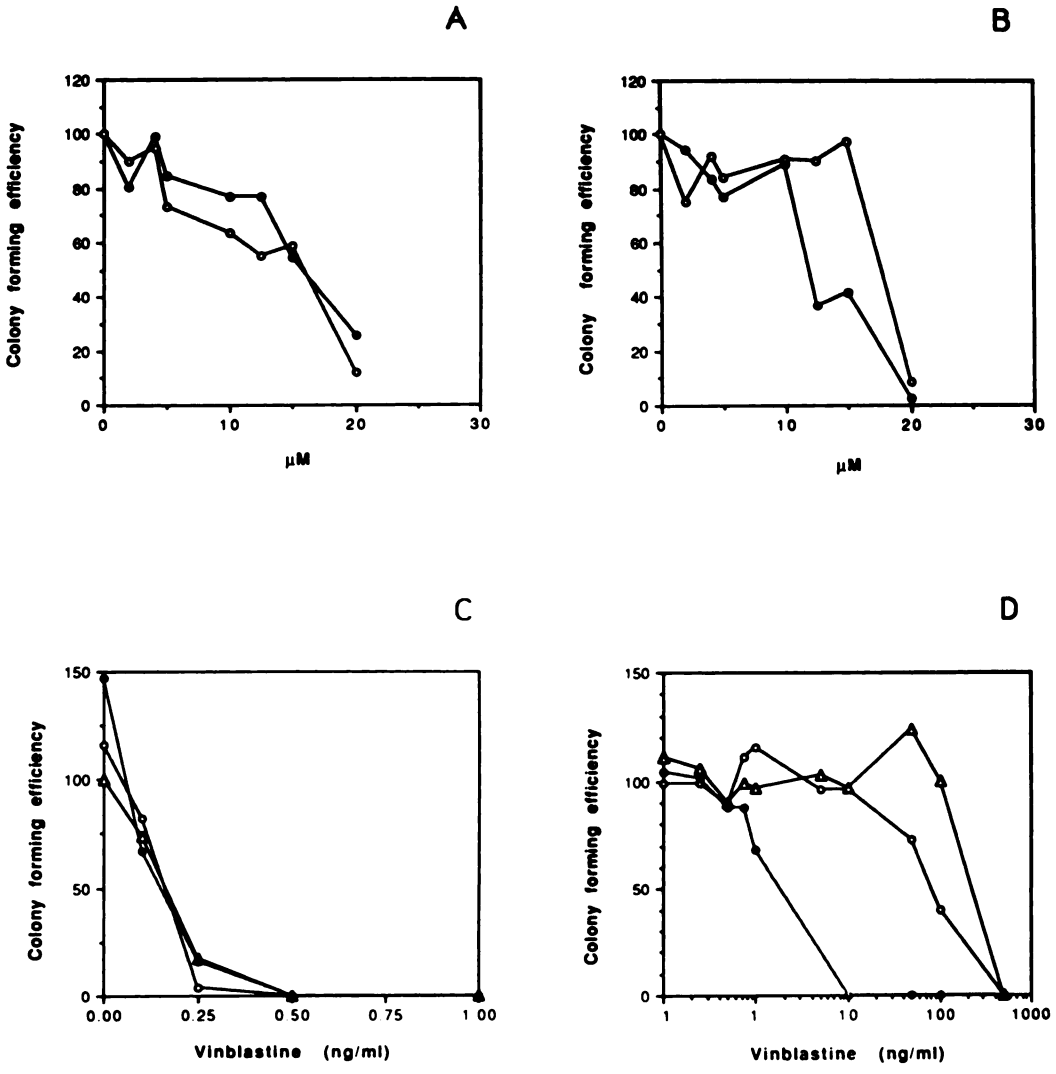


Fig. 3. Cell survival, measured by colony formation, of KB cell lines. KB-3-1 (A) and KB-V1 (B) were plated, as described in Materials and Methods, in the absence of any drug for 16 hr and were grown for 10 days after addition of various concentrations of either verapamil (●) or Bodipy-verapamil (○). After determination of the level of toxicity of Bodipy-verapamil, another set of KB-3-1 (C) and KB-V1 (D) cells were plated under the same conditions and were grown in the presence of a constant 10 μM concentration of either verapamil (●) or Bodipy-verapamil (○), or without either of these compounds (△), in combination with various concentrations of vinblastine.

