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HIV protease inhibitors interact with ABCC4/MRP4: a basis for unanticipated enhanced cytotoxicity

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Running title: HIV protease inhibitors and MRP4

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Abbreviations: PI, protease inhibitor; NFV, Nelfinavir (aka viracept); ABCC4/ MRP4, ATP-binding cassette transporter/multidrug resistance transporter 4; WT, wild-type; KO, knockout; HAART, Highly active antiretroviral therapy

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Abstract

HIV-pharmacotherapy, by combining different drug classes such as nucleoside analogues and HIV protease inhibitors (PI), has increased HIV-patient life expectancy. Consequently, among these patients, an increase in non-HIV associated cancers has produced a patient cohort requiring both HIV and cancer chemotherapy. We hypothesized that MRP4/ABCC4, a widely expressed transporter of nucleoside-based antivirals as well as cancer therapeutics, might interact with PI. Among the PI evaluated (nelfinavir, ritonavir, amprenavir, saquinavir, and indinavir), only nelfinavir both effectively stimulated MRP4 ATPase activity and inhibited substrate-stimulated ATPase activity. Saos2 and HEK293 cells engineered to overexpress MRP4 were then used to assess transport and cytotoxicity. MRP4 expression reduced intracellular accumulation of nelfinavir and consequently, conferred survival advantage to nelfinavir cytotoxicity. Nelfinavir blocked Mrp4 mediated export, which is consistent with its ability to increase the sensitivity of MRP4 expressing cells to methotrexate. In contrast, targeted inactivation of Abcc4/Mrp4 in mouse cells specifically enhanced nelfinavir and PMEA cytotoxicity. These results suggest that nelfinavir is both an inhibitor and substrate of MRP4. Because nelfinavir is a new MRP4/ABCC4 substrate we developed a MRP4/ABCC4 pharmacophore model, which showed that the nelfinavir-binding site is shared with chemotherapeutic substrates such as adefovir and methotrexate. Our studies reveal, for the first time, that nelfinavir, a potent and cytotoxic PI, is both a substrate and inhibitor of MRP4. These findings suggest HIV-infected cancer patients receiving nelfinavir might experience both enhanced anti-tumor efficacy and unexpected adverse toxicity given MRP4/ABCC4 role in exporting nucleoside-based anti-retrovirals and cancer chemotherapeutics.

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Introduction

The incidence of non-AIDS-defining cancers (e.g. Hodgkin's lymphoma, lung, testicular germ-cell, breast) has increased significantly as patients with HIV/AIDS achieve longer life expectancy (Deeken et al., 2012; Rudek et al., 2011). These individuals are a therapeutic challenge because concurrent treatment with anti-neoplastic drugs and highly active antiretroviral therapy (HAART) might increase the potential for drug-interactions (Rudek et al., 2011) . The interactions between cancer chemotherapeutics and HAART drugs have the potential to increase the therapeutic benefit by increasing tumoricidal activity (De Clercq et al., 1999). Despite this, mechanistic evidence is lacking for direct interactions between cancer chemotherapeutics and drugs in the HAART regimen.

Acyclic nucleoside phosphonates like tenofovir and adefovir (PMEA; 9-(2-phosphonylmethoxyethyl) adenine) are acyclic nucleotide analogs of adenosine monophosphate that, due to their capacity to inhibit viral polymerases, are very effective against a variety of viruses (e.g., hepatitis B and HIV) and have become integral to the success of HAART regimens. Nonetheless, they also possess potent tumoricidal properties (De Clercq et al., 1999). Tenofovir is structurally similar to adefovir only differing by a methyl-group addition in the sugar-like aliphatic linker. *In vitro* studies and studies in knockout mice indicate that adefovir and tenofovir are exported by the ABC transporter, Abcc4/Mrp4 (Imaoka et al., 2007; Ray et al., 2006; Takenaka et al., 2007). Notably, absence of Abcc4/Mrp4

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enhances tenofovir toxicity thereby indicating ABCC4/MRP4 export is crucial to preventing acyclic nucleoside phosphonate toxicity (Imaoka et al., 2007).

The HAART regimen typically includes HIV protease inhibitors (PIs). While some PIs (ritonavir, nelfinavir) increase the toxicity of acyclic nucleoside phosphonates used in antiretroviral therapy (PMEA, adefovir, tenofovir) (Kiser et al., 2008), the basis for this is unknown. Because adefovir and tenofovir are substrates of MRP4, we hypothesized that PIs might inhibit MRP4 and increase not just their cytotoxicity, but also cancer chemotherapeutics. We tested the possibility that PIs interact with ABCC4/MRP4 by assessing their impact on substrate-stimulated ATPase, inhibition of basal ATPase and transport activity using genetic models of ABCC4/MRP4 overexpression and newly developed knockout cell lines. We show that the therapeutically important HIV protease inhibitors, nelfinavir (NFV) and ritonavir, modulate substrate-stimulated ATPase activity, which correlates with their potential as MRP4 substrates. These studies were extended to show that ABCC4/MRP4 overexpression reduces NFV uptake and protects against NFV cytotoxic effects. Moreover, absence of ABCC4/MRP4 renders cells more sensitive to NFV. Finally because NFV is an ABCC4 substrate, we developed a pharmacophore to further identify potential substrates and/or inhibitors of ABCC4/MRP4. These findings suggest among HIV-infected cancer patients that inhibition of ABCC4/MRP4 by nelfinavir may alter anti-tumor efficacy.

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Materials and Methods:

Reagents

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: nelfinavir, ritonavir, amprenavir, saquinavir, and indinavir. Generation of wild-type and Mrp4 KO MEFs from C57BL/6J mouse embryos were described previously (Sinha et al., 2013).

ATPase Assays

ATPase activity of MRP4 in crude membranes (10 μg of protein per assay) of insect cells was measured by the end-point P_i assay as previously described (Ambudkar, 1998; Sauna et al., 2004; Wu et al., 2005), with minor modifications. MRP4-specific activity was recorded as the beryllium fluoride (BeFx)-sensitive ATPase activity, where the amount of P_i released was quantified using a colorimetric method (Ambudkar, 1998)(Supplementary data).

Cell proliferation assay

Saos2, HEK293, wild-type or Abcc4/Mrp4 knockout MEFs were incubated in MTT medium (DMEM containing 10% dialyzed serum) containing various concentrations of Bis(POM)-PMEA, NFV or MTX for 4-6 hr. Following three days of culture in fresh MTT medium, cell proliferation was measured using MTT assay according to the manufacturer's instructions (Promega, Madison, WI).

Intracellular accumulation of ABCC4 substrates

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Saos-2 and HEK293 stably expressing either vector or MRP4 and wild-type and ABCC4/ Mrp4 knockout MEFs were incubated with 10 μM Bis(POM)-PMEA (with a trace amount of [^{3}H]-Bis(POM)-PMEA) for 1 to 6 hr with 30 min pre-incubation with NFV or MK571 as indicated. The intracellular accumulation of PMEA was measured as previously described (Takenaka et al., 2007)(Supplementary data). Intracellular amounts of nelfinavir in HEK293 cells expressing either vector or MRP4 were determined using LC-MS/MS.

