JPET Fast Forward. Published on January 5, 2005 as DOI: 10.1124/jpet.104.072033

Efflux of Depsipeptide FK228 (FR901228, NSC-630176) is mediated by both P-glycoprotein and MRP1

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Running title: Depsipeptide FK228 efflux mediated by P-glycoprotein, MRP1

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Number of text pages: 26 (including abstract and references)

Number of figures: 12

Number of tables: 1

Number of references: 40

Number of words: *Abstract*: 246; *Introduction:* 514; *Discussion*: 976

Abbreviations: MRP1, multidrug resistance-associated protein 1; MRP2, multidrug resistance-associated protein 2; P_{app}, apparent permeability coefficient; T_{eff}, efflux transport efficiency; HAT, histone acetyl transferase; HBSS, Hanks' balanced salt solution; DMEM, modified Eagle's medium, D-PBS, Dulbecco's phosphate-buffered saline; TEER, transepithelial electrical resistance; ESI, electrospray ionization

RECOMMENDED SECTION ASSIGNMENT: Absorption, distribution, metabolism, and excretion

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Abstract

Depsipeptide FK228, a novel histone deacetylase (HDAC) inhibitor, was previously reported to be a P-glycoprotein (Pgp) substrate. We now expand the investigation to demonstrate that FK228 is a substrate for both Pgp and multidrug resistance-associated protein 1 (MRP1). Transport of FK228 across the Caco-2 cell monolayer in both apical to basolateral (AP→BL) and basolateral to apical (BL→AP) directions in the absence and presence of Pgp and MRP inhibitors were investigated. An *in vitro* uptake study in human red blood cells (RBC) and a cytotoxicity assay in MRP1(-) HL60 and MRP1(+) HL60Adr cells were conducted to show that FK228 is a MRP1 substrate. A FK228 resistant cell line (HCT-15R) was developed from HCT-15 colon carcinoma, and characterized using a 70-oligomer cDNA microarray, RT PCR, western blot, histone acetyltransferase (HAT) and HDAC activity assays, and cytotoxicitiy assays. FK228 showed a nearly unidirectional flux across the Caco-2 cell monolayer, with the BL→AP apparent permeability coefficient 32x that of AP→BL without apparent saturation. Pgp inhibition decreased the BL→AP P_{app} and increased the AP→BL P_{app}. RBC showed a concentration-dependent uptake and a saturable efflux of FK228. HL60Adr cells were 4-fold more resistant to FK228 than HL60 cells and the resistance was reversed by MRP inhibition. Upregulation of Pgp, but not changes of MRPs or HAT/HDAC enzymatic activities, was the major mechanism for the acquired FK228 resistance. These studies demonstrate that FK228 is a substrate for both Pgp and MRP1, and reversible Pgp upregulation is predominantly involved in FK228 resistance *in vitro*.

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Introduction

Depsipeptide FK228, formerly FR901228 and NSC 630176 (Fig. 1), is a novel, naturally occurring bicyclic peptide with a disulfide linkage isolated as a fermentation product of *Chromobacterium violaceum* (Shigematsu et al., 1994; Ueda et al., 1994a; Ueda et al., 1994b). It was originally developed as an anti-ras compound (Ueda et al., 1994c; Wang et al., 1998), later found to interfere with mitogen induced signaling pathways (Rajgolikar et al., 1998; Sandor et al., 2000a; Sandor et al., 2000b), and more recently has been found to be a potent histone deacetylase (HDAC) inhibitor (Nakajima et al., 1998; Yoshida and Horinouchi, 1999; Yoshida et al., 2001). Because of its observed preclinical antitumor activity, selectivity, and passage in its preclinical toxicology evaluation, FK228 has entered into several phase I clinical trials against various refractory cancers (Marshall et al., 2002; Sandor et al., 2002), and now being planned into phase II clinical trials.

Preclinical pharmacokinetics studies in rats showed a low oral bioavailability of FK228 (Chan et al., 1997; Li and Chan, 2000). Later, it was reported that FK228 is a P-glycoprotein (Pgp) substrate, but not a Pgp inhibitor, during a NCI screening project (Scala et al., 1997). However, due to the screening nature of the study, no kinetic or quantitative transport data of FK228 was reported.

Multidrug resistance-associated protein 1 (MRP1) is highly expressed in RBC membrane, responsible for the efflux of oxidized GSH (GSSG) to maintain a reducing intracellular environment (Rychlik et al., 2000). MRP1 is also associated with drug resistance and functions as an efflux pump in cancer cells for drug GSH conjugates and

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a variety of structurally unrelated drugs, including some neutral compounds (Paul et al., 1996; Rychlik et al., 2000; Wijnholds, 2002). The overlap of substrates between Pgp and MRP1 (van Zuylen et al., 2000) suggests the possibility of FK228 being a MRP1 substrate. Moreover, expression of MRP2 on the apical membrane of the Caco 2 monolayer (Hirohashi et al., 2000; Sun et al., 2002; Cooper et al., 2004) provides a model to investigate if FK228 shares affinity for MRP2.

Herein, we aimed to further characterize Pgp and/or MRP1 mediated FK228 transport and uptake kinetics using multiple models, including the Caco 2 cell monolayer, human RBC (Rychlik et al., 2000), and Pgp(-)/MRP1(-) HL60 and Pgp(-) /MRP1(+) HL60Adr cell lines (Gollapudi and Gupta, 1992).

HDAC inhibitors cause histone hyperacetylation (Weidle and Grossmann, 2000) and upregulate the expression of various genes including MDR1 (or ABCB1, both gene names of Pgp) (Jin and Scotto, 1998; El-Osta et al., 2002). Since FK228 is a Pgp substrate and a potent HDAC inhibitor, we were interested in determining whether FK228 induces Pgp and MRP1 expression, which in turn, leads to FK228 resistance. For this reason, we utilized human colon carcinoma HCT15 cell line and its resistant daughter cell line, HCT-15R, developed in our laboratory, to study the nature of FK228 resistance. Both cell lines were characterized by a custom 70-oligomer cDNA microarray, RT PCR, western blot, cytotoxicity assay and HAT/HDAC activity assays. Reversibility of the Pgp induction and cross-resistance of HCT-15R cells to other Pgp substrate drugs were also studied.

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MATERIALS AND METHODS

Materials

Non-formulated depsipeptide FK228 (Figure 1, purity >99%) was supplied by the Drug Synthesis and Chemistry Branch, the National Cancer Institute (Bethesda, MD) and used without further purification. N-t-Boc-Met-Leu-Phe (BMLP, purity >97%). Indomethacin were purchased from Sigma Chemical Co. (St Louis, MO). Potassium phthalate buffer (pH 4, 50 mM) was obtained from Van Water and Rogers Scientific (Chicago, IL). MK571 (L 660711), a specific MRP inhibitor, was purchased from BioMol Research Lab., Inc. (Plymouth Meeting, PA). Cyclosporin A (CsA), (±)-verapamil hydrochloride (Ver) and Lucifer Yellow CH were purchased from Sigma. All organic solvents were obtained from Fisher Scientific (Pittsburgh, PA) and were of HPLC grade. HPLC-Grade water (>18 mΩ) was generated with an E-pure water purification system (Barnstead, Dubuque, IA). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM) non-essential amino acids (NEAA), sodium penicillin G, Streptomycin, HEPES buffer, Hanks' balanced salt solution (HBSS), D-PBS and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). Caco 2 and HCT-15 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). HL60 and HL60Adr cells were a generous gift from Dr. Hans Hinderman, Roswell Park, NY.

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Cell cultures

Caco 2 cells were cultured in high glucose DMEM, supplemented with 10% FBS, 25 mM HEPES, 0.1 mM NEAA, 4 mM L-glutamine, 100 U/mL sodium penicillin G and 100 μg/mL streptomycin. HCT-15, HL60 and HL60Adr cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL sodium penicillin G and 100 μg/mL streptomycin. Cells were grown in 75-cm^2 tissue culture flasks at 37°C in a 5% CO_2 atmosphere, and the culture medium was replaced every other day. The adhesive cells were harvested at 80% confluence by exposure to a trypsin-EDTA solution (0.25% trypsin and 0.002% EDTA in Hanks' balanced salt solution). For transport studies, Caco-2 Cells of passages 44 ~ 46 were seeded on collagen-coated 6-well Transwell inserts (0.4-μm pore size, 4.7 cm^2 growth area, Corning Costar Corp., Cambridge, MA) at densities of 5×10^4 cells/cm^2. The medium was changed every other day for the first week and every day thereafter until the transport study (24-27 days post-seeding).