in the presence of both 10 μM verapamil (corresponding to its ID_{50} for NIH3T3 cells) and 1 μM Bodipy-verapamil, the MDR cells showed bright lysosomal labeling (Fig. 2, B and B′), suggesting that the multidrug transporter was blocked by the excess of verapamil, allowing the fluorescent Bodipy-verapamil to remain in the cytosol. Evidence for lysosomal accumulation of Bodipy-verapamil in the presence of verapamil was achieved by increasing the intralysosomal pH with chloroquine. The bright lysosomal pattern observed in the control experiment (NIH3T3-MDR cells incubated with a combination of verapamil and Bodipy-verapamil, followed by an incubation with verapamil alone) disappeared when chloroquine was added to the second incubation (data not shown). The same accumulation of Bodipy-verapamil in lysosomes was seen with a combination of Bodipy-verapamil (1 μM) and vinblastine (10 μM) (Fig. 2, C and C′), consistent with competition of the two compounds for the transport process.

Biological effects of Bodipy-verapamil. The biological effects of the Bodipy derivative of verapamil have also been studied in two different types of MDR cells (KB-V1 and NIH3T3-MDR) and their drug-sensitive counterparts (KB-3-1 and NIH3T3, respectively) (Figs. 3 and 4). Killing curves to determine toxicity of both compounds show that, for both NIH3T3 cell lines, the Bodipy derivative is more toxic than verapamil (Fig. 4, A and B), whereas the opposite effect is noted for the KB MDR cell line (Fig. 3B).

A concentration of Bodipy-verapamil resulting in 75% cell survival (2 μM and 10 μM for NIH3T3 and KB cells, respec-