Detection of MRP4 proteins by immunoblotting

Homogenates prepared from tissues harvested from C57BL/6J WT and KO adult female mice (Leggas et al., 2004) were analyzed by immunoblotting as previously described (Takenaka et al., 2007). Antibodies used were M4I10 (MRP4; Abcam, Cambridge, MA), MRP1 (Abcam), P-gp (JDS), MRP5 (a gift from Dr. George Scheffer), BXP53 (ABCG2; Kamiya, Seattle, WA), and β-actin (Sigma Aldrich, St. Louis, MO).

PMEA efflux in the presence of NFV

PMEA efflux was measured over indicated time in wild-type or Abcc4/Mrp4 knockout MEFs as described previously using a trace amount of [^{3}H]-Bis(POM)-PMEA in the absence or presence of 50 μM NFV (Nagai et al., 2011)(Supplementary data).

MRP4 pharmacophore models

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PI MRP4 pharmacophore: Computational molecular modeling studies were performed using Discovery Studio 2.5.5. and 3.5.5. (Acers, San Diego, CA). Common feature pharmacophore models describe the arrangement of key features important for biological activity (Clement and Mehl, 2000; Ekins et al., 2007). A common features pharmacophore was developed for PIs using nelfinavir as the most active molecule, followed by ritonavir and then amprenavir, indinavir and saquinavir as inactive (**Supplementary Table S1**). Up to 255 molecule conformations were generated with the FAST conformer generation method, with the maximum energy threshold of 20 kcal/mol.

This common features pharmacophore was applied to screen the several databases (FDA Drugs from CDD (www.collaborativedrug.com (Hohman et al., 2009)), Human metabolize database (Wishart et al., 2009), SCUT (Ekins et al., 2005), Microsource US drugs (http://msdiscovery.com/) and KEGG (Kanehisa and Goto, 2000)) using the FAST search method as previously described (Ekins et al., 2005). The quality of the molecule mapping to the pharmacophore was determined by the FitValue, which is dependent on the proximity of a compound to the pharmacophore feature centroids and the weights assigned to each centroid, where a higher FitValue represents a better fit.

MRP4 inhibitor pharmacophore: A more diverse training set of 10 MRP4 inhibitors were selected from the literature (Russel et al., 2008) and used for both a common features model and a quantitative pharmacophore (**Supplementary Table S2**). The common features model was described as above but instead

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used the CAESAR conformer generation algorithm. Dipyridamole and Quercetin were used as the most active molecules with 10 μM as the cutoff for activity. The quantitative MRP4 pharmacophore used the IC_{50} data associated with each molecule. Both pharmacophores were then used to search the SCUT database.

Mapping PGE_2 and quercetin to the PI MRP4 substrate pharmacophore: The common features pharmacophore developed with the 5 PIs was used to map PGE_2 and quercetin using the ligand pharmacophore mapping protocol with rigid fitting.

Statistical Analysis

For proliferation assays, cytotoxicity for each drug was expressed as IC_{50} values calculated with ADAPT II modeling software (Biomedical Simulations Resource, Los Angeles, CA). Non-linear and linear regression analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA).

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Results:

MRP4 ATP-hydrolysis and HIV-protease inhibitors

Previous studies have classified MRP4 substrates into the following: 1) cyclic nucleotide or nucleoside phosphate analogs; 2) anti-cancer agents; 3) steroids; and 4) prostaglandins (Russel et al., 2008). Previously, we demonstrated that some substrates (e.g., PGE_2) stimulate ATPase activity, while others show concentration-dependent biphasic kinetics (stimulation and inhibition) (Sauna et al., 2004). ATP hydrolysis mediated by ABC transporters is a useful surrogate assay to identify potential transport substrates based on the premise that the transport of substrates is powered by ATP-binding and hydrolysis; however, not all transport substrates stimulate ATP hydrolysis (Sauna et al., 2004). We evaluated a panel of HIV protease inhibitors (PIs) (amprenavir, indinavir, saquinavir, ritonavir, and nelfinavir; **Supplementary Fig. S1**) for their ability to affect ABCC4/MRP4 ATPase activity. Each of the PIs tested produced some stimulation (~50%) of ATP hydrolysis by MRP4 (**Fig. 1A)** with the positive control, PGE_2 showing >100% stimulation. A notable feature is the inability of these PIs to inhibit ATP hydrolysis, unlike cGMP, which stimulates and inhibits ATPase (Sauna et al., 2004). To monitor whether these PIs compete for the same binding site as known MRP4 substrates (Sauna et al., 2004; Wu et al., 2005), PGE_2 and quercetin were used to stimulate ATP hydrolysis. In **Fig. 1B**, we show that, among the PIs, only nelfinavir (NFV) and ritonavir (RTV) inhibit the PGE_2-stimulated ATP hydrolysis ($p< 0.0005$). We extended these studies to determine if these PI affected quercetin-stimulated activity. None of the PI inhibited

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quercetin-stimulated activity suggesting that, NFV and ritonavir share a common binding site with PGE_2, but not quercetin.

PI modulation of MRP4 transport of PMEA

To directly determine if the PIs inhibit MRP4 mediated transport we used the PMEA prodrug, [^{3}H]Bis(POM)-PMEA. Bis(POM)-PMEA bypasses the PMEA uptake carrier OAT1 (Adachi et al., 2002; Hatse et al., 1998; Schuetz et al., 1999) and is hydrolyzed to the MRP4 substrate, PMEA, by intracellular esterases. Two different cell lines of different histotype (Saos-2 (osteosarcoma) and Hek293 (kidney)) were engineered to express MRP4 because each cell line has different endogenous levels of transporters (**Fig. 2A**). Each cell type was co-incubated with 10 μM Bis(POM)-PMEA and either the positive control MRP4 inhibitor, indomethacin (100 μM) or a PI (at 50 μM) for 6 hours followed by determination of total intracellular radioactivity. Neither amprenavir, indinavir, nor saquinavir consistently increased PMEA accumulation in both cell types. In contrast, while ritonavir modestly increased PMEA concentration NFV strongly increased intracellular PMEA in HEK293 with a more modest effect in Saos2 (**Fig. 2B**). These studies suggest NFV is a good inhibitor of MRP4.

Nelfinavir is an MRP4 substrate

To determine if NFV inhibits MRP4 at concentrations that are achievable clinically (7-10 μM (Markowitz et al., 1998)), we incubated both HEK293 and Saos-2 cells with various concentrations of NFV (**Fig. 3A**) before adding

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Bis(POM)-PMEA. NFV dose-dependently increased PMEA accumulation with an estimated IC_{50} of ~ 24 and 15.8 μM, for Saos2 and Hek293, respectively. We extended these studies to determine if NFV (15 μM) was capable of reversing MRP4 mediated resistance to PMEA (**Fig. 3B**). NFV reduced the PMEA IC_{50} over 3-fold from 3.5 μM to 1.1 μM in MRP4 expressing cells. In contrast, only a modest shift in IC_{50} was observed for the empty vector cells (from 0.8 to 0.5 μM). These studies suggest NFV, by inhibiting MRP4, increases PMEA accumulation producing greater cytotoxicity.

NFV impacts MRP4 ATPase activity (**Fig. 1**) and inhibits its function to enhance accumulation of a well-known substrate, PMEA (**Figs 2B and 3A**). To test if NFV is an MRP4 substrate, either vector or MRP4 expressing cells were incubated with NFV followed by determination of intracellular concentrations of NFV by LC-MS/MS (**Fig. 3B, middle**). The uptake of NFV was lower in MRP4 cells with an estimated 40% lower steady-state accumulation of NFV.

