Cell Preparation:

Cryopreserved cells were thawed quickly by placing them in a 37°C water bath. After thawing, cells were transferred to 15ml tubes and the volume brought up to 15 mls of media that contained RPMI 1640 media, 10% FCS, 4μM $MgSO_4$, 5u/ml Heparin and 70u/ml DNAse. After allowing the tubes to sit at room temp for 10 minutes, the samples were then centrifuged for 4 minutes at 2500rpm and 18ºC, decanted, and resuspended in 2ml fresh media supplemented with 10% FCS.
Cells were then counted using a Coulter counter, and viability was determined using the Trypan Blue exclusion assay. Samples with viabilities less than 40% were considered inevaluable and discarded.

Drugs and modulators

Amonafide malate was provided by Xanthus, Inc. Daunorubicin, Idarubicin, Mitoxantrone, and Rhodamine 123 were purchased from Sigma-Aldrich, St Louis, MO. $DiOC_2(3)$ was obtained from Molecular Probes (Carlsbad, California) and Pheophorbide A from Dr Alan Oseroff (RPCI, Buffalo NY).

Stock solutions of all six drugs were prepared in the appropriate solvent at a concentration of 1 mg/ml, and with the exception of $DiOC_2(3)$, were then frozen at -20ºC. $DiOC_2(3)$ was stored at 4ºC. Solvents included DMSO for Amonafide Malate and $DiOC_2(3)$, 100% Ethanol for Rhodamine 123, and PBS for Mitoxantrone, Idarubicin, and Daunorubicin. Compounds were used at the following final concentrations; Amonafide 10μm, Daunorubicin, Idarubicin and Mitoxantrone all at 3μm, PheophorbideA 10μm, and $DiOC_2(3)$ at 6ng/ml and Rhodamine-123 at 200ng/ml.

Modulators were also prepared at stock concentrations of 1mg/ml and frozen at -20ºC. PSC-833 (Novartis, East Hanover, NJ), specific for Pgp was dissolved in 100% Ethanol and used at a final concentration of 2.5μm. MK-571 (Calbiochem, San Diego, CA), specific for MRP-1, in DMSO, was used at a final concentration of 15μm. Fumitremorgin C (FTC) (from Dr. Susan Bates, National Cancer Institute, Bethesda, MD), specific for BCRP, was also prepared in DMSO, and used at 10μm. Lastly, cyclosporin A (CsA) (Sigma-Aldrich, St Louis, MO), which modulates all three proteins, was prepared in 100% Ethanol and used at a final concentration of 2.5μm.

MDR protein expression and function:

Cellular uptake

Briefly, uptake of the six fluorescent substrates was performed by incubating $1 \_ 10^6$ cells in 2ml of RPMI-1640 medium (supplemented with 10% FBS) containing each drug alone, or in combination with the modulator at the desired final concentrations, for 30 min at 37°C. Cells were then washed twice using 2mls ice-cold PBS, spun down, resuspended in 500µl cold PBS and placed on ice. Drug-associated fluorescence was measured by flow cytometry using a FacScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped in standard fashion with an Argon laser for 488 nm excitation and a 585/42 band-pass filter (FL2) or a 670 long-pass (FL3) filter for emission collection. Data were analyzed with WinList software (Verity Software House, Topsham, ME).

Expression

Pgp expression was studied on unfixed cells with the MRK16 monoclonal antibody and by comparing it to it's Isotype control IgG2a (Kamiya Biomedical Company, Tukwila, WA). MRP-1 and BCRP expression was determined in fixed cells with the MRPm6 and BXP-21 monoclonal antibodies (Kamiya) and compared to their Isotype controls which are IgG1 and IgG2a respectively. The concentrations of antibodies used were determined by previous titers (serial dilutions of antibody are made, above and below the manufacturers recommended concentration, and are tested on cell lines expressing the protein of interest, and the concentration that results in less than 10% reduction of the maximum achievable signal is used. Isotype concentrations are matched to the antibody titer by IgG protein concentrations).

Briefly, for Pgp, $1 \_ 10^6$ cells in 50µls PBS were incubated on ice for 20 min with antibody or Isotype, then washed with 2mls PBS, spun down, decanted, and resupended in the residual volume. Following blocking with normal Goat IgG (Caltag, California) for 10 minutes the secondary antibody, Phycoerythrin- goat anti-mouse was added for 20 minutes. Cells were then washed as previously with PBS, resuspended in 500µl PBS, and kept for analysis on ice, in the dark, by flow cytometry.

As both MRPm6 and Bxp21 antibodies bind to internal epitopes, cells needed to be first fixed and permeabilized. This was accomplished by incubating cells in 1 ml of 4% formaldehyde at room temperature for 10 minutes, washing in 2 mls wash buffer (PBS, 0.1% triton x-100) centrifuging, decanting , and followed by 10 minute incubation in 1 ml of 90% methanol at room temperature. Cells were washed again in 2 mls wash buffer, as before, at room temperature, spun down and pelleted, and resuspended in residual volume. Antibodies or isotypes were added according to previous titer and incubated for 40 minutes at room

temperature, washed in 2mls PBS, spun down, pelletted, and resuspended in the residual volume, and a secondary antibody FITC goat anti mouse added for 40 minutes (again the amount was determined by previous titer). Cells were then washed and resuspended in 500µl PBS. Fluorescence was measured using the same FacScan flow cytometer as previously described.