FK228 transport

The Caco-2 cell monolayers were washed twice with warm (37°C) Dulbecco's phosphate-buffered saline (D-PBS, 1×, pH 7.4). For FK228 transport studies without Pgp inhibition, the D-PBS was replaced by the warm HBSS bathing solution (1× HBSS containing 25 mM D-glucose and 10 mM HEPES, pH 7.4). The volumes of HBSS bathing solution at the apical and the basolateral sides of the cell layer were 1.5 mL and 2.5 mL, respectively. The Transwell plates were returned to the incubator to equilibrate at 37 °C for 30 min. The HBSS bathing solution was removed and the Transwell inserts were transferred to a clean 6-well plate. Warm HBSS bathing solution alone or HBSS

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bathing solutions containing FK228 at appropriate concentrations were added to the acceptor and donor chamber, with 1.5 mL on the apical side and 2.5 mL on the basolateral side, respectively, followed by incubation of the plate at 37°C. A 200 μL sample was taken from the acceptor side at pre-selected time points of 0, 15, 30, 45, 60, 90, 120, 150 and 180 min, and 200 μL warm blank HBSS bathing solution was added each time to the acceptor side to maintain the constant volume. FK228 concentrations in the donor-chamber evaluated for both apical to basolateral (AP→BL) and basolateral to apical (BL→AP) transport were 0.5, 1, 2, 5, 10 and 20 μM. This range included the plasma steady state concentrations in patients receiving 4 hr FK228 infusion at 13 mg/m^2 (Sandor et al., 2002). Three inserts were used for each treatment. The integrity of the cell monolayer was monitored by measuring the transepithelial electrical resistance (TEER) prior to and after the transport. Only monolayers with TEER values >800 Ω·cm^2 were utilized. Lucifer Yellow CH was used as a paracellular transport marker during the experiments, and no leak was observed. For FK228 transport with pretreatment and co-incubation with the Pgp (CsA and Ver) or MRP (MK571 and indomethacin) inhibitors, the same procedures as described above were followed, except that the HBSS bathing solutions were added 5 μM CsA, 100 μM Ver, 50 μM MK571, or 20 and 40 μM indomethacin during both the pretreatment and transport study. FK228 concentrations were determined by HPLC/MS/MS as described previously (Li and Chan, 2000).

Calculation of Apparent Permeability Coefficient P_{app}

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Apparent permeability coefficients P_{app} were calculated from concentration-time profiles as measured in the receiver compartment according to Fick's first law using the following equation:

$$P_{app} = \left(\frac{dC}{dt}\right) \cdot \frac{V}{A \cdot C_0} \qquad \text{(Eq. 1)}$$

where *dC/dt* represents the appearance of FK228 in the receiver chamber (pmol/mL/min), V is the volume of the receiver compartment (mL), A is the cross-section area, and C_0 is the initial donor concentration (pmol/mL) at time = 0. The flux across the monolayer was determined by linear regression from individual FK228 concentration versus time curves. Comparison of P_{app} in the BL→AP direction (P_{app}, BL→AP) to P_{app} in the AP→BL direction (P_{app}, AP→BL) was used to assess efflux transport efficiency (T_{eff} = P_{app}, BL→AP/P_{app}, AP→BL). The statistical significance of differences between treatments was evaluated using two-tailed, paired, student t tests.

Uptake studies

Freshly obtained heparinized blood samples from 8 healthy volunteers were used to test FK228 uptake at two concentrations, 1.8 μM and 18 μM. At 1.8 μM of FK228 the uptake study in the presence of 50 μM MRP inhibitor MK571 was also carried out. Following addition of appropriate drugs, the blood samples were incubated at 37°C, and the plasma FK228 concentrations were followed for up to 60 min.

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Development of FK228 resistant HCT-15R cell line

HCT-15 cells were treated with FK228 started from a sub-IC50 concentration, 100 nM. The medium was changed every other day with an increment of 100 nM of FK228 each time. Within a month, the HCT-15 cells acquired FK228 resistance and were cultured in a medium containing 1000 nM FK228 thereafter. The daughter cell line was designated HCT-15R.

To study the reversibility of the FK228 induced Pgp upregulation, HCT-15R cells were cultured either in medium containing 1000 nM FK228 or in FK228-free medium for 1 to 6 weeks. After 6 weeks, the cells were then treated with 500 nM FK228 for one week. Cells were harvested and analyzed for Pgp expression by western blot analysis.

Cytotoxicity assay

HCT-15 and HCT-15R cells were seeded on 96-well plates at 2000 cells/well, and allowed to adhere overnight before drug treatment. The cells were treated with FK228, paclitaxel or doxorubicin at a series of concentrations alone or in combination with 5 μM CsA or 50 μM MK571. After 24hr treatment, the medium was replaced by fresh drug-free medium followed by a 72-hr incubation. The cytotoxicity was determined by standard SRB assay (Skehan et al., 1990) for attached cells. HL60 and HL60Adr cells were also seeded on 96-well plates at 5000 cells/well, and treated with FK228 at appropriate concentrations in the absence or presence of 50 μM MK571 continuously for 72 hr. The cytotoxicity was determined by standard XTT assay, which is generally used for suspension cells (Goodwin et al., 1995). The IC50 was defined as the concentrations of the tested compounds, at which the number of living cancer cells was

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reduced by 50% as compared with the untreated controls. To avoid possible intracellular retention of FK228 from the resistance development, HCT-15R cells were cultured in FK228-free medium for three days before seeding. All cytotoxicity assays were conducted in triplicates.

Custom cDNA Microarray

We used a 70-oligomer custom cDNA microarray previously developed in our laboratories, which comprises 1070 probes targeting 640 transporter (including all known 48 ABC transporters) and ion channel genes, as well as 430 genes belonging to families of growth factors and receptors, cell adhesion molecules and signal transduction factors (Anderle et al., 2004; Huang et al., 2004). For some genes of special interest (e.g. all ABC transporter genes), two different probes were designed and printed on the arrays. Total RNA was extracted from the parental and daughter cells individually with TRIzol (Invitrogen, Carlsbad, CA, USA), and further purified using the RNeasy Mini column (Qiagen, Carlsbad, CA, USA). Eighteen μg total RNA was used for cDNA synthesis and the cDNA labeled with Cy5 or Cy3 by amino-allyl coupling. The protocol is available at http://derisilab.ucsf.edu/pdfs/amino-allyl-protocol.pdf. A paired and dye-swap design was applied in that the cDNA samples of parent cell lines were in one experimental group labeled with Cy3 dye and the samples of resistant daughter cells were labeled with Cy5 dye; and vice versa in another experimental group. The samples were then mixed, and the labeled cDNA was resuspended in 20 μL HEPES buffer (25 mM, pH 7.0) containing 1 μL of tRNA, 1.5 μL of polyA$^+$, and 0.45 μL of 10% SDS. The mixture was hybridized to the slides for 16 h at 65 ºC. Slides were

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washed, dried and scanned in an Affymetrix 428 scanner to detect Cy3 (green) and Cy5 (red) fluorescence.