NFV is capable of killing cells by multiple mechanisms (Gills et al., 2007; Xie et al., 2011). To extend these studies, we evaluated if MRP4 impacted NFV cytotoxicity. We cultured cells in various concentrations of NFV for 4 hr. Subsequently, cell survival was determined 72 hr post-NFV treatment. Cells expressing MRP4 had a 3 fold shift in NFV IC_{50} from 28.6 to 84.6 μM relative to vector cells (**Fig. 3B, right**).

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NFV reduces MRP4-mediated resistance to methotrexate

In total, these studies support our proposition that NFV is an MRP4 substrate. Based on these studies and the potential for HIV patients to develop cancers that are typical of the non-HIV infected population (Deeken et al., 2012; Rudek et al., 2011), we tested if NFV would affect methotrexate (MTX) cytotoxicity, as MTX is an MRP4 substrate (Chen et al., 2002) that is widely used in combination therapy to treat multiple cancers from acute lymphoblastic leukemia to breast cancer (Bonadonna et al., 1995; Pui and Evans, 2006). Like NFV, methotrexate (MTX) inhibits PGE_2-stimulated MRP4 ATP hydrolysis (Sauna et al., 2004) suggesting these compounds occupy a similar or identical substrate-binding site. Based upon these findings, we tested if NFV modulated sensitivity of MRP4 overexpressing cells to MTX (**Fig. 3C**, **left**). Cells were incubated with various concentrations of MTX for 4 hr in the absence and presence of NFV followed by incubation in drug-free medium for 72 hrs. As shown in **Fig. 3C** the IC_{50} for Saos2 empty vector cells was 1.0 μM and overexpression of MRP4 shifts the IC_{50} to 1.8 μM. Addition of NFV during the MTX incubation had minimal effect on the IC_{50} for the empty vector cells (1.0 vs. 1.1), whereas the IC_{50} for MRP4 is shifted to 1.5-fold (1.2 μM, **Fig. 3C, right**) which indicates that NFV enhances MTX cytotoxicity by MRP4 inhibition.

Constitutive MRP4 protects against NFV

To assess if constitutive levels of Abcc4/Mrp4 were sufficient to protect cells from NFV cytotoxicity and affect Abcc4/Mrp4 mediated export, we developed cell lines

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from Abcc4/Mrp4 knockout and wild-type mouse embryo fibroblasts (MEF) (**Fig. 4**). Immunoblot analysis of three independent wild-type (WT) and Abcc4/Mrp4 knockout (KO) MEF lines shows comparable amounts of Abcc4/Mrp4 among WT MEF cell lines (**Fig. 4A**,**left**). Unlike our previous studies showing compensation for Abcc4/Mrp4 absence by upregulation of Abcg2 in some tissues (e.g., spleen and brain), the knockout MEFs do not display upregulation of Bcrp/Abcg2, nor do they exhibit upregulation of Mrp5, a transporter also capable of exporting PMEA (Fukuda and Schuetz, 2012). The level of expression of Mrp4/Abcc4 is comparable to spleen but less than kidney (**Fig. 4A**, **middle**). Our previous studies have demonstrated MRP4 absence impacts accumulation of its substrates in spleen (Takenaka et al., 2007). We next determined Mrp4 function in the WT MEFs by evaluating PMEA accumulation as described above (**Fig. 2 and 3**). In WT MEFs, incubation with the ABCC4/MRP4 inhibitor Mk571 (25 μM) produced a strong increase in PMEA accumulation in WT MEFs (2.1 fold) with a small effect in KO MEFs demonstrating MRP4 is functional in WT MEFs (**Fig. 4A, right**). In addition, while Bcrp/Abcg2 transports PMEA (Takenaka et al., 2007), inhibition of Bcrp/Abcg2 with the specific inhibitor fumitremorgin C (FTC) (Rabindran et al., 2000), revealed only a small increase in PMEA accumulation indicating Abcg2 levels in MEFs are insufficient to impact PMEA accumulation. To confirm that NFV blocks Abcc4/Mrp4 mediated export of PMEA, KO and WT MEFs were pre-loaded with Bis(POM)-PMEA in media containing deoxyglucose (to inhibit glycolysis), but also lacking glucose to block regeneration of ATP, thereby depriving MRP4 the energy to fuel export as previously described

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(Schuetz et al., 1999). Subsequently, cells were washed in ice-cold media to stop PMEA accumulation. Export was restored by re-addition of warmed media (37^{o}C) containing glucose (5 mM) with or without NFV (50 μM). The rate of PMEA export from WT MEFs was 21 pmol/mg/min vs. 5.6 pmol/mg/min for KO MEFs, a 3.8-fold difference in rate and confirming a highly functional Mrp4 in WT MEFs. Further support for NFV blocking Abcc4/Mrp4 mediated export is demonstrated by NFV suppression of PMEA export from WT MEFs which is essentially complete after 30 min exposure to NFV (**Fig. 4B, left**). The total amount of PMEA exported from WT MEFs (over a 2h interval) was 60% of the total PMEA. In contrast, KO MEFs exported was <20% of total PMEA (**Fig. 4B, right**). Notably, NFV strongly reduced export of PMEA from WT MEFs to a level comparable to KO MEFs.

Enhanced MRP4 levels reduce NFV accumulation and cytotoxicity (**Fig. 3**). We assessed if *Abcc4/Mrp4* absence altered NFV cytotoxicity in KO MEFs (**Fig. 4C**). Prior to conducting these studies, we assessed sensitivity to Bis(POM)-PMEA in WT and KO MEFs by determining a viability 3 days after 4 h Bis(POM)-PMEA treatment. The absence of MRP4/ABCC4 dramatically sensitizes MEFs to Bis(POM)-PMEA producing a shift in the IC_{50} from 4 μM in WT to 0.33 μM in KO MEFs (**Fig. 4C**, **left**). The NFV IC_{50} was 2.4 μM for Abcc4/Mrp4 KO MEFs whereas it was 18.3 μM for WT MEFs, a 7.6-fold increase (**Fig. 4C, middle**). This indicates endogenous Mrp4 protects against NFV cytotoxicity. Moreover, KO

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MEFs are not generally sensitized to cytotoxic agents because WT and KO MEFs are equally sensitive to etoposide (**Fig. 4C**, **right**).

Pharmacophore models for MRP4 substrates and inhibitors

Ten diverse MRP4 inhibitors from the literature were used to generate a common feature (**Supplementary Fig. S2A**) and a quantitative pharmacophore (**Supplementary Fig. S2B**). These different approaches have been widely used for infer pharmacophores for multiple transporters such as: P-gp (Ekins et al., 2002a; Ekins et al., 2002b), OATP (Chang et al., 2005), and MATE1(Astorga et al., 2012). Notably, using a method that does not require a rigid alignment of all molecules appears advantageous when substrates and/or inhibitors are structurally diverse(Kortagere and Ekins, 2010). The diverse MRP4 common features model was used to search the SCUT database and resulted in 227 hits while the diverse MRP4 quantitative pharmacophore resulted in 192 hits (selected compounds shown in **Supplementary Tables S3 and S4**). After ranking by fit score potentially interesting molecules were highlighted along with known substrates. The diverse MRP4 common features model found 9 known MRP4 drug substrates (as well as rediscovering 5 of the training compounds) and the diverse MRP4 quantitative pharmacophore found 7 substrate hits (and rediscovered 2 of the training compounds). The array of features suggests MRP4 inhibitors are structurally promiscuous.

