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Fluorescence methods to assess multidrug resistance in individual cells

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Abstract *Purpose*: Microscopic methods to measure the activity of drug extrusion systems important in multidrug resistance in individual cells were developed. *Methods*: Multidrug-resistant (MDR) and parental lines of hamster CHO and pituitary GH_3 cells were incubated with the acetoxymethylester (AM) forms of several fluorescent calcium-sensing dyes, fura2, indo1 and fluo3. The AM forms of these compounds are hydrolyzed by intracellular esterases and then trapped in cells, and the AM forms of the dyes are excellent substrates for P-glycoprotein (Pgp). *Results*: The fluorescent free acid forms of fura2, indo1 and fluo3 did not accumulate in MDR lines unless a chemosensitizer such as cyclosporin A, *R*(+)verapamil, quinidine, or progesterone was included during loading to prevent the cells from extruding the AM forms of the dyes before they could be hydrolyzed. Cyclosporin A increased the fluorescence due to intracellularly trapped fura2 free acid from 8- to 20-fold and was maximally effective at $< 5\ \mu M$. Fluorescence microscopy was employed to measure fura2 free acid accumulation by parental and MDR cell lines using excitation at the Ca^{2+}-insensitive wavelength. When MDR cells were incubated with rhodamine 123 and fura2/AM, no fluorescence was detectable. Cellular fluorescence was dramatically increased by inclusion of cyclosporin A, quinidine, progesterone, or *R*(+)verapamil. There was no measurable decline in the fura2 free acid fluorescence in 1 h while the fluorescence due to rhodamine 123 diminished rapidly in cells overexpressing Pgp. *Conclusions*: These fluorescence methods detect drug-extruding activity in individual cells and therefore have the potential to provide complementary information to studies quantifying protein or mRNA levels of Pgp or other efflux pumps. In addition, they provide a rapid and quantifiable method for screening multidrug resistance reversing agents.

Key words Drug transport · Fura2 · Multidrug resistance · P-glycoprotein

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Introduction

Intrinsic or acquired high levels of expression of P-glycoprotein (Pgp), the human MDR1 gene product, is an important cause of resistance to chemotherapeutic drugs [1, 4, 9, 10, 17]. Pgp, a 170-kDa membrane glycoprotein, mediates this resistance by preventing the accumulation of drugs to cytotoxic concentrations. Substrates for the ATP-driven Pgp pump include some endogenous molecules such as steroid hormones and numerous structurally unrelated xenobiotics. In cell culture models, multidrug resistance conferred by Pgp can be reversed by drugs, termed chemosensitizers or reversing agents, such as verapamil and other calcium channel blockers and cyclosporin A [9, 23]. Overexpression of another group of proteins, multidrug resistance associated proteins or MRPs, can also cause multidrug resistance [4, 17]. Preferred substrates for Pgp are positively charged or neutral hydrophobic molecules, and the pump displays no stereoselectivity, whereas MRP appears preferentially to extrude organic anions from cells.

There have been many studies correlating the overexpression of Pgp with clinical drug resistance, and the limitations in existing methods have been reviewed recently [2]. Pgp protein has been quantified by Western blot or immunocytochemistry, Pgp mRNA by in situ hybridization, Northern blot, RNase protection or RT-PCR, and Pgp activity by the uptake of drugs, most often rhodamine 123. All of these methods have

limitations, and to date, identification of cells with very high overexpression has proven more reliable than identification of cells with lower levels of Pgp.

Several fluorescent dyes normally used for calcium imaging or viability studies have been used to quantify drug extrusion activity in cell lines overexpressing Pgp or MRP by both fluorimetry and fluorescence activated cell sorting [11–14, 22]. Both of these methods require relatively large numbers of cells and provide population data. We have extended these studies and used microscopy to assess Pgp activity in individual cells. We used both a pituitary cell line, which expresses relatively high endogenous Pgp activity, and CHO cells, which have very low activity, and multidrug resistant (MDR) clones overexpressing Pgp. We showed that drug extrusion activity can be measured microscopically in individual cells, and that the accumulation of intracellularly trapped fluorescent dyes can provide a rapid, simple and quantifiable way to measure potential resistance-reversing agents.

Materials and methods

Materials

Fura2/AM, indo1/AM, fluo3/AM, and the respective free acids were obtained from Molecular Probes (Eugene, Ore.). Tissue culture media and sera were from Grand Island Biological Co. (Grand Island, N.Y.), and tissue culture plasticware was from Corning (Corning, N.Y.). Colchicine, rhodamine 123, quinidine, progesterone, and buffers were obtained from Sigma Chemical Co. (St. Louis, Mo.). *R*(+)verapamil was from Research Biochemicals (Natick, Mass.) and cyclosporin A (Sandimmune™) from Sandoz Pharmaceutical (East Hanover, N.J.).