Background subtraction and calculation of medians of pixel measurements per spot was carried out using GenePix Software 3.0 (Foster City, California). Spots were filtered out if they had both red and green intensity less than 500 units after background subtraction, or if they were flagged for any visual reason (odd shapes, background noise, etc). Data normalization was carried out using the statistical software package R (www.r-project.org). This method is based on transformations:

$R/G \rightarrow \log_2 R/G - c_j(A) = \log_2 R/k_j(A) * G \rightarrow (1/a_j) * \log_2 R/k_j(A) * G$,

where R and G represent the green fluorescence intensity of Cy5 and the green fluorescence intensity for Cy3. The parameter cj(A) is the Lowess fit of the M vs. A plot for spots on the jth grid of each slide ($M = \log_2 R/G$ and $A = \log2(R*G)^{1/2}$), and aj is the scale factor for the jth grid (to obtain equal variances along individual slides). To identify differentially expressed genes, we calculated the R/G ratios for each primer (4 prints/primer) and for each dye-swap group (2 groups/primer). The cDNA level ratios between HCT-15R and HCT-15 cells were calculated based on the R/G ratios and then averaged and standard deviation calculated. In cases when 2 primers were used for one gene, the R/G values were individually calculated for each primer.

RT-PCR

Total RNA was extracted from HCT-15 and HCT-15R cells by TRIsol. The RNA was further purified by isopropyl alcohol precipitation and 70% ethanol washing. Single-strand cDNA was prepared from the extracted RNA using oligo-dT priming

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(Thermoscript RT Kit, invitrogen, Carlsbad, CA, USA). PCR was performed using primers for MDR1 (5'-CAGCAAAGGAGGCCAACATAC-3' and 5'-TGAGGCTGTCTAACAAGGGCA-3') and for β-actin (5'-CCTGGCACCCAGCACAAT-3' and 5'-GCCGATCCACACGGAGTACT-3'). The PCR conditions were as follows: 95°C for 10 min, 94°C for 45 sec, 60°C for 45 sec, 72°C for 45 sec, and 72°C for 7 min, 20 cycles.

Western immunoblotting

For western blot analysis, cells were washed twice with phosphate-buffered saline (PBS) and lysed in lysis buffer containing 950 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.15% Igepal CA-630 and 1.5 mM PMSF. Equal amounts of proteins (100 μg) were size fractionated on 6% (Pgp) or 15% (acetylated histone protein H3 and H4) SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane. The membrane was blocked with blocking buffer (5% nonfat milk, 200 mM NaCl, 50 mM Tris and 0.05% Tween-20) at room temperature for 2 hr. The blocked membrane was then incubated with primary antibodies at 4°C overnight. After washing the membrane with TBS-T buffer (20 mM Tris, 500 mM NaCl and 0.05% Tween-20) for 3×15 min, the membrane was incubated with secondary antibody at room temperature for 1 hr. The detection of specific protein binding was performed with the ECL chemiluminescent Western blotting detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden). The antibodies used were mouse monoclonal JSB-1 anti-human P-glycoprotein antibody (Research Diagnostics Inc., Flanders, NJ, 1:50), mouse monoclonal AC-15 anti-β-actin antibody (Abcam Inc., 1:5000), peroxidase-conjugated

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AffiniPure donkey anti-mouse IgG (Research Diagnostics Inc., Flanders, NJ, 1:10,000), rabbit polyclonal anti acetylated histone H3 antibody (upstate, 1:500), rabbit anti acetylated histone H4 antibody ChIP grade (Upstate, 1:500), and peroxidase-conjugated donkey anti-rabbit IgG (Upstate, 1:2000).

HAT and HDAC activity assays.

Nuclear contents were extracted from the 8×10^6 HCT-15 and HCT-15R cells using a nuclear extract kit (Upstate Biotechnology, Inc., Waltham, MA). The resistant cells were cultured in FK228-free medium for 3 days before nuclear extraction. The HAT activity contained in the nuclear extracts was determined using non-radioactive colorimetric kit (Upstate Biotechnology, Inc., Waltham, MA). And the HDAC activity was determined using a fluorescent kit (Upstate Biotechnology, Inc., Waltham, MA). The experiments were conducted according to the manufacture's protocols.

RESULTS

FK228 is a Pgp substrate

AP→BL and BL→AP transport of FK228 was investigated at concentrations ranging from 0.5 to 20 μM. Figure 2 shows the amount of FK228 transported across Caco-2 monolayers over time for both AP→BL and BL→AP directions (Fig. 2a). For AP→BL transport at 0.5 μM of FK228, only trace amounts of FK228 were found, and the levels were below the quantification limit and thus not included in the calculation. FK228 transport was found to be linear with time for up to 180 min for both directions.

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The flux (*J*) was found to be proportional to the donor-side FK228 concentration (Fig. 2b). The calculated P_{app} values were $4.07 \pm 0.74 \times 10^{-6}$ cm/s (n = 18) and $1.27 \pm 0.73 \times 10^{-7}$ cm/s (n = 15) for BL→AP and AP→BL, respectively. The BL→AP transport was 32 times faster than that of AP→BL ($p<0.005$). No apparent Pgp saturation was observed.

In the inhibition studies, pretreatment and co-incubation with CsA (5 μM) or Ver (100 μM) caused dramatic decreases in the BL→AP transport rate (Fig. 3). Between the two inhibitors, CsA showed higher inhibition, causing a 13.6-fold decrease ($p<0.005$) in P_{app}, as compared with 8.4 times by Ver ($p<0.005$). For AP→BL direction, both inhibitors caused small but significant increases of P_{app} ($p<0.05$). In the presence of the inhibitors, the T_{eff} ratios decreased from 32 to 1.65 and 2.47 for CsA and Ver, respectively, suggesting essentially complete inhibition of efflux.

FK228 is a MRP1 substrate

To investigate the contribution of MRP1 to FK228 transport and uptake, FK228 was incubated in human blood from 8 healthy volunteers. FK228 showed a concentration dependent uptake (Fig. 4) and was taken up by RBC more rapidly and extensively at the higher concentration (18 μM) than at the lower concentration (1.8 μM). MRP1 inhibition by MK571 at 50 μM in the blood significantly increased the rate of FK228 removal from the plasma at 1.8 μM.

HL60 cells are Pgp(-)/MRP1(-), while the derivative HL60Adr cells are Pgp(-) /MRP1(+) (Bhalla et al., 1985; Gollapudi and Gupta, 1992). If FK228 is indeed a MRP1 substrate, HL60Adr cells should be more resistant to FK228 than HL60 cells. For this reason we conducted cytotoxicity assays in these two cell lines. Our results showed that

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in the absence of the MRP inhibitor MK571, the IC50 values of FK228 were 5.6 ± 0.56 and 19.4 ± 1.8 nM for HL60 and HL60Adr cells, respectively. In the presence of MK571, the IC50 did not change appreciably for HL60 cells (5.3 ± 0.59 nM), but for HL60Adr cells, it was reduced by more than 3 folds (6.5 ± 0.46 nM) (Fig. 5). This confirmed that FK228 is a MRP1 substrate.

To further investigate whether FK228 is also a substrate of another major member of MRP family, MRP2, the effects of MRP inhibitors MK571 and indomethacin on FK228 transport across the Caco 2 cell monolayer were studied. MK571 at 50 μM significantly decreased BL→AP transport ($p<0.005$), but increased AP→BL transport ($p<0.005$) across the Caco 2 monolayer. Indomethacin at 20 or 40 μM showed no significant effect (Fig. 6) on FK228 transport of either direction ($p>0.05$). The change of FK228 transport in the presence of MRP inhibitors was consistent with the apical localization of MRP2 in the differentiated Caco 2 cell monolayer (Hirohashi et al., 2000; Sun et al., 2002; Cooper et al., 2004).

Development of FK228 resistant HCT-15R cell line

The HCT-15 colon carcinoma cells acquired significant FK228 resistance fast (within a month) with no observable massive cell kills (Fig. 7a, Table 1). The HCT-15R cells did not show apparent morphological change (data not shown), however. In the absence of CsA, the IC50 of FK228 on HCT-15 cells was 378 ± 49.5 nM, as compared with 7139 ± 813 nM on HC-15R cells. This 19-fold difference in the IC50 was diminished by 5 μM CsA, which dramatically reduced IC50 values to 7.44 ± 1.1 nM and 11.4 ± 0.4 nM for HCT-15 and HCT-15R cells, respectively. HCT-15R cells also showed cross-

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resistance to other two Pgp substrates, paclitaxel and doxorubicin (Fig. 7b and c Table 1). Similar to the case of FK228, CsA caused sensitization of both HCT15 and HCT15R cells and resulted in essentially superimposable IC50 curves. These results suggests that Pgp may play an important role in the resistance and cross resistance, since paclitaxel and doxorubicin are well known Pgp substrates.