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Because ritonavir also appears to be a MRP4 substrate (albeit weak-**Supplementary Fig. S3**) and HIV-patients concurrently take other medicines especially during cancer chemotherapy, we extended the current pharmacophore modeling and database screening to identify other potential Mrp4 substrates. The 5 protease inhibitors produced a common feature pharmacophore model exhibiting 4 hydrogen bond acceptors (green), one hydrogen bond donor (purple) and 3 hydrophobes (cyan) (**Fig. 5**). Because NFV inhibits PGE_2 stimulated MRP4/ABCC4 ATPase, we hypothesized that the pharmacophore of NFV and PGE_2 would share similar features. The common pharmacophore of NFV was used to map PGE_2 using the ligand pharmacophore mapping protocol. PGE_2 was allowed to miss 2 features and had a fit value of 1.63. NFV (in yellow) is shown fitting to the pharmacophore, and indicates that PGE_2 and NFV, by sharing most features, share a binding site in MRP4 (**Fig. 5 B, left**). In contrast, quercetin was difficult to map and even after allowing for 3 missing features, the fit value was only 0.056 (**Fig. 5 B, middle**). Moreover, quercetin does not appear to map to the same central hydrogen bond donor as PGE_2 (**Fig. 5, right**). The strong similarity of the NFV and PGE_2 pharmacophore suggests either a similar binding mode or site on MRP4. In contrast, quercetin's markedly different pharmacophore suggests either a different binding mode or distinct binding site.

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Discussion:

The success of HAART therapy in the treatment of HIV has increased the life expectancy among those infected with HIV such that, among some, prolonged survival now leads to acquisition of non-HIV, but age associated cancers (e.g., lung and breast cancer). This HAART maintenance regimen could enhance the efficacy of cancer therapy because these agents (e.g., tenofovir, adefovir) (Gallant and Deresinski, 2003) also exhibit antitumor activity (De Clercq et al., 1999). Thus, their enhanced accumulation might increase cytotoxicity in cancers where ABCC4/MRP4 is the major route of egress. We hypothesized that some PIs (another class of drugs in HAART) might interact with ABCC4/MRP4 as either substrates or inhibitors. Among those tested (ritonavir, nelfinavir (NFV), amprenavir, indinavir, and saquinavir) only NFV was highly effective. By screening with ATPase assays on ABCC4/MRP4 programmed membrane vesicles as well as multiple cell lines overexpressing ABCC4/MRP4 and Abcc4/Mrp4 knockout MEFs, we show that NFV is an ABCC4/MRP4 substrate. This is further supported by findings demonstrating that ABCC4/MRP4 has a role in protecting against the cytotoxic effects of NFV, a recently reported cytotoxin and potential chemotherapeutic (Gupta et al., 2005; Shim et al., 2012; Yang et al., 2005). MRP4 overexpression and absence both protects and sensitizes to the cytotoxic effects of NFV, respectively. Because NFV enhances the cytotoxicity of both PMEA and methotrexate, our studies have strong implications for treating HIV-patients on HAART with cancer chemotherapeutic regimens. To

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further characterize the features of drugs interacting with ABCC4/MRP4, we developed ABCC4/MRP4 pharmacophore models. Such models have been described for other transporters as a technique to assist in identifying salient properties of substrates and inhibitors (Ekins et al., 2012). Our ABCC4/MRP4 pharmacophores included a model based upon NFV and other PIs, as well as models derived from literature described MRP4 inhibitors. There was overlap between these pharmacophores as each was dominated by multiple hydrogen bond acceptors (**Fig. 5, Supplementary Fig. S2**). The PI MRP4 substrate-pharmacophore included NFV and is likely useful to predict other drugs, especially anti-cancer and anti-viral, that could provoke enhanced cytotoxicity among HIV-infected cancer patients secondary to MRP4 inhibition.

ABCC4/MRP4 and ABCG2 share a number of common substrates from endogenous compounds (e.g. cyclic GMP) (de Wolf et al., 2008; Russel et al., 2008) to antiretrovirals (PMEA, tenofovir (Takenaka et al., 2007) to anti-cancer chemotherapeutics (methotrexate and irinotecan)(Chen et al., 2002; Volk and Schneider, 2003). However, with respect to PI, none of those tested were good ABCG2 substrates (Kis et al., 2010). Notably, NFV is only an inhibitor of ABCG2 (Gupta et al., 2004). Considering ABCG2 and ABCC4/MRP4 are broadly expressed (Takenaka et al., 2007) these findings suggest that in cells (both normal and cancer infected with HIV) that co-express both transporters, ABCG2 inhibition by NFV might be over-ridden by ABCC4/MRP4 mediated export of NFV. This suggests ABCC4/MRP4 has not only the potential to disarm

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nucleoside-based anti-viral inhibitors (e.g., PMEA, tenofovir, etc), but also protease inhibitors like NFV that share a pharmacophore recognized by ABCC4/MRP4 (**Fig. 6**). It is notable that our PI MRP4 substrate pharmacophore analysis suggests that atazanavir (**Supplementary Table S4**) is a potential ABCC4/MRP4 substrate. Although not formally tested, this seems to be supported by recent studies of Bierman et al (Bierman et al., 2010). Validating this *in vitro* may represent future work.

After screening a number of clinically relevant HIV drugs, our studies find that unlike several other common PIs, only NFV strongly interacts with human and murine Abcc4/Mrp4. NFV appears to be both a substrate and an inhibitor of MRP4. The ability of ABC transporter substrates to act as both an inhibitor and substrate is not unique to either NFV or MRP4/ABCC4. Notably, tyrosine kinase inhibitors in their interactions with ABCB1 or ABCG2 display both substrate and inhibitor properties (Brozik et al., 2011).

How does NFV inhibit MRP4/ABCC4? Because NFV was recently shown to interact with multiple kinases (Xie et al., 2011) we might infer that it interacts with the ABCC4/MRP4 nucleotide binding domain producing a reduction in ATPase activity. However, this molecular mechanism seems unlikely for two reasons: first NFV only stimulates and does not inhibit ABCC4/MRP4 ATPase activity, and second the predicted ATP binding site that NFV reportedly interacts with on

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EGFR bears little sequence resemblance to the ABCC4/MRP4 nucleotide binding domains (Kool et al., 1997; Xie et al., 2011).