Methods

Lines from the GH_4C_1 subclone of the GH_3 line were selected for resistance to colchicine at 1.5 or 3.0 μg/ml. These lines, which are designated GH_4C_1/RC1.5 or GH_4C_1/RC3.0, have been described previously [16]. CR1R12 cells, CHO cells selected for resistance to colchicine [21], were obtained from Dr. Alan E. Senior at the University of Rochester. The MDR lines and parental lines, GH_3 and Aux B1 line of CHO cells, were grown as previously reported [16, 21]. Cells were grown on 100-mm petri dishes or on #1 glass coverslips for microscopy. For use with the pituitary cell lines, coverslips were coated with 13 μg of CellTac (Collaborative Biomedical Products, Bedford, Mass.) for 30 min at room temperature and cells were plated 24–48 h prior to use.

To load cells with dye for fluorimetry, cells on petri dishes were rinsed twice and gently scraped or pipetted into 5 ml Hanks' balanced salt solution supplemented with 15 m*M* HEPES, pH 7.4 (HBSS). Cells were pelleted by centrifugation and resuspended in HBSS containing fluorescent dye and drugs at the concentrations noted in the text for 30 min at room temperature or 37 °C, as noted. Dye-loaded cells were washed in 10 ml HBSS and then resuspended in 2 ml fresh HBSS at 37 °C and transferred to a thermostatted cuvette with a magnetic stirbar. Fluorescence was recorded and cells were then lysed with 50 μ*M* digitonin to obtain F_{max}, and 7.5 m*M* EGTA (pH 8.3) was added to obtain F_{min}. Total dye loading was calculated as $F_{max} - F_{min}$. In all cases the results shown were obtained from replicate plates measured in a single experiment. Experiments routinely included controls of cells incubated with no drug or with 42 μ*M* (50 μg/ml) cyclosporin A, a maximally effective concentration, and all experiments were replicated with essentially the same results.

For fluorimetric experiments, the excitation and emission wavelengths were 340/493, 355/410 and 488/535 nm for fura2, indo1 and fluo3. Effects of Pgp inhibitors were found to be the same using 340, 360 and 380 nm excitation for fura2 and using 410, 440 and 475 nm emission for indo1. For microscopy, calcium-independent wavelengths were used. Autofluorescence was very low and was subtracted. Intracellular free calcium concentrations were calculated as described previously [15] and were found to be the same in control and MDR pituitary cells (217 ± 41 vs 243 ± 47 n*M*) and not significantly altered by 42 μ*M* cyclosporin A (331 ± 51 n*M* for MDR cells).

For intracellular imaging, cells on coverslips were loaded with dyes as described in the text, and the coverslips were rinsed, placed in a chamber maintained at 25 °C, and bathed in HBSS + HEPES with or without cyclosporin A. Images were captured on a Nikon inverted fluorescence microscope using a Dage CCD 72 camera (Dage MTI, Michigan City, Ind.) and a Geniisys image intensifier system and Image I/AT image processing software from Universal Imaging (Media, Pa.). Fura2 fluorescence was measured using an excitation filter at the calcium-independent wavelength (360 nm) and a standard fura2 emission filter. Rhodamine 123 and fluo3 fluorescence were measured using a standard narrow bandpass rhodamine filter set. Bleedthrough was negligible. All fura2 images and all rhodamine 123 images for a given cell line were directly comparable. In every figure, control and experimental images were obtained identically from replicate coverslips in the same experiment. All of the results were replicated in multiple independent

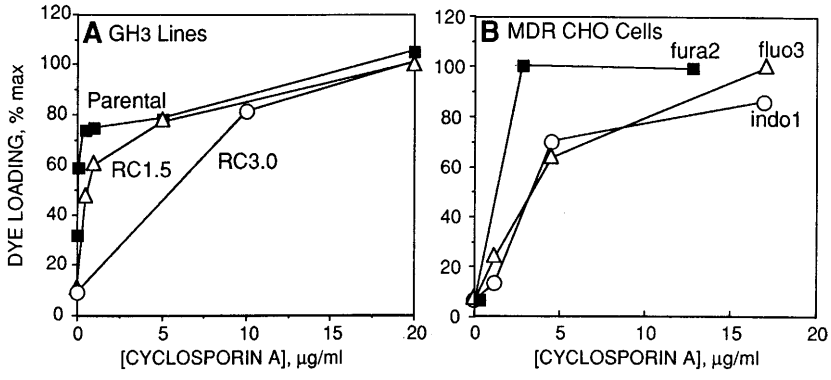


Fig. 1A,B Effect of cyclosporin A on dye loading. Cells were incubated with 2 μ*M* fura2/AM, 5 μ*M* indo1/AM or 5 μ*M* fluo3/AM for 30 min at 37 °C in HBSS containing the concentrations of cyclosporin A shown. Fluorescence is expressed relative to the maximum, 100%, obtained in each experiment for an identical sample incubated with 42 μ*M* cyclosporin A. **A** ■ Parental GH_3 cells, Δ GH_3 cells resistant to 1.5 μg/ml colchicine (RC1.5), or ○ GH_3 cells resistant to 3.0 μg/ml colchicine (RC3.0). **B** Accumulation of the free acid forms of (■) fura2, (○) indo1 or (△) fluo3 by CR1R12 cells (MDR CHO cells)