In contrast to Pgp inhibition, MRP1 inhibition by 50 μM MK571 resulted in parallel IC50 shifts for both HCT-15 and HCT-15R cells (Fig. 8), suggesting that the MRP1 expressions are essentially the same in HCT-15 and HCT-15R cells.

Further characterization of FK228 resistant HCT-15R cell line

The resistance of HCT-15R cells to FK228 was found to be reversed by CsA (Fig. 7a, Table 1). This suggests that Pgp upregulation is a major mechanism for the acquired resistance. However, this resistance may also be derived from other factors such as upregulation of MRPs. For this reason, we used a custom 70-oligomer cDNA microarray (the list of included genes is available upon request) to screen possible up or down regulation of genes. The results showed that Pgp (gene name ABCB1), but not other ABC transporter genes, was predominantly upregulated among all the tested genes (Fig. 9). The Pgp upregulation was further confirmed at the mRNA level by semi-quantitative RT PCR (Fig. 10a) and at the protein level by western immunoblotting (Fig. 11 panels 0 and 7). The lack of MRP1 upregulation (gene name ABCC1) (Fig. 9) suggests that expressions of Pgp and MRP1 may be controlled by different mechanisms.

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FK228 is a HDAC inhibitor and upregulates Pgp through histone hyperacetylation (Jin and Scotto, 1998; El-Osta et al., 2002). However, treatment with a Pgp substrate drugs, such as paclitaxel (Schondorf et al., 2003) or doxorubicin (Marie et al., 1993; Schondorf et al., 2003), can also lead to rapid Pgp upregulation. To find out if FK228 induces Pgp through HDAC inhibition, we determined the histone acetylation status in HCT-15 and HCT-15R cells (Fig.10b). Global increases of histone H3 and H4 acetylation suggested that Pgp induction is associated with HDAC inhibition by FK228.

FK228 has been reported to be a reversible inhibitor of class I HDACs (Marks et al., 2000). Consistent with this result, we found that FK228 reversibly upregulates Pgp, and the sustained upregulation is dependent on continuous FK228 exposure (Fig. 11). Pgp expression of HCT-15R cells decreased over time in the absence of FK228, and fell to the baseline Pgp level within 6 weeks. Re-treatment of HCT-15R cells after 6 weeks with 500 nM FK228 rapidly restored the Pgp expression (Fig. 11). The relatively slow decrease of Pgp expression following FK228 cessation of treatment was probably due to a long turnover time of Pgp protein.

Histone acetylation status is controlled by both HDAC and HAT enzymes, and deregulation of HAT and HDAC may also contribute to the acquired FK228 resistance. For this reason, we determined the HDAC and HAT activities in HCT-15 and HCT-15R cells (Fig. 12). However, no significant change of HAT or HDAC activity was detected.

DISCUSSION

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FK228 is a substrate for both Pgp and MRP1

Pgp and MRP1 are associated with resistance of many anticancer drugs (Lee, 2000). An NCI screening project showed that FK228 is a Pgp substrate but not a Pgp inhibitor (Scala et al., 1997). However, no quantitative data regarding FK228 transport kinetics were available, and it was not clear if other membrane transporters are associated with FK228 efflux.

Our current study confirmed that FK228 is a Pgp substrate, with BL→AP transport more than 30 times faster than AP→BL direction across the Caco 2 cell monolayer. The very low AP→BL P_{app} values helps to explain the low oral bioavailability of FK228 (Chan et al., 1997; Li and Chan, 2000). However, no apparent Pgp saturation was achieved throughout the rather wide FK228 concentration range from 0.5 to 20 μM. A possible explanation would be that FK228 is not a strong Pgp substrate and its K_m value for Pgp binding is higher than 20 μM, the highest concentration we tested. This explanation is consistent with the data published by Scala *et al.* (Scala et al., 1997), which showed that FK228 did not competitively inhibit cellular efflux of several model Pgp substrates by SW620 Ad300 cells. On the other hand, CsA at 5 μM significantly blocked FK228 BL→AP transport at 5 μM, and the resulting transport rates for both directions became similar, with T_{eff} ratio of 1.65 (Fig. 3). This could be readily explained if FK228 is not a strong Pgp substrate, since CsA, as a moderate competitive Pgp inhibitor at the same concentration, seemed to bind Pgp more efficiently than FK228.

Paradoxically, being a relatively weak Pgp substrate, FK228's cellular efflux is expected to be minimal; however, our data showed an almost unidirectional FK228 transport across the Caco 2 monolayer. We hypothesize that FK228, being highly

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lipophilic (Chan et al., 1997), is likely to be trapped within the cell membrane. It has been reported that Pgp works as a flippase or a 'hydrophobic vacuum cleaner', which pumps out substrate drugs from within the inner layer of cell membrane rather than from cytosol (Teodori et al., 2002). Thus, although FK228 is a relative weak Pgp substrate, its high concentration within the cell membrane may increase the efficiency of its Pgp-mediated efflux. This efflux is expected to decrease the FK228 concentration in the inner layer of cell membrane, as well as the FK228 concentration gradient between the inner membrane and cytosol. Since the concentration gradient is the driving force for FK228 to diffuse into the cell, Pgp-mediated efflux is thus expected to result in significantly decreased FK228 concentration in cytosol.

The above findings may have clinical significance. FK228 can be potentially used against Pgp positive cancers in combination with a Pgp inhibitor, since Pgp-related efflux can be readily reverted by Pgp inhibition.

The concept of FK228 functioning as a MRP1 substrate was first established by a human RBC uptake study (Fig. 4). Human RBC not only expresses high concentrations of MRP1 (Rychlik et al., 2000), but also the membrane structure is relatively simple and contains fewer interfering membrane transporters (e.g. P-gp). For these reasons, RBC is a good model to study MRP1 related uptakes (Zaman et al., 1996; Evers et al., 1997; Klokouzas et al., 2001). The saturable RBC uptake kinetics and the effect of MRP1 inhibition on the uptake were consistent with FK228 being a MRP1 substrate (Fig. 4). The Pgp(-)/MRP1(-) HL60 and Pgp(-)/MRP1(+) HL60/Adr cell pair served as another good system to study MRP1-mediated cellular uptake, and

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showed an inverse correlation between MRP1 expression and FK228 cytotoxicity (Fig. 5). Taken together, these evidences indicated that FK228 is a MRP1 substrate.

Caco 2 cell monolayer is widely used to study Pgp related transport. However, its use to study MRP-mediated transport and uptake has been limited due to debates on whether differentiated Caco 2 cells express functional MRP proteins. Recently, it was reported that MRP2 is the major functional MRP transporter on the apical membrane of Caco 2 cell, while MRP1 expression is minimal (Hirohashi et al., 2000; Sun et al., 2002; Cooper et al., 2004). Using Caco 2 cell monolayer as a model, we found that MK571 at 50 μM significantly increased FK228 AP→BL but decreased BL→AP transport (Fig. 6), suggesting that MRP2, or other related transporters that are expressed on the apical membrane, may play a role in FK228 transport across the Caco2 monolayer. Another commonly used MRP inhibitor, indomethacin, at either 20 or 40 μM, showed little on FK228 transport profile across the Caco 2 cell monolayer, probably due to its weaker intrinsic inhibitory activity against MRP2 as compared with MK571. The phenomenon that either Pgp or MRP1 inhibition reversed the net BL→AP flux of FK228 across the Caco2 monolayer was rather difficult to explain, probably due to non-specificity of CsA and Ver as Pgp inhibitors. For this reason, we use a more specific Pgp inhibitor PSC833 (Valspodar) at 1 μM to better determine the effect of Pgp on FK228 transport. Due to between-experiment variations, we were only able to determine the P_{app} values in the BL→AP direction of 75.4 ± 13 and $4.98 \pm 1.2 \times 10^{-7}$ cm/s in the absence and presence of PSC833, respectively ($p<0.005$). This 15-fold decrease in $P_{app,\ BL\rightarrow AP}$ suggests Pgp plays a major role in FK228 transport. However, due to the lack of PSC833 inhibition data in the AP→BL direction, as well as the fact that Caco2 is not a dedicated model for

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MRP transporters, the contribution of MRP2 or related transporters to FK228 transport cannot be quantitatively determined.