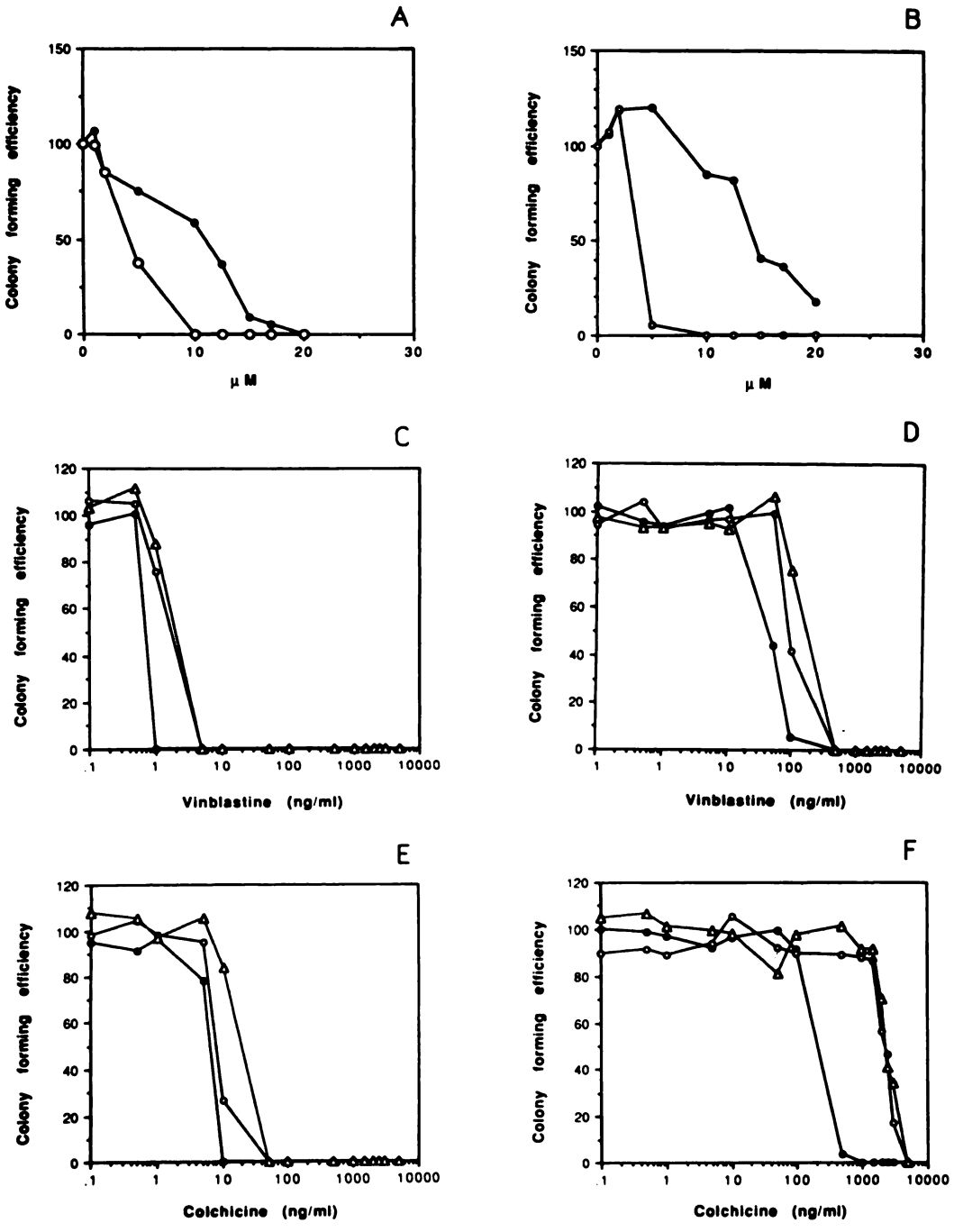


Fig. 4. Cell survival, measured by colony formation, of NIH3T3 cell lines. NIH3T3 drug-sensitive (A) and NIH3T3-MDR cells (B) were plated, as described in Materials and Methods, in the absence of any drug. After 16 hr of growth, various concentrations of either verapamil (●) or Bodipy-verapamil (○) were added, to determine the level of toxicity. A second set of drug-sensitive (C and E) and drug-resistant (D and F) cell cultures were grown in the presence of 2 μM verapamil (●) or Bodipy-verapamil (○), or without either of these drugs (△), in combination with various concentrations of vinblastine (C and D) or colchicine (E and F).

tively) was chosen to test its ability to reverse resistance of MDR cells to various concentration of *Vinca* alkaloids. The same concentration of verapamil was used as a reference standard. Cells were treated with either verapamil or its Bodipy derivative, in combination either with vinblastine (KB and NIH3T3 lines) or with colchicine (NIH3T3 lines). Control killing curves were determined with Bodipy-verapamil or with verapamil alone. The results show that, whereas in the sensitive KB cell line there was no difference in cell survival in the presence of either verapamil or its Bodipy derivative (Fig. 3C), verapamil reversed resistance of the MDR cell line, but Bodipy-verapamil had only a small reversing effect. For the NIH3T3-MDR cells, Bodipy-verapamil did not reverse drug resistance to either vinblastine or colchicine (Fig. 4, D and F). This difference in reversing ability may be related to the fact that NIH3T3-MDR cells were transfected with an *MDR*1 cDNA carrying a mutation at position 185 (Gly→Val), which affects substrate specificity of the multidrug transporter (26).

Conclusion

Taken together, these results suggest that the addition of the bulky Bodipy group to verapamil still allows its efflux by MDR cells. However, this Bodipy group must interfere with the ability of the compound to serve as an effective competitive inhibitor of the transporter for cytotoxic drugs such as colchicine and vinblastine. One hypothesis accounting for this result is that Bodipy-verapamil has a lower affinity for the drug binding site, resulting from steric hindrance from the Bodipy group.

The higher cytotoxicity exhibited by Bodipy-verapamil *per se* may result from either lysosomal (amidases) or microsomal (cytochrome P-450 multienzymatic system) metabolism of this compound. The lysosomal accumulation of a metabolite may occur in experiments performed over a long period of time, such as killing curves. Because fibroblastic cells, such as NIH3T3, and most of the transformed cells do not possess the cytochrome P-450 drug metabolism system, production of a metabolite could only result from lysosomal hydrolysis. However, for fluorescence measurements, which are performed in a short period of time, hydrolysis of Bodipy-verapamil by amidases seems unlikely, because these enzymes have a low hydrolytic activity, which should not allow significant metabolism of Bodipy-verapamil during the incubation time used to detect lysosomal fluorescence.

Taken together, these data show that Bodipy-verapamil is a useful agent for imaging of lysosomes in drug-sensitive cells. The most interesting feature of this fluorescent compound, when used in combination with verapamil, is its accumulation in lysosomes, which allows a simple and rapid screening of various cell types for expression of a multidrug transport system.

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