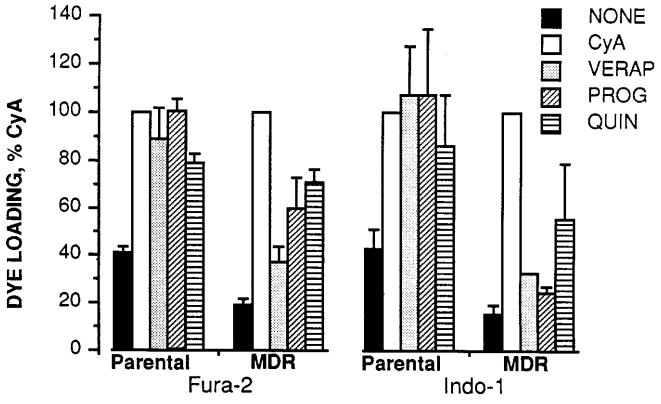


Fig. 2 Effect of multidrug resistance reversal agents on fura2 and indo1 free acid accumulation by rat pituitary cells. GH_3 (*parental*) or GH_4C_1/RC3.0 cells (*MDR*) were loaded with 4 μ*M* fura2/AM or 6 μ*M* indo1/AM for 30 min at 37 °C in HBSS containing: no drug (*NONE*), 42 μ*M* cyclosporin A (*CyA*), 50 μ*M* *R*(+)verapamil (*VERAP*), 50 μ*M* progesterone (*PROG*), or 50 μ*M* quinidine (*QUIN*). Fluorescence (mean ± range or SE of two or three determinations) is expressed relative to the maximum, 100%, obtained in the presence of 42 μ*M* cyclosporin A

experiments. Fluorescence in individual cells was measured on a scale of 0–255 after transferring images into Adobe Photoshop and subtracting the background of adjacent areas without cells.

Results

The neutral acetoxymethylester (AM) forms of calcium-sensing dyes such as fura2 cross the plasma membrane by passive diffusion and are hydrolyzed by intracellular esterases to anionic, calcium-sensitive forms that become trapped in the cells [15]. The AM forms of these drugs, which are not fluorescent, are pumped out of the cell by Pgp [13]. Accumulation of the fluorescent, anionic fura2 free acid provides a stable measure of Pgp activity and can be measured at wavelengths where fluorescence intensity is independent of calcium concentration.

We have described MDR lines derived from rat pituitary GH_3 cells which display typical properties of MDR cells: sensitivity to multiple drugs that is reversed by verapamil, reduced accumulation and accelerated efflux of rhodamine 123, and overexpression of Pgp measured immunocytochemically [16]. MDR pituitary cells failed to accumulate the intracellular Ca^{2+} indicator fura2 free acid unless cyclosporin A, an effective inhibitor of Pgp, was included in the incubation with fura2/AM (Fig. 1A). Cyclosporin A increased fura2 free acid fluorescence between 5- and 12-fold for colchicine-resistant GH_3 cell lines. Like many endocrine cells [3], pituitary cells display endogenous Pgp activity, and