The divergent substrate-dependent effect of NFV on substrate stimulated ATPase suggests either distinct binding sites or a different binding mode in the same binding pocket. For example, NFV inhibits PGE_2 stimulated MRP4/ABCC4 ATPase. This suggests NFV and PGE_2 share either a common binding site or have a similar biding mode. Because pharmacophore modeling revealed shared properties of both PGE_2 and NFV on ABCC4/MRP4 it is likely a similar binding mode. This proposition is supported by PGE_2 fit value of 1.62 with respect to NFV pharmacophore. In contrast, quercetin poorly fits the NFV pharmacophore and just minimally overlaps (Fit value 0.056). This agrees with NFV inability to affect quercetin stimulated ATPase. As quercetin can stimulate ABCC4/MRP4 ATPase, but is not inhibited by NFV (Fig1B, right panel), it is likely quercetin binding mode or site are distinct. At this point, we cannot distinguish if different MRP4/ABCC4 substrates and inhibitors have distinct binding sites or binding modes. However, we note that van Aubel et. al. (Van Aubel et al., 2005) showed that ABCC4/MRP4 concurrently transports urate and either cAMP or cGMP. This suggests MRP4/ABCC4 has a large binding pocket that might allow occupation of multiple substrates/inhibitors that adopt different binding modes. Moreover, the propensity of a substrate to assume different binding modes on MRP4 might increase the likelihood of drug-drug interactions among cytotoxic substrates relying on MRP4 export. This is supported, in part, by our studies

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showing NFV increases the cytotoxicity of methotrexate in MRP4 overexpressing cells a finding consistent with the evidence that, like NFV, methotrexate shares a binding mode with PGE_2 as shown by its inhibition of PGE_2 stimulated MRP4/ABCC4 ATPase activity (Sauna et al., 2004).

NFV has shown effectiveness as a potential chemotherapeutic against several different tumor cell lines, possibly by suppressing activity of the Akt pathway (Gills et al., 2007). Consistent with this, a recent proteome-screen predicted that NFV was capable of binding Akt as well as other members in the protein kinase superfamily (Xie et al., 2011). While Akt activity may impact the sensitivity of tumor cells to NFV, our study reveals MRP4/ABCC4 amounts determine the cellular concentration of NFV. Consequently the accumulation of NFV in target cells, tissues or organs will be determined by the amount of MRP4/ABCC4. These findings suggest that agents impairing MRP4/ABCC4 export might enhance cytotoxicity by increasing intracellular concentration of NFV. Conversely, we show NFV, as a new ABCC4/MRP4 inhibitor, reduces export thereby increasing the toxic effects of known ABCC4/MRP4 substrates (e.g., adefovir and methotrexate, respectively) by way of drug-drug interactions. A further extension of these findings is that inhibition of MRP4/ABCC4 mediated drug export has the potential to alter metabolism of drugs, especially in the kidney, which has high levels of MRP4/ABCC4 (Leggas et al., 2004; Takenaka et al., 2007). In addition, our computational modeling provides new insights into the pharmacophore of drugs with the potential to interact with ABCC4/MRP4,

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enabling us to predict which drugs might alter ABCC4/MRP4 function. In *toto*, these *in vitro* and computational pharmacophore findings highlight an important therapeutic mechanism that might explain both unexpected enhancements in anti-tumor efficacy, but also host toxicities that could occur when treating HIV-infected cancer patients on HAART regimens.

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Conducted experiments: Fukuda, Takenaka, Wu, and Ekins

Contributed new reagents or analytical tools: Sparreboom and Cheepala

Performed data analysis: Fukuda, Takenaka, Sparreboom, Wu, Ekins, Ambudkar, and Schuetz

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Figure Legends

Figure 1. Nelfinavir and Ritonavir modulate MRP4 ATPase activity. (**A**) The BeFx-sensitive ATPase activity of ABCC4/MRP4 was determined using the P_i release assay in the presence of various concentrations of nelfinavir (NFV), ritonavir (RTV), amprenavir (APV), saquinavir (SQV) or indinavir (IDV). PGE_2 a known MRP4 substrate (Reid et al., 2003) that stimulates ATPase activity (Sauna et al., 2004) was used as a positive control. (**B**) The effect of indicated compounds on (left) PGE_2– and (right) quercetin (QCE)-stimulated ATPase activity was evaluated.

Figure 2. Among common HIV-Protease Inhibitors only nelfinavir is an inhibitor. (**A**) Immunoblot analysis of MRP4, P-gp, or ABCG2 expression in either HEK293 or Saos2 cells programmed with either empty vector of an MRP4 expression vector. (**B)** Bis(POM)-PMEA uptake by Saos2 or HEK293 cells containing either empty vector or an MRP4 expression vector was determined in the presence of the indicated PI's.

Figure 3. Nelfinavir is a substrate and inhibitor of MRP4. (**A**) Bis(POM)-PMEA uptake was determined in Saos2 or HEK293 cells containing either empty vector or an MRP4 expression vector in the presence of various NFV concentrations. (**B**) NFV strongly increases Bis(POM)-PMEA cytotoxicity in MRP4 expressing cells (**left**). NFV uptake is strongly reduced in MRP4

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expressing cells (**middle**). MRP4 expression reduces NFV cytotoxicity (**right**). (**C**) Presence of 50 μM NFV (**right**) increases methotrexate (MTX) cytotoxicity in MRP4 expressing-Saos2 cells compared to MTX alone (**left**).

Figure 4. MRP4/ABCC4 absence reveals nelfinavir is a substrate and Inhibitor. (**A**) Immunoblot analysis of 3 independent clones for each genotype reveals no upregulation of ABCC1, ABCC5, and ABCG2 in MRP4 KO MEFS (**left**). MEFs express MRP4 at levels comparable to normal tissues containing functional MRP4 (**middle**). BCRP/ABCG2 is only minimally functional in MRP4 KO MEFs (**right**). (**B**) NFV blocks MRP4/ABCC4 mediated export of PMEA. KO and WT MEFs were incubated with Bis(POM)-PMEA under energy-depleted conditions. Subsequently, energy containing media was restored and export of PMEA determined (**left**). 120 min after PMEA export was initiated the proportion of PMEA in the media and cells was determined and expressed as % of Total (**right**). (**C**) MRP4 KO MEFs were more sensitive to the cytotoxic effects of Bis(POM)-PMEA (**left**) and NFV (**middle**) but not to etoposide (**right**).

Figure 5. A pharmacophore model of MRP4 reveals distinct substrate properties. (**A**) PI MRP4 substrate common features pharmacophore showing nelfinavir mapped. Features include hydrogen bond acceptors (green) with vectors, hydrogen bond donor (purple) with vectors and hydrophobic features (cyan). (**B**) Mapping (**left**) PGE_2 and (**middle**) quercetin onto the common pharmacophore with NFV shown in yellow. (**Right**) NFV (yellow), PGE_2 (red),