Pgp induction is the major mechanism for acquired FK228 resistance

HCT-15 cells readily acquired FK228 resistance. As shown by our data, reversible Pgp upregulation seemed to be a major resistance mechanism. This was confirmed by a series of cytotoxicity assays (Fig. 7, Table 1), microarray (Fig. 9), RT PCR (Fig. 10a), and western blot (Fig. 11). However, there seemed to be no MRP1 upregulation in HCT-15R cells according to our cytotoxcitiy (Fig. 8) and microarray (Fig. 9) results. This suggests that expressions of Pgp and MRP1 may be controlled by different mechanisms. Moreover, we found the HAT/HDAC machinery is not deregulated in HCT-15R cells.

To date, FK228 is the only known HDAC inhibitor being a Pgp substrate, and it is not clear if Pgp induction is associated with the overall low response rate during several FK228 clinical trials (Marshall et al., 2002; Sandor et al., 2002). However, potential Pgp induction by FK228, and maybe by other HDAC inhibitors of clinical interest (e.g. SAHA and valproic acid), should be taken into consideration when these drugs are used to pre-treat patients receiving Pgp-substrate drugs.

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Footnotes:

Financial supports:

This work was supported by grant NIH 1R21CA 96323 and by BioMedical Mass Spectrometry Laboratory at the Ohio State University

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Legend for Tables and figures:

Table 1: IC50 values of FK228, paclitaxel and Doxorubicin on HCT-15 and HCT-15R cells in the presence or absence of 5 μM CsA.

Figure 1. Structure of depsipeptide FK228.

Figure 2. Transepithelial flux of FK228 across the Caco-2 cell monolayer.

(a) FK228 flux was linear with time up to 180 min for both BL→AP and AP→BL directions as measured at 5 μM (n = 3). (b) Flux was proportional to the FK228 concentration throughout the concentration range with no apparent saturation for both BL→AP and AP→BL directions (n = 3).

Figure 3. Effects of CsA and Ver on FK228 flux across the Caco-2 cell monolayer.

Transport of FK228 was studied with pretreatment and co-incubation of 5 μM CsA or 100 μM Ver. Transport of FK228 in the absence of CsA and Ver was used as a control. For AP→BL direction, inhibitors caused small but significant increase in P_{app} ($p < 0.05$); For BL→AP direction, the inhibitors caused dramatic drop in P_{app} ($p < 0.005$).

Figure 4: Human RBC uptake/metabolism of FK228.

Blood samples from 8 healthy volunteers were spiked with FK228, incubated at 37 °C, and the RBC uptake/metabolism was followed over time up to 60 min. RBCs appeared to uptake/metabolize FK228 more rapidly at higher FK228 concentrations. Treatment of

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blood with a MRP inhibitor MK571 at 50 μM significantly increased the rate of FK228 removal from plasma.

Figure 5: Cytotoxicity assays of FK228 in Pgp(-)/MRP1(-) HL60 and Pgp(-)/MRP1(+) HL60Adr cells.

In the absence of the specific MRP inhibitor MK571, the IC50s of FK228 were 5.6 ± 0.56 nM and 19.4 ± 1.8 nM for HL60 and HL60Adr cells, respectively. Addition of MK571 at 50 μM caused little change of the IC50 in HL60 cells (5.3 ± 0.59 nM), while totally reversed the IC50 value in HL60Adr cells (6.5 ± 0.46 nM, $p<0.05$). (a), cytotoxicity curves; (b), comparison of IC50s of FK228 between HL60 and HL60Adr cells in the presence or absence of MK571.

Figure 6: Effects of MRP inhibition on FK228 transport across the Caco 2 cell monolayer in AP-BL and BL-AP directions.

MRP inhibitor MK571 at 50 μM increased AP-BL transport ($p<0.005$) as well as decreased BL-AP transport ($p<0.005$) of FK228, while the other MRP inhibitor indomethacin (IN) had less influence on FK228 transport at either 20 or 40 μM ($p>0.05$).

Figure 7: Cytotoxicity assays in HCT-15 and HCT-15R cells.

(a) FK228, (b) Doxorubicin and (c) paclitaxel in HCT-15 and HCT-15R cells. IC50s in HCT-15 cells treated with the drug alone (•) or in combination with 5 μM CsA (o) were compared with those in HCT-15R cells treated with the drug alone (▼) or in combination with 5 μM CsA (∇).

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Figure 8: Cytotoxicity assays of FK228 in HCT-15 and HCT-15R cells.

In the absence of MK571, the IC50s of FK228 were 371 ± 4 and 5770 ± 549 nM for HCT-15 and HCT-15R cells, respectively. In the presence of 50 μM MK571, the IC50s shifted to 8.7 ± 1.0 and 140 ± 10 nM for the two cell lines, respectively. The IC50 shifts for both cell lines were about 40 folds, suggesting the MRP1 levels in these two cell lines are essentially the same.

Figure 9: The mRNA level comparison between HCT-15R and HCT-15 cell lines using a custom 70-oligomer cDNA microarray.

ABCB1 (gene name for Pgp) was the only gene that was upregulated among all the genes included in the array. Two 70-mer probes were used for Pgp, and showed similar results. No ABCC1 or ABCC2 (gene names for MRP1 and MRP2) upregulation was observed.

Figure 10: Characterization of HCT-15 and HCT-15R cell lines by RT PCR and western blot.

(a) RT PCR showed Pgp upregulation at the mRNA level in HCT-15R cells using beta actin for normalization. (b) Western analysis showed a global increase of acetylation of histone proteins H3 and H4 using beta actin as the loading control.

Figure 11: Reversible Pgp induction in HCT-15R cells as measured by Western blot.

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HCT-15R cells were either cultured in medium containing 1000 nM FK228 (lane 0) or in FK228-free medium for 1 to 6 weeks (lanes 1- 6). The Pgp expression level decreased over time. HCT-15 cells were used as a negative control (lane 7). Treating the resulting 6-week HCT-15R cells with 500 nM FK228 readily restored the high Pgp expression (lane 8). Beta actin was used as the loading control.

Figure 12: HDAC and HAT activity assays of HCT-15 and HCT-15R cells.

Nuclear extracts from HCT-15 and HCT-15R cells were determined for relative HDAC (a) and HAT (b) activities. The relative HDAC activities in HCT-15 and HCT-15R cells were 150.7 ± 5.2% and 150.0 ± 5.0%, respectively; and the relative HAT activities were 82.9 ± 37.5% and 64.5 ± 31.1%, respectively. Neither HDAC nor HAT activity was significantly changed in the two cell lines ($n = 3$, $p>0.05$).