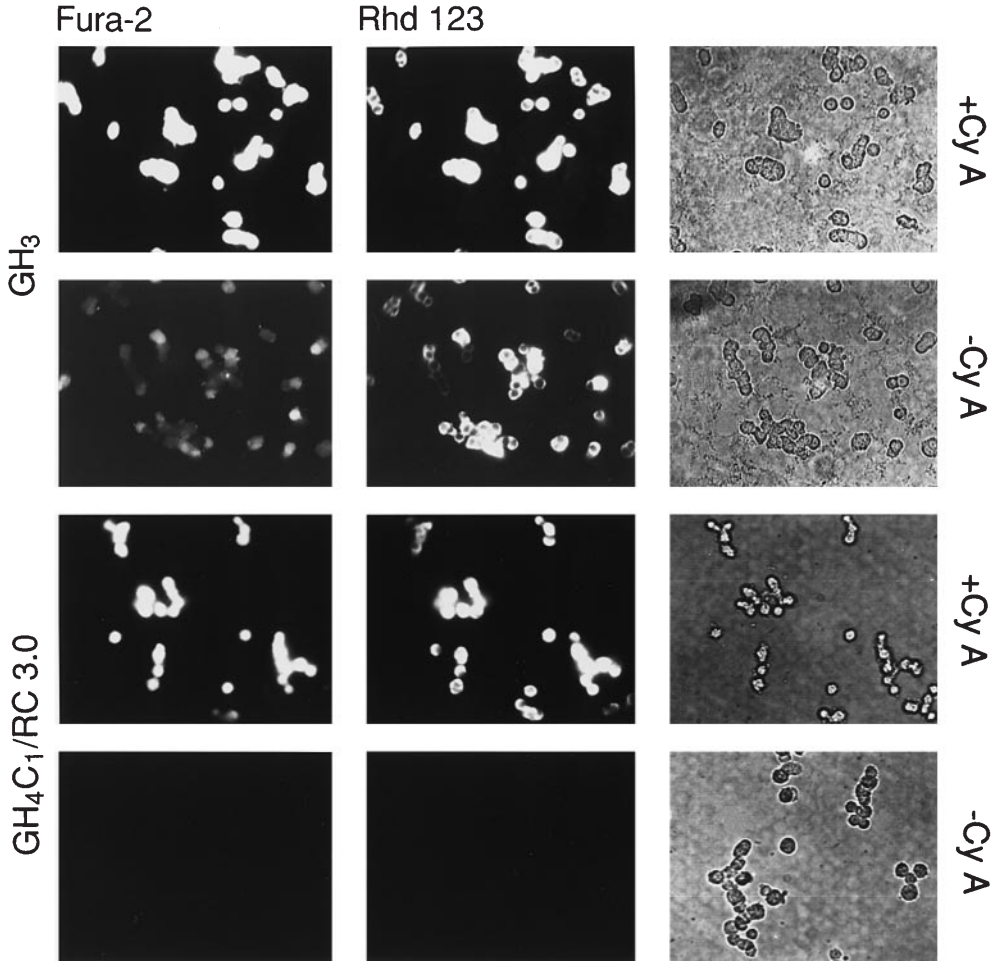


Fig. 3 Effect of cyclosporin A on fura2 and rhodamine 123 fluorescence in parental and multidrug resistant cells. Cells grown on coverslips were loaded with 250 ng/ml rhodamine 123 and 10 μ*M* fura2/AM for 30 min at 37 °C, with or without 42 μ*M* cyclosporin A. GH_3 and MDR GH_4C_1/RC3.0 cells were washed and fura2 fluorescence images were captured using excitation at 360 nm, the isofluorescence point and calcium-insensitive wavelength, and standard fura2 emission filters. Rhodamine images were obtained using narrow bandpass rhodamine filters. Each row depicts the same field of cells

cyclosporin A increased fura2 free acid accumulation two- to threefold even in the parental cell line. Since fura2 free acid can be extruded from some types of cells by an organic anion exchanger [5], we tested the effect of 2 m*M* probenecid, an inhibitor of the exchanger, and showed that it had no effect on fluorescence in MDR pituitary cells incubated with fura2/AM (data not shown). Together, these results indicate that cells overexpressing Pgp were extruding fura2/AM, but not fura2 free acid. Known chemosensitizers [9, 23], including cyclosporin A, *R*(+)verapamil (which is inactive at calcium channels), quinidine, and progesterone all increased loading of the fluorescent free acid forms of fura2 and another ratiometric Ca^{2+} indicator, indo1 (Fig. 2). *R*(+)verapamil, quinidine and progesterone were equally effective at increasing dye loading in the parental GH_3 cells, which express a low level of Pgp, but cyclosporin A was the most effective in the colchicine-resistant line. Without a pump inhibitor, a MDR line derived from CHO cells that expresses a high level of Pgp, CR1R12, accumulated essentially no fura2 free acid. Cyclosporin A increased fluorescence due to the free acids of fura2, indo1, and fluo3, a non-ratiometric Ca^{2+} dye, 5–16-fold for the MDR CR1R12 cells (Fig. 1B).

Fura2 free acid accumulation also served as a stable marker of Pgp activity at the single cell level. We incubated cells simultaneously with rhodamine 123, which is frequently used as a fluorescent substrate for the Pgp pump [6, 19, 24], and fura2/AM, and then measured the fluorescence of the two dyes at appropriate wavelengths (Fig. 3). When the drug-resistant GH_4C_1/RC3.0 cells were incubated without a pump inhibitor, there was no visible rhodamine 123 or fura2 fluorescence. Loading was dramatically increased by inclusion of cyclosporin A. In the absence of cyclosporin A, the parental GH_3 cells accumulated rhodamine 123 and fura2 free acid, although there was marked cell to cell heterogeneity. Addition of cyclosporin A (Fig. 3) or *R*(+)verapamil (data not shown) during loading increased the accumulation of both dyes and produced more uniform staining. Several Pgp inhibitors (cyclosporin A, quinidine, *R*(+)verapamil and progesterone) increased the accumulation of fura2 free acid by CRIR 12 cells (Fig. 4) or GH_4C_1/RC3.0 cells (data not shown). Similar findings were obtained with parental and MDR CHO cells (data not shown).