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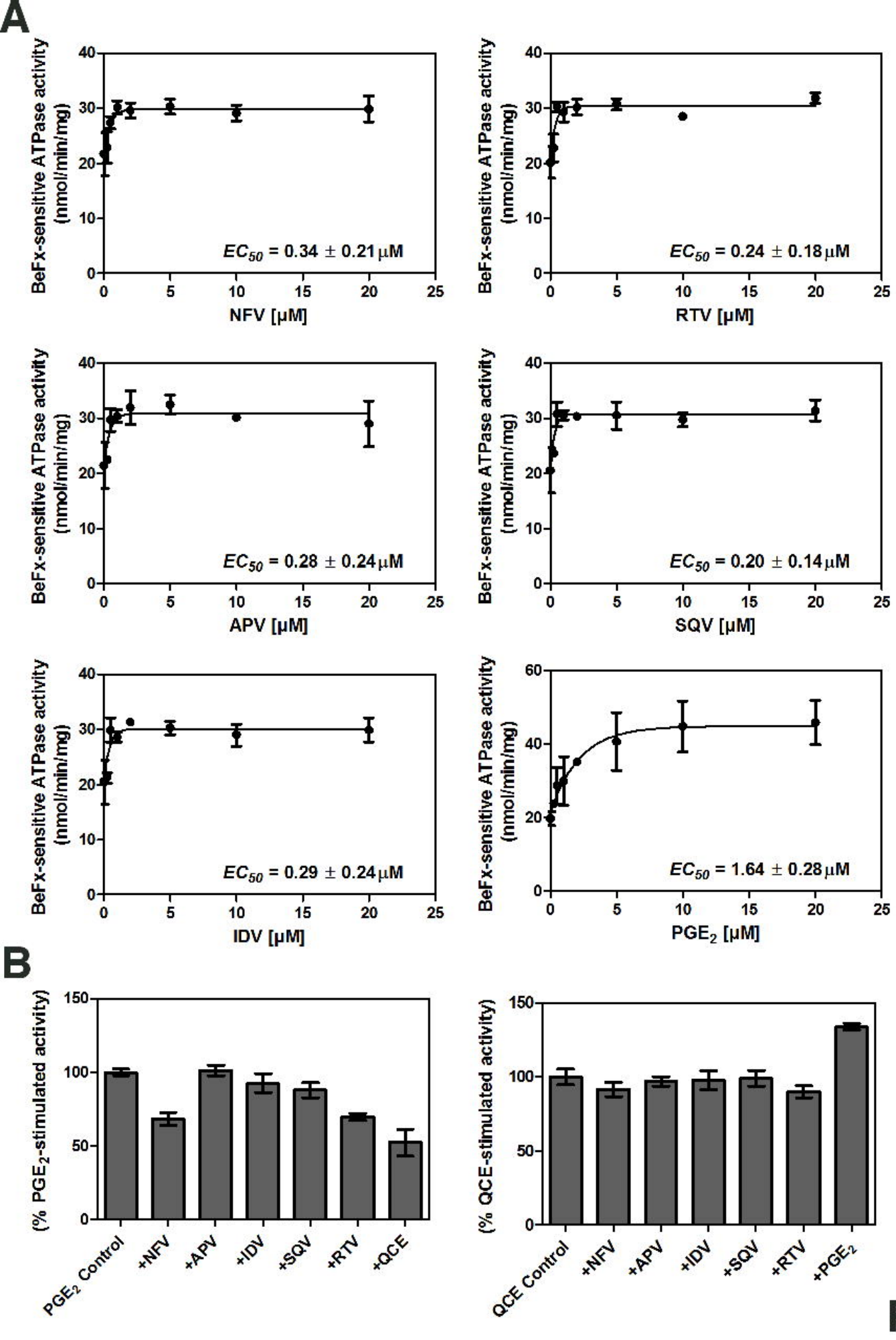
and quercetin (grey) are mapped to show that quercetin does not overlap with other two compounds.

Figure 6. Schematic showing two potential drug binding pockets in MRP4 and possible drug-drug interactions. (**A**) Quercetin (QCE) and PGE_2 have distinct binding sites. (**B**) PMEA, methotrexate (MTX), and NFV compete for the same binding pocket as PGE_2. The black arrow on panel **A** shows the direction of transport.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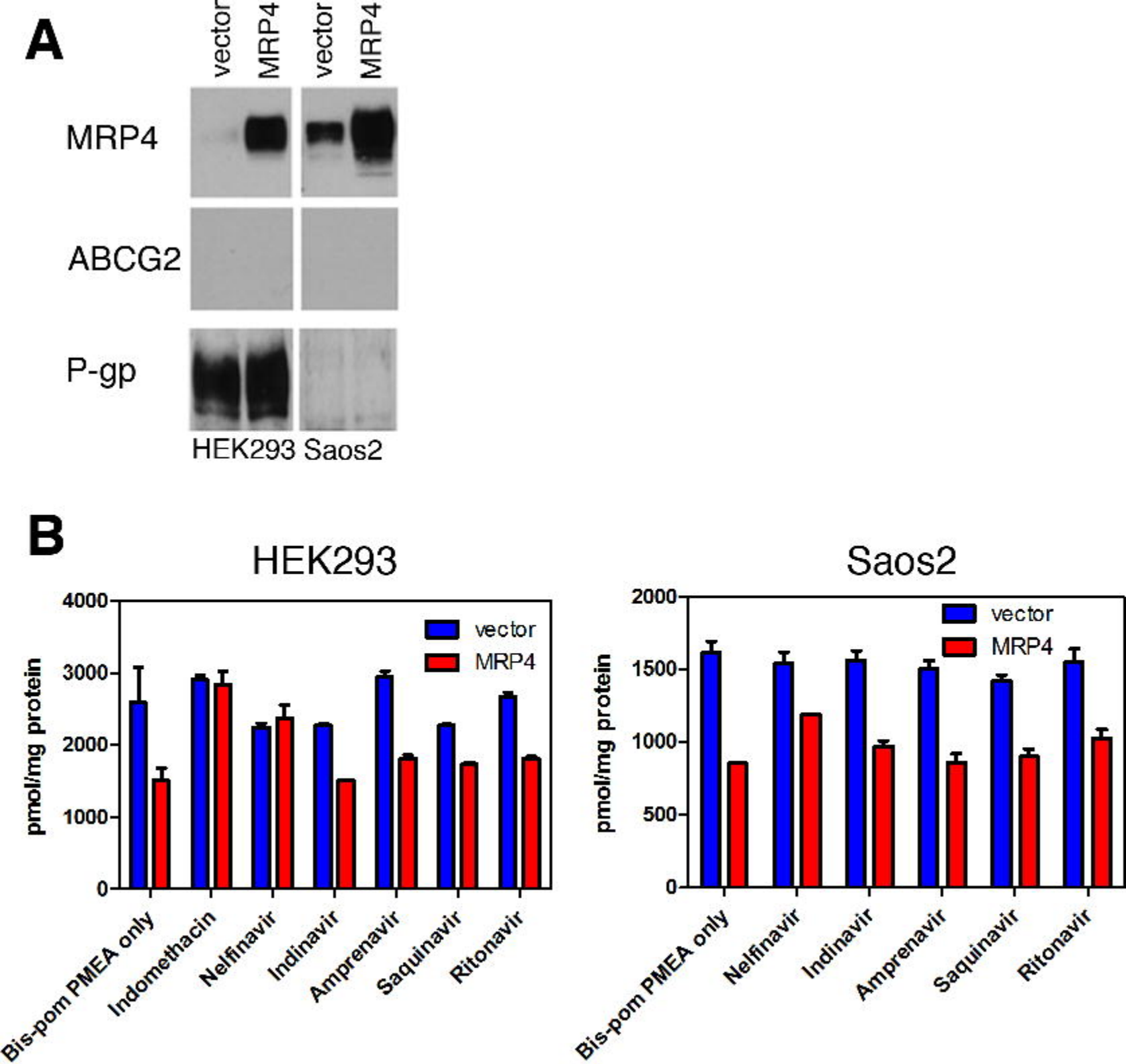
BeFx-sensitive ATPase activity (nmol/min/mg)
EC50 = 0.34 ± 0.21 μM
NFV [μM]
EC50 = 0.24 ± 0.18 μM
RTV [μM]
EC50 = 0.28 ± 0.24 μM
APV [μM]
EC50 = 0.20 ± 0.14 μM
SQV [μM]
EC50 = 0.29 ± 0.24 μM
IDV [μM]
EC50 = 1.64 ± 0.28 μM
PGE2 [μM]
B
(% PGE2-stimulated activity)
PGE2 Control
+NFV
+APV
+IDV
+SQV
+RTV
+QCE
(% QCE-stimulated activity)
QCE Control
+NFV
+APV
+IDV
+SQV
+RTV
+PGE2