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Table 1

Cell lines	Treatment with 5 μM CsA	IC50 values (mean ± SD, nM), n = 3		
		FK228	paclitaxel	Doxorubicin
HCT-15	no	378.1 ± 49.5	168.6 ± 5.9	250.5 ± 6.1
	yes	7.4 ± 1.1	2.54 ± 0.3	30.2 ± 2.0
HCT-15R	no	7139 ± 813	964.9 ± 47.7	2901 ± 203
	yes	11.4 ± 0.4	3.52 ± 0.6	55.1 ± 4.2

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Figure 1

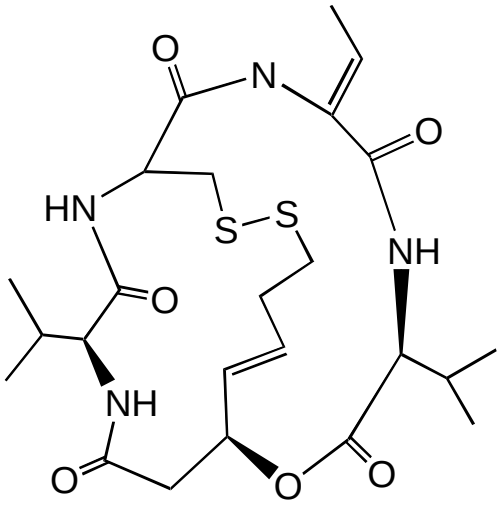

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Figure 2

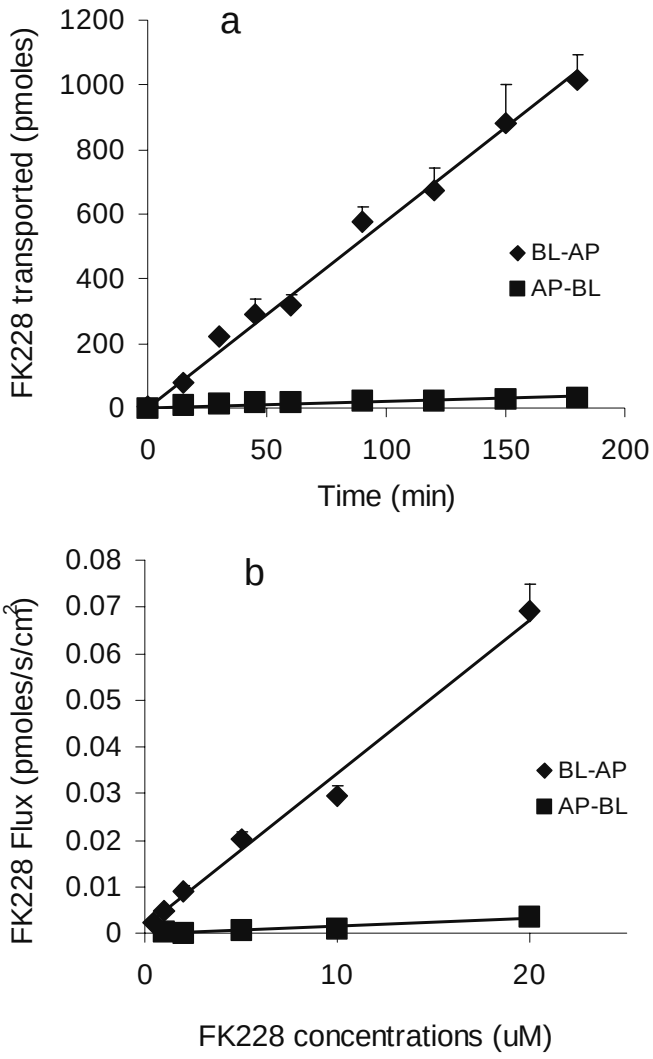

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Figure 3

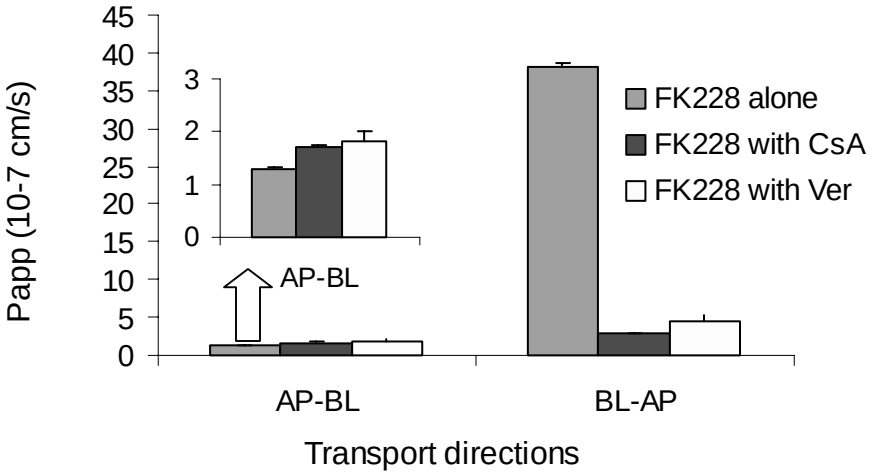

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Figure 4

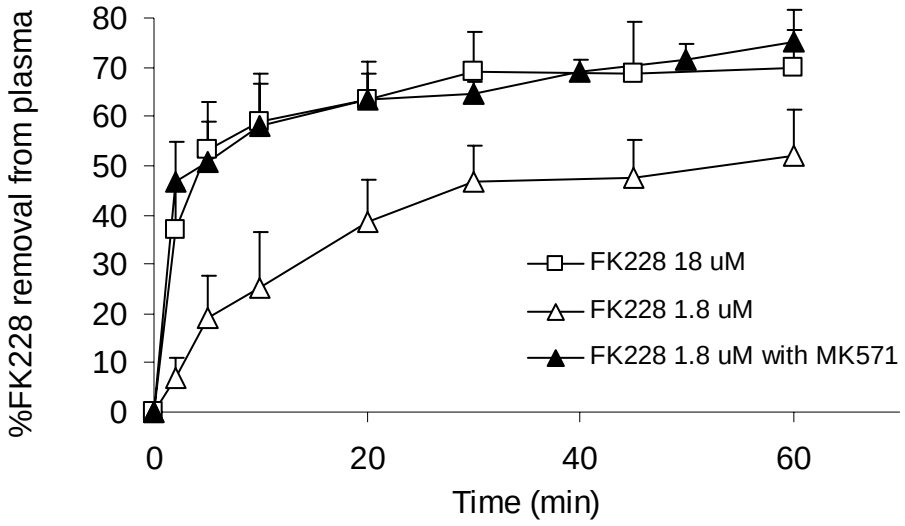

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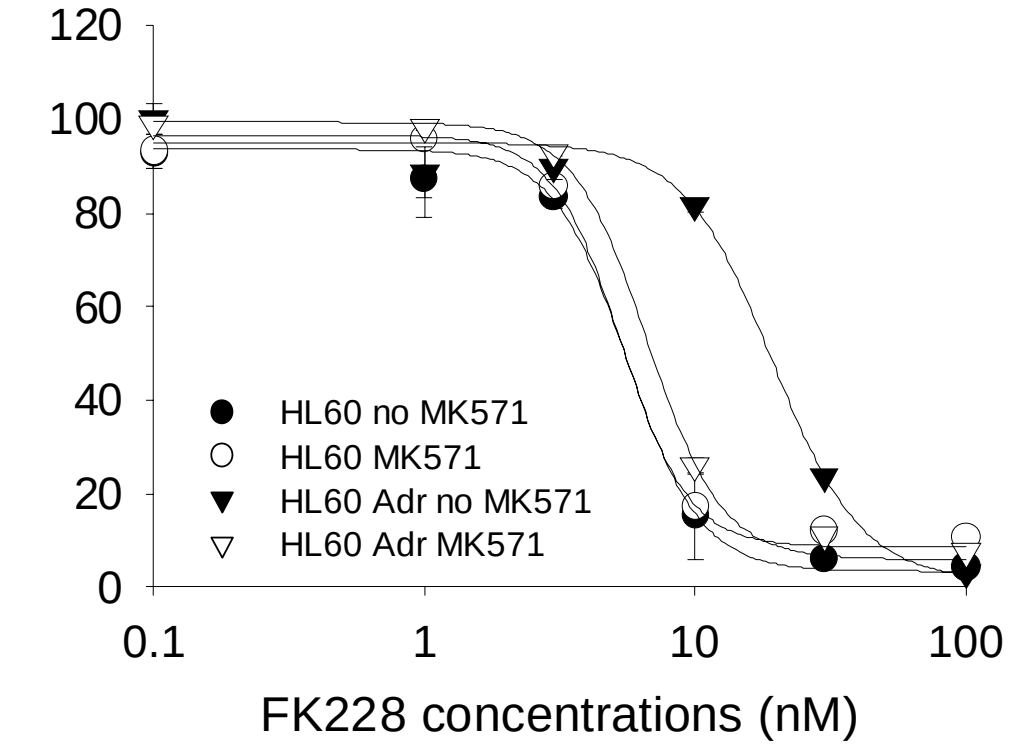
Figure 5