One potential complication to using trapped dye as an index of drug extrusion activity is the possibility that a cell lacking the cytoplasmic esterase necessary to hydrolyze the AM form of the dye or a leaky cell will be scored as Pgp-positive. This possible problem was avoided by exposing cells sequentially to two different esterified dyes, the first (fura2/AM) in the absence of a chemosensitizer, and the second (fluo3/AM) in the presence of cyclosporin A. The spectra of the free acid forms of these two indicators permit dual fluorescence imaging. Leaky or esterase-defective cells should not

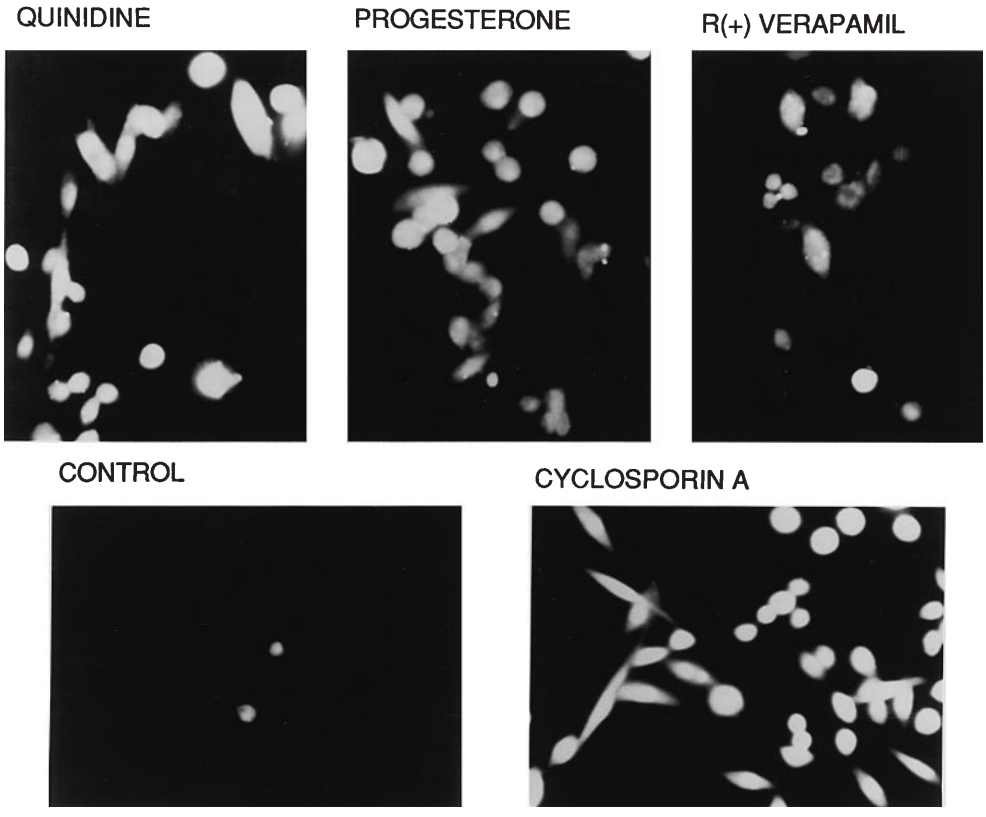


Fig. 4 Effect of multidrug resistance reversal agents on fura2 fluorescence of CR1R12 cells. CR1R12 cells grown on coverslips were loaded with 4 μ*M* fura2/AM for 30 min at 37 °C together with: no drug, 42 μ*M* cyclosporin A, 50 μ*M* *R*(+)verapamil, 50 μ*M* quinidine, or 50 μ*M* progesterone. Cells were washed and images captured following excitation at 360 nm, the calcium-insensitive wavelength

accumulate either dye. Non-MDR cells accumulated both dyes, although there was considerable variability in the intensity of fluorescence in individual cells. For control cells, fluo3 fluorescence averaged 102 ± 49 (mean ± SD, $n = 20$, arbitrary 0–255 scale) with a range of 52–241 in untreated dishes and was not significantly different (84 ± 48, range 35–238, $n = 18$) in cyclosporin A-treated dishes. MDR cells did not accumulate fura2 free acid, because Pgp extruded the AM form of the dye, but did accumulate the second dye (fluo3 free acid) when Pgp was blocked (Fig. 5). For MDR cells, fluo3 fluorescence averaged 45 ± 32 (range 12–115, $n = 16$) in untreated dishes and 213 ± 24 (range 115–226, $n = 24$) in cyclosporin A-treated dishes ($P < 0.001$). The ability of a chemosensitizer to increase accumulation of Pgp substrates in an individual cell over a short period of time would not be expected to vary with differences in cell viability or intrinsic esterase activity.