Fig. 1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MRP4
vector
MRP4
MRP4
ABCG2
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HEK293 Saos2
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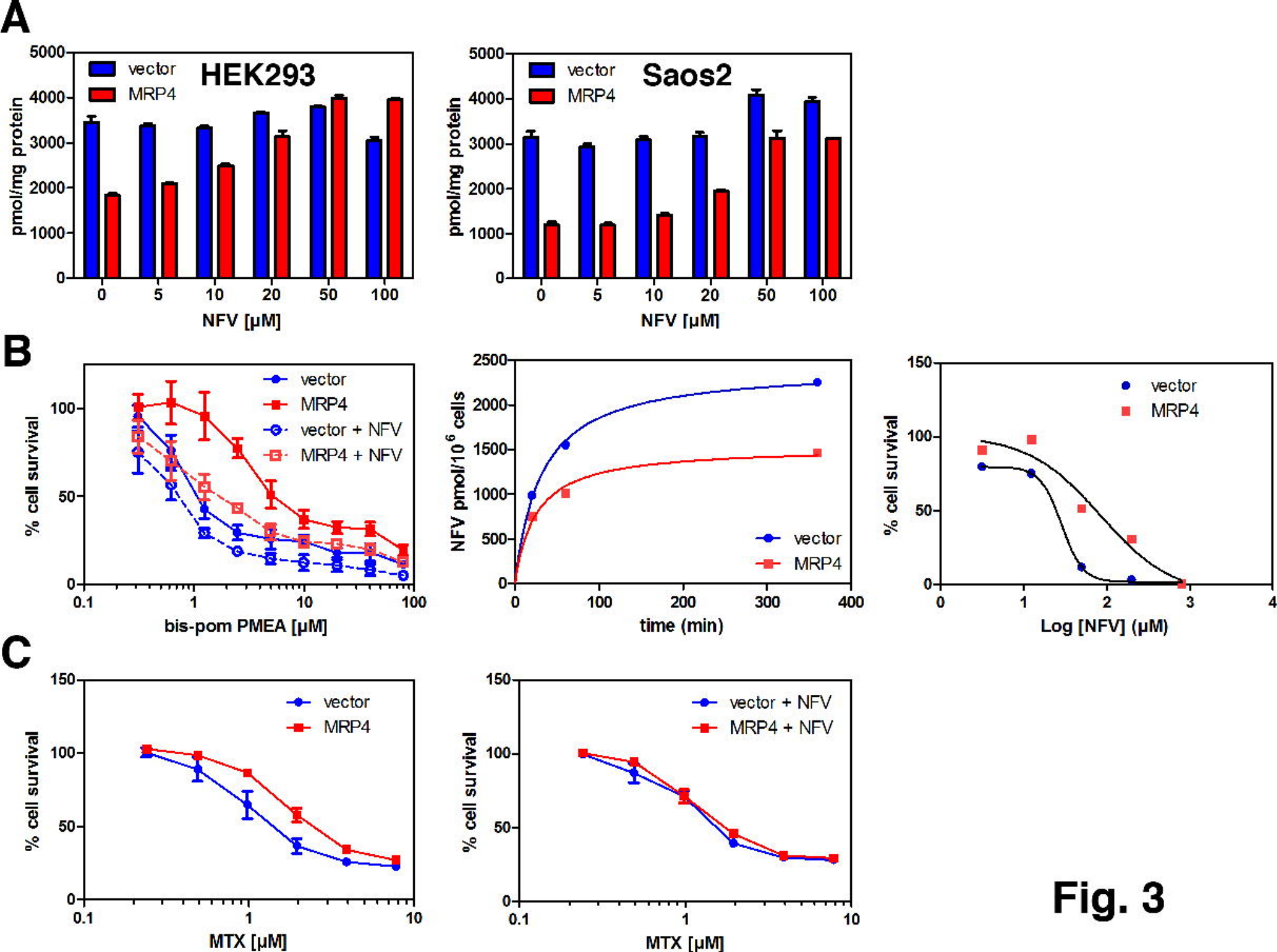
HEK293
Saos2
pmol/mg protein
4000
3000
2000
1000
0
2000
1500
1000
500
0
vector
MRP4
Bis-pom PMEA only
Indomethacin
Nelfinavir
Indinavir
Amprenavir
Saquinavir
Ritonavir

Fig. 2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vector
MRP4
pmol/mg protein
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4000
3000
2000
1000
0
0
5
10
20
50
100
NFV [μM]
Saos2
vector
MRP4
pmol/mg protein
NFV [μM]
B
vector
MRP4
vector + NFV
MRP4 + NFV
% cell survival
bis-pom PMEA [μM]
NFV pmol/10^6 cells
time (min)
vector
MRP4
% cell survival
Log [NFV] (μM)
vector
MRP4
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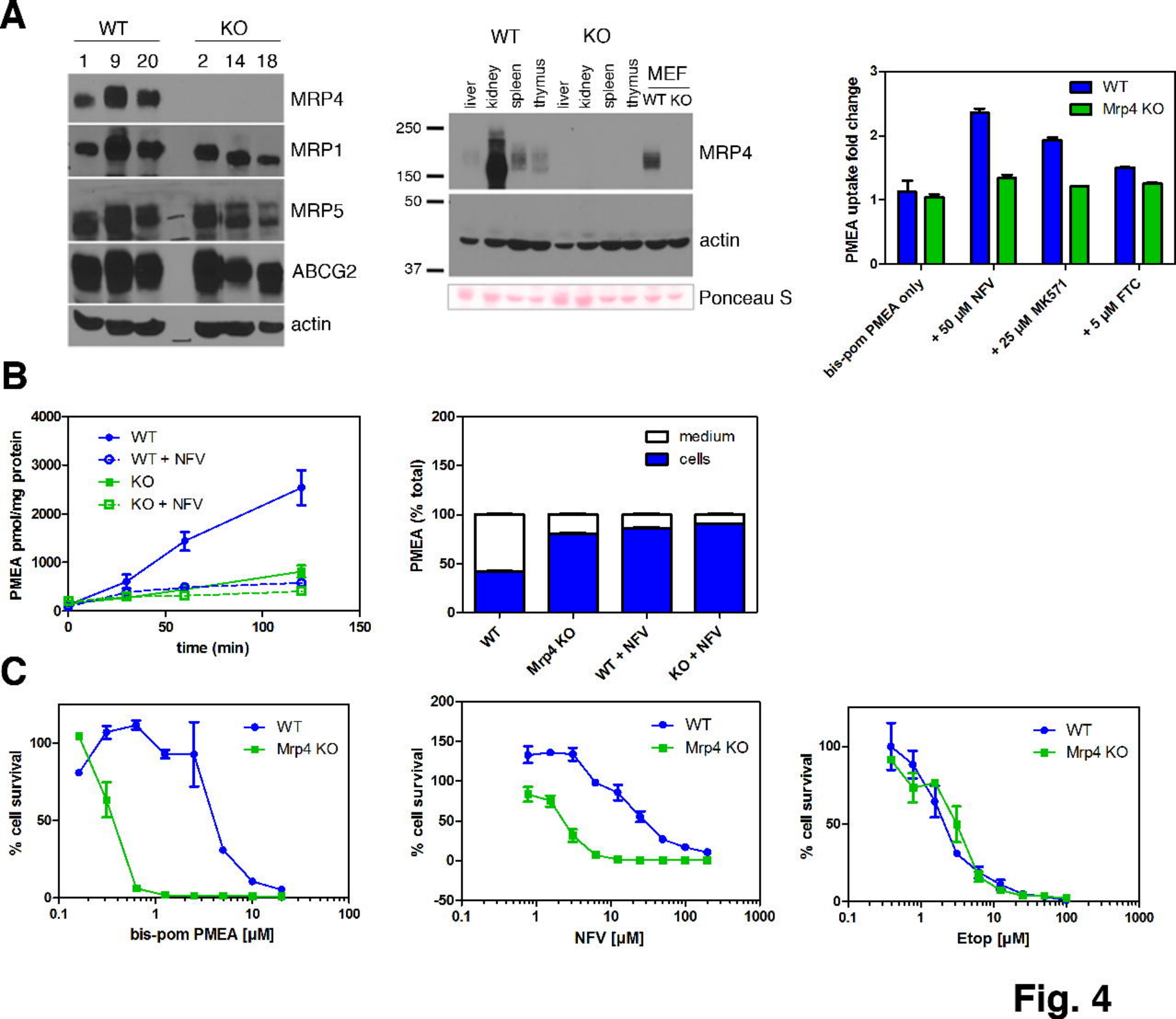
% cell survival
MTX [μM]