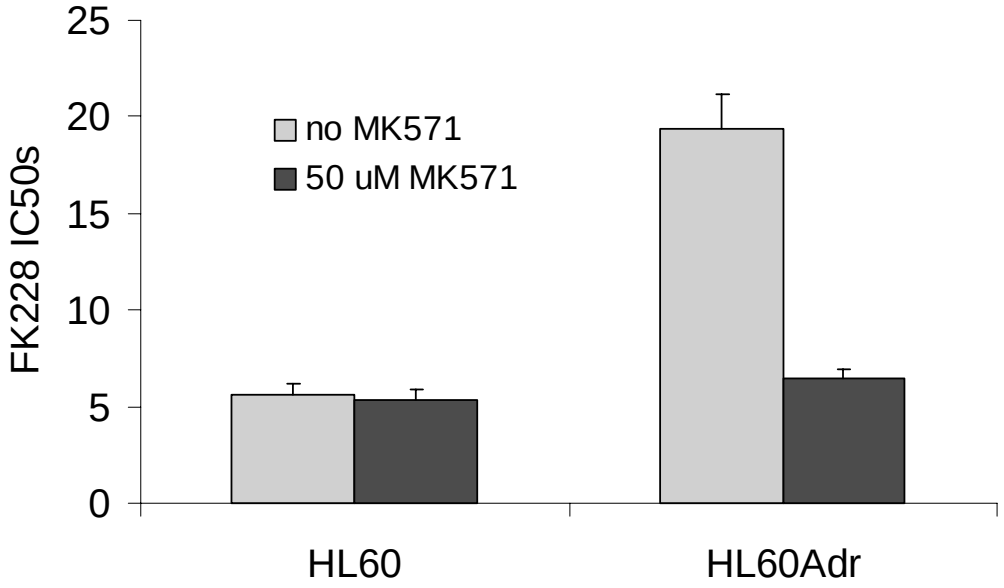


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Figure 6

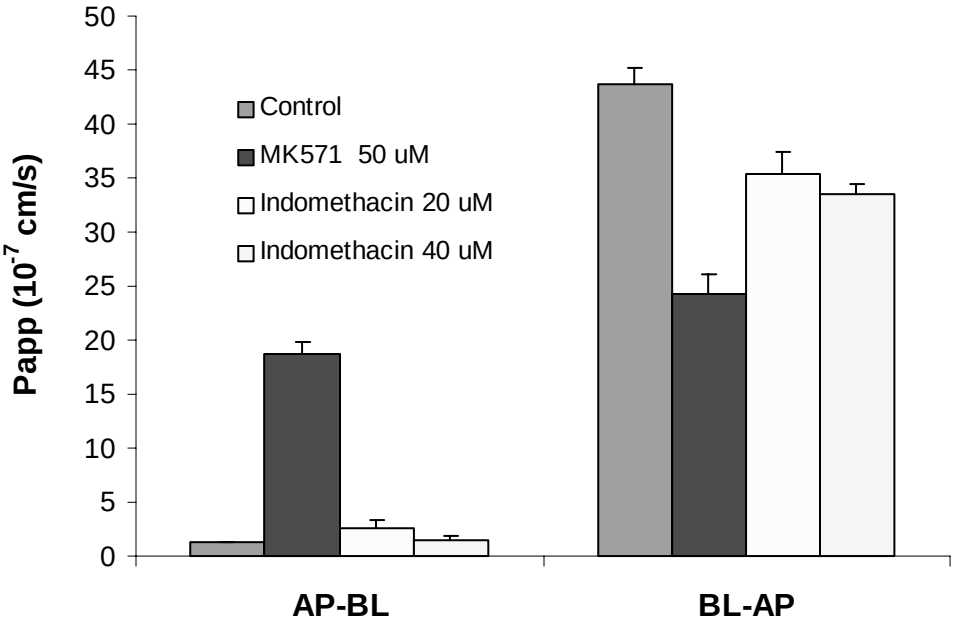

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Figure 7

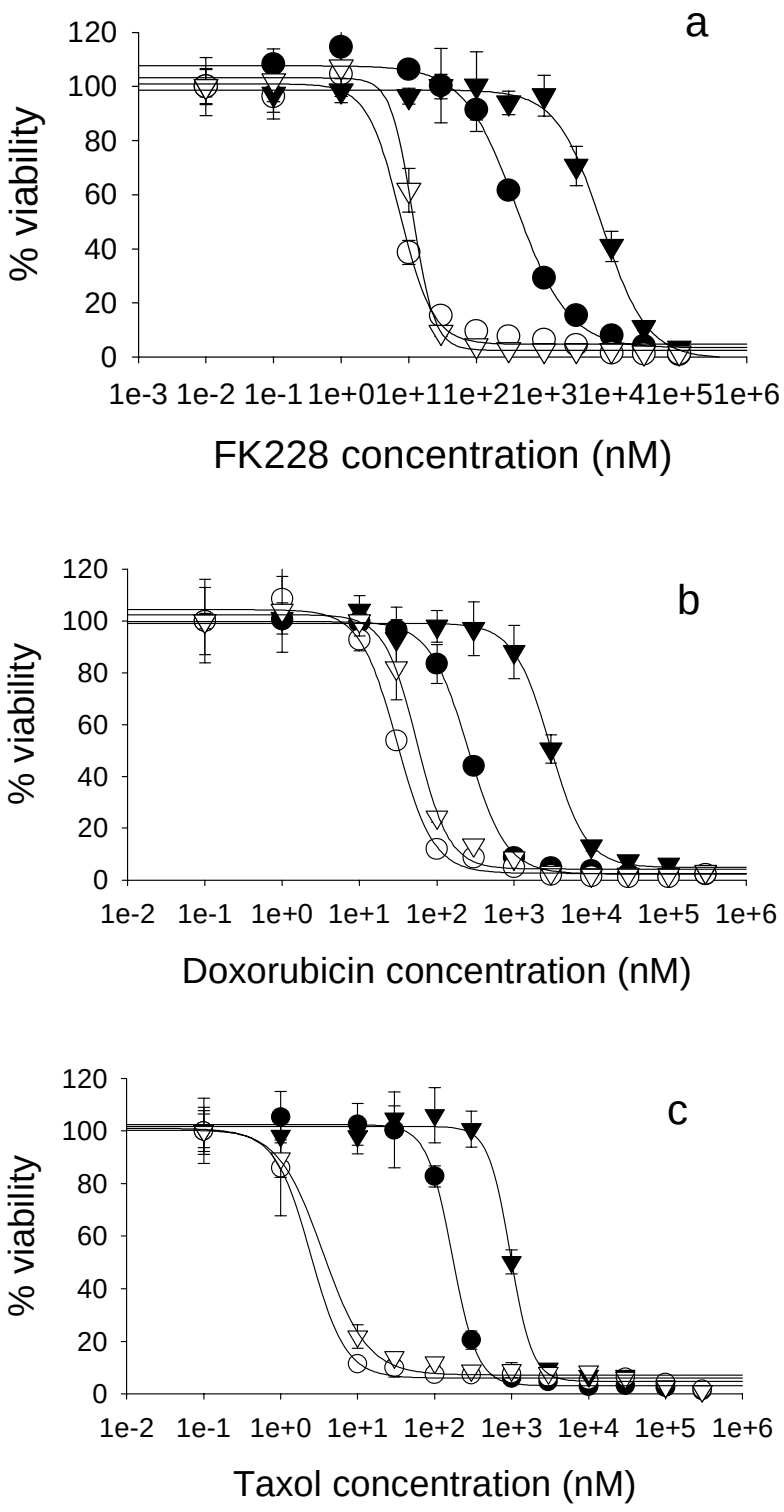

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Figure 8

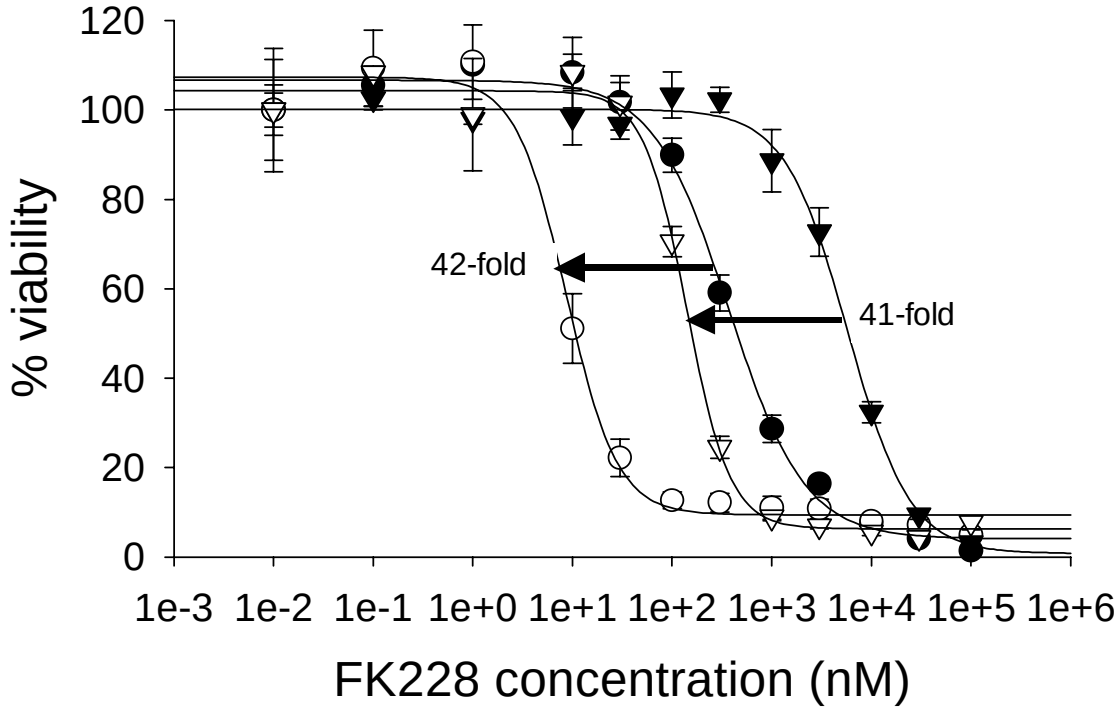