The advantage of a trapped dye over rhodamine 123, which is also a Pgp substrate, is evident in Fig. 6, which shows rates of decline of fluorescence from intracellular fura2 free acid and rhodamine 123, presumably reflecting drug efflux. MDR cells were loaded with rhodamine 123 and fura2/AM in the presence of cyclosporin A, since there was no measurable fluorescence otherwise. Cells were then incubated in buffer with or without cyclosporin A. Rhodamine 123 fluorescence diminished rapidly in CR1R12 cells, while over the same time period there was no measurable decline in the fura2 free acid fluorescence (Fig. 6). Inclusion of cyclosporin A in the observation period largely prevented the decrease in rhodamine fluorescence, but did not alter the fluorescence due to fura2 free acid. Similar results were seen for MDR rat pituitary cells (data not shown). This again shows that once fura2/AM is hydrolyzed it is no longer a substrate for Pgp, and also shows that the method allows the assessment of drug extrusion activity over a period of at least several hours, whereas rhodamine 123 fluorescence is only measurable over minutes.

Discussion

Intracellularly trapped fluorescent dyes have properties that make them useful markers for drug resistance caused by overexpression of drug extrusion pumps. The AM forms of fura2, BCECF, calcein and fluo3 are substrates for Pgp encoded by the human MDR1 [11, 13], and both the ester and free acid forms of calcein are reported to be substrates for MRP [12]. It has previously

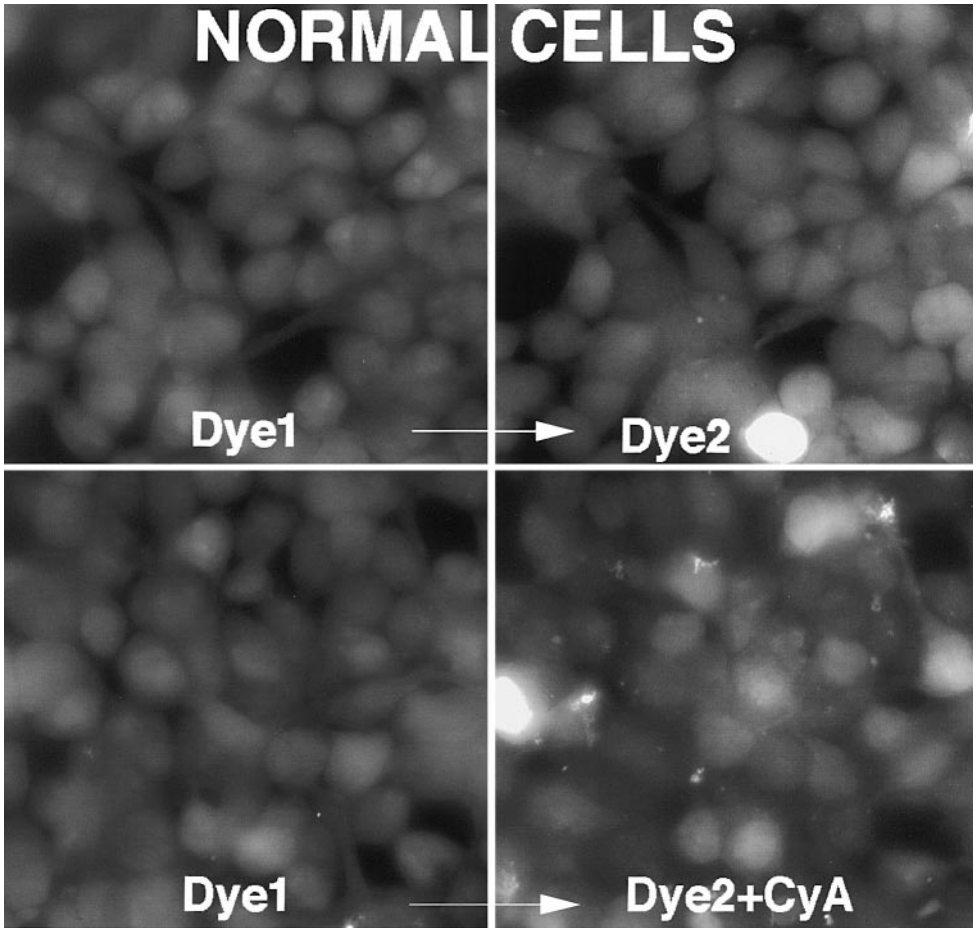
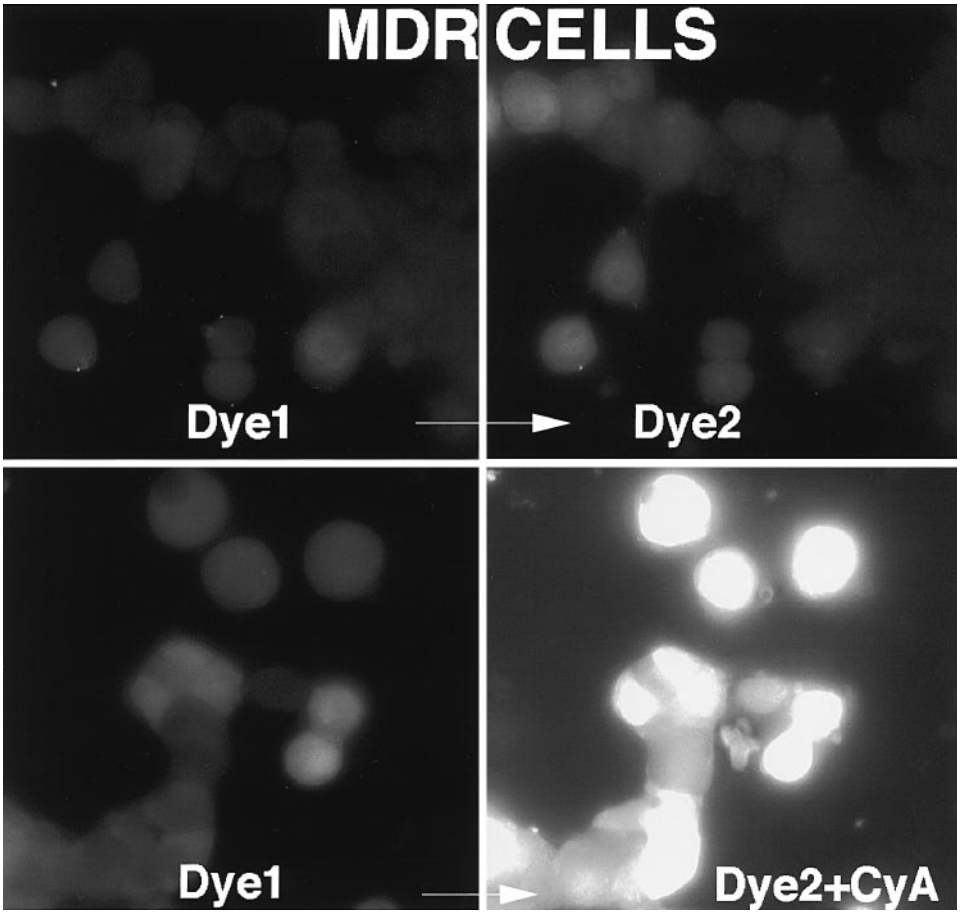


Fig. 5 Sequential staining for drug extrusion activity. Either parental or drug-resistant GH_3 cells on coverslips were loaded with 4 μ*M* fura2/AM for 30 min at room temperature, and washed, and fura2 images were captured using excitation at 360 nm and standard fura2 emission filters. The cells were then incubated with 5 μ*M* fluo3/AM in the presence of 5 μg/ml cyclosporin A for 30 min at room temperature, and washed, and fluo3 free acid fluorescence images were captured using narrow bandpass rhodamine filters. There was no appreciable bleedthrough

Fig. 5 (*Contd.*)



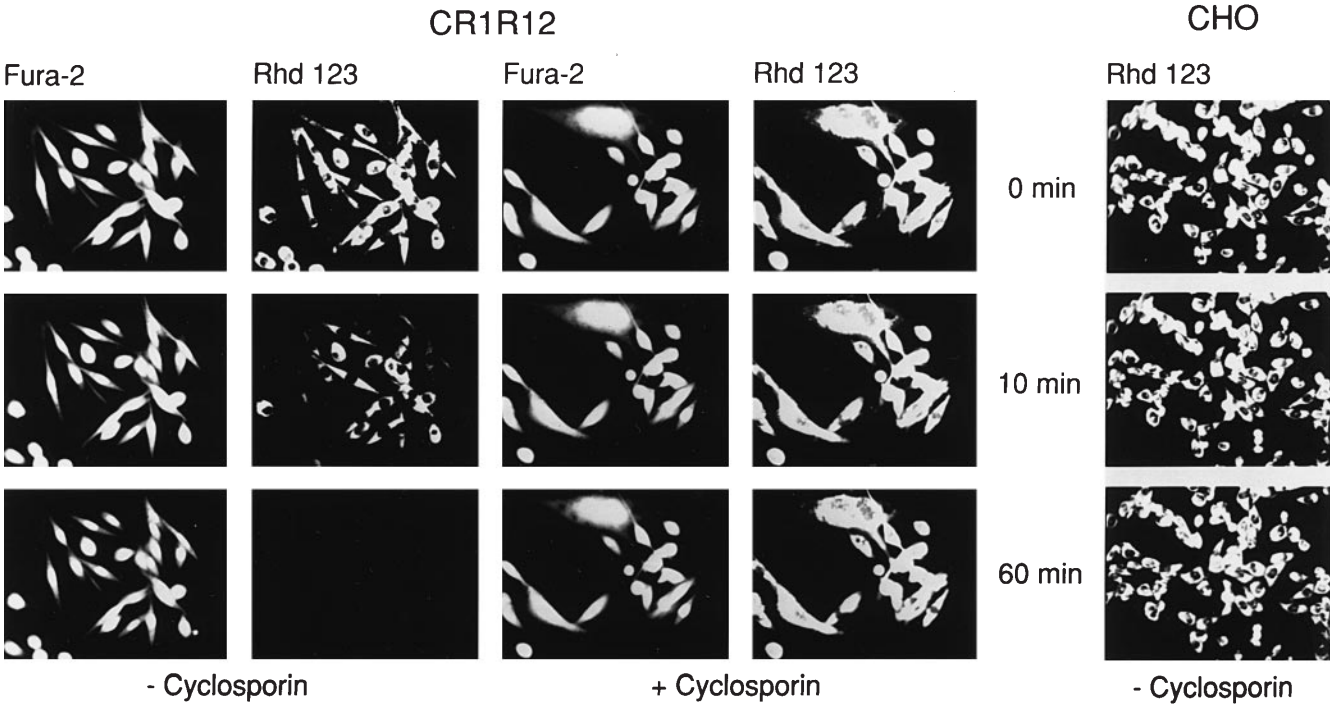


Fig. 6 Efflux of fura2 free acid and rhodamine 123 from CHO and CR1R12 cells. CHO or CR1R12 cells were loaded with 250 ng/ml rhodamine 123 and 10 μ*M* fura2/AM for 30 min at 37 °C with 42 μ*M* cyclosporin A. Cells were washed and then incubated at 25 °C for 0–60 min, as noted on the figure, in HBSS or HBSS containing 42 μ*M* cyclosporin A. Images were captured at intervals; those obtained at 0, 10, and 60 min are shown

been shown that accumulation of these dyes can be used to quantify the activity of Pgp or MRP in cell populations [11–14]. We have shown that the use of these dyes in conjunction with fluorescence microscopy can provide information about Pgp activity on a single cell level, and drug extrusion activity can be correlated with morphology or other characteristics of individual cells. The method was applicable in two model systems, one expressing significant endogenous Pgp activity (a model for tissues such as kidney and adrenal tissue) and one expressing very little (a model for most other tissues) [7, 18, 20]. Effects on intracellular calcium were not noted, but in any case they would not have complicated interpretation as long as data were collected at calcium-insensitive wavelengths. Sequential staining without and with a chemosensitizer can be carried out to rule out either the lack of a cytoplasmic esterase or general cell leakiness as causes of a failure to accumulate the fluorescent free acid forms of the dyes. In principle, it should be possible to determine whether the failure of a cell to accumulate a dye is due to overexpression of Pgp or MRP by using selective inhibitors of the two pumps in sequential staining protocols. Multidrug resistance can also be inferred if cells fail to accumulate normal amounts of a dye such as rhodamine 123. Since rhodamine 123 is an excellent substrate for Pgp, it does not accumulate in cells overexpressing Pgp unless a chemosensitizer is present. However, use of rhodamine123 accumulation to assess drug resistance is limited because it is extruded rapidly from cells and monitoring must be done quickly, because it is sensitive to membrane potential and because it eventually accumulates in mitochondria. The use of trapped dyes, as shown here, largely circumvents these problems. Nonetheless, estimation of drug extrusion activity will always require a preparation of live cells, which is easier to attain with leukemias and lymphomas than with solid tumors.

The potency of a compound to reverse drug resistance is measured by determining the concentrations of the drug needed to reduce the LD_{50} of a cytotoxic agent in cultured MDR cells. Screening potential chemosensitizers in this fashion is labor intensive and time consuming, and data interpretation can be complicated by drug effects at sites other than Pgp. The ability of a compound to increase ATPase activity attributable to Pgp provides a direct measurement of drug interaction at the pump, but this approach requires highly enriched preparations containing Pgp. Furthermore, a number of drugs known to be extruded by Pgp do not increase ATP hydrolysis by purified Pgp [21]. The dose-response curve for increased accumulation of an intracellularly trapped fluorescent dye in MDR cells provides a rapid, simple, inexpensive, and quantitative method to screen compounds for potency as inhibitors of Pgp activity in an intact cell.

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