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Figure 9

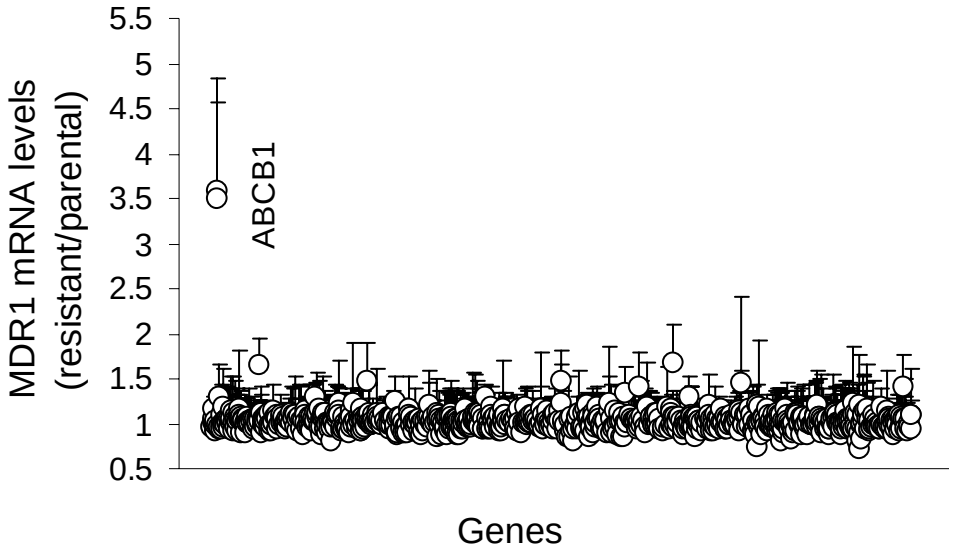

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Figure 10

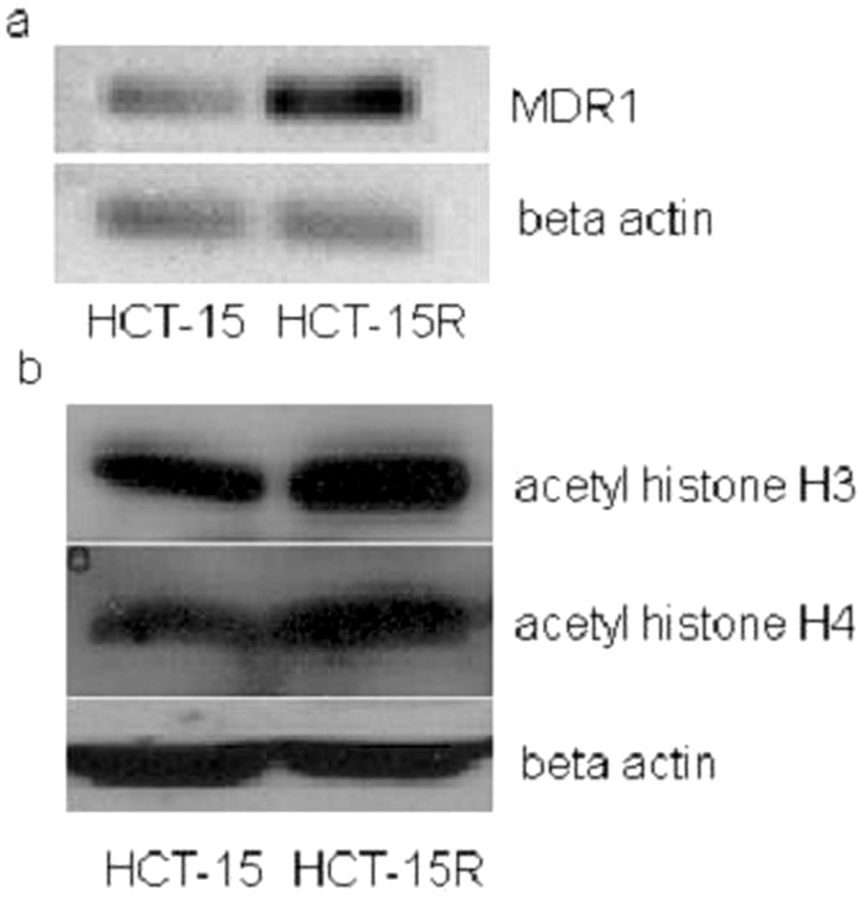

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Figure 11

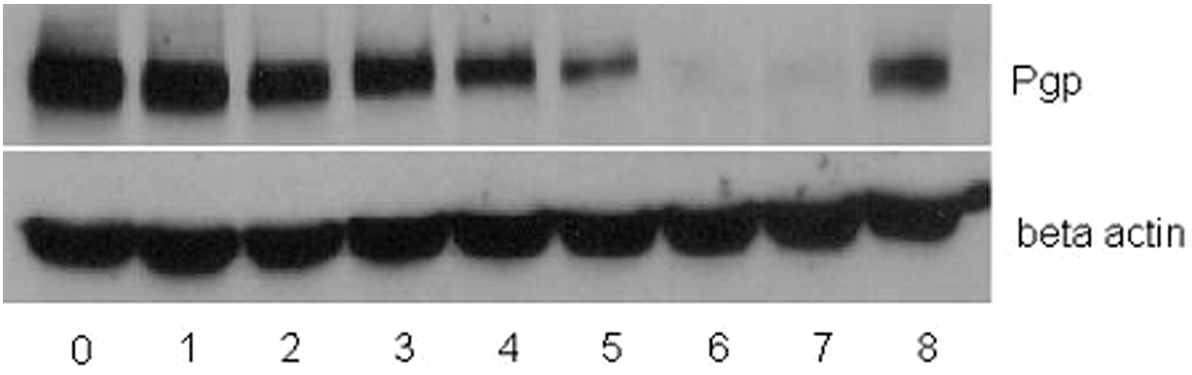

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Figure 12