Fig. 3


A
WT
KO
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MRP4
MRP1
MRP5
ABCG2
actin
WT
KO
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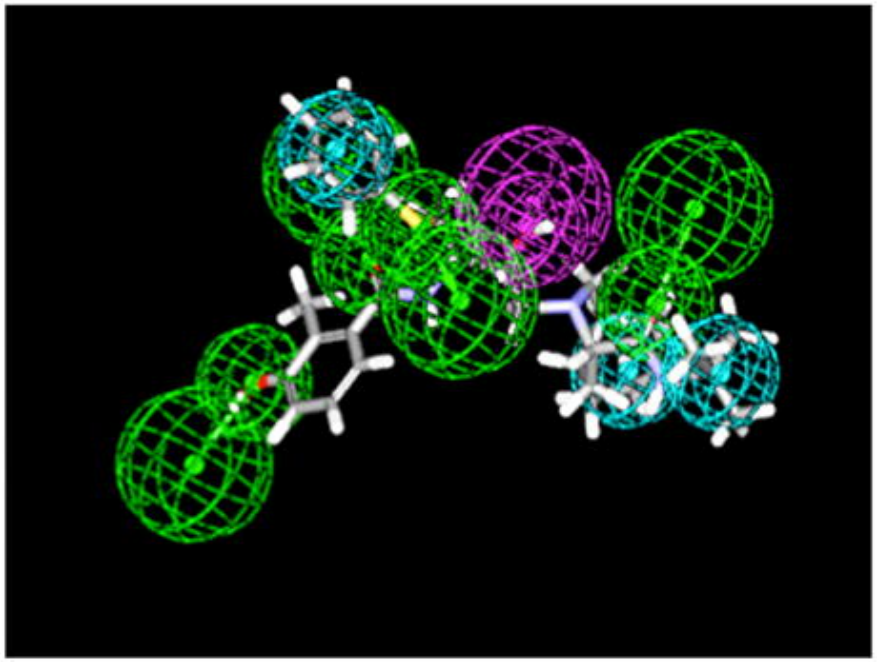
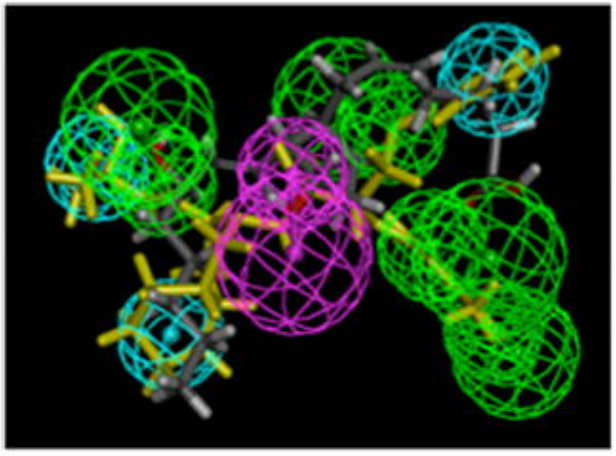
250
150
50
37
MRP4
actin
Ponceau S
PMEA uptake fold change
WT
Mrp4 KO
bis-pom PMEA only
+ 50 µM NFV
+ 25 µM MK571
+ 5 µM FTC
B
PMEA pmol/mg protein
WT
WT + NFV
KO
KO + NFV
time (min)
PMEA (% total)
medium
cells
WT
Mrp4 KO
WT + NFV
KO + NFV
C
% cell survival
WT
Mrp4 KO
bis-pom PMEA [µM]
NFV [µM]
Etop [µM]

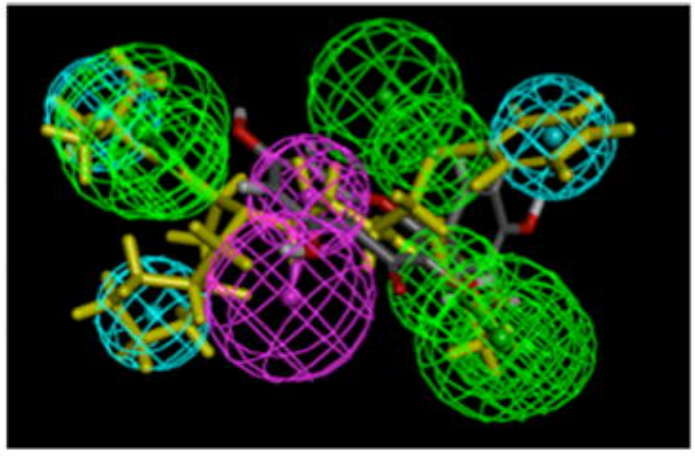
Fig. 4

A



B





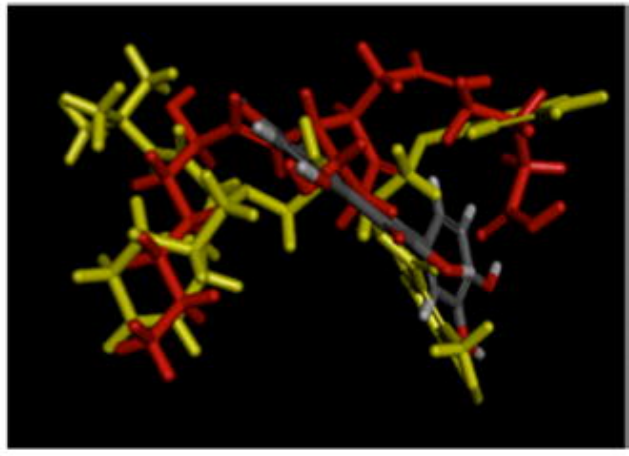


Fig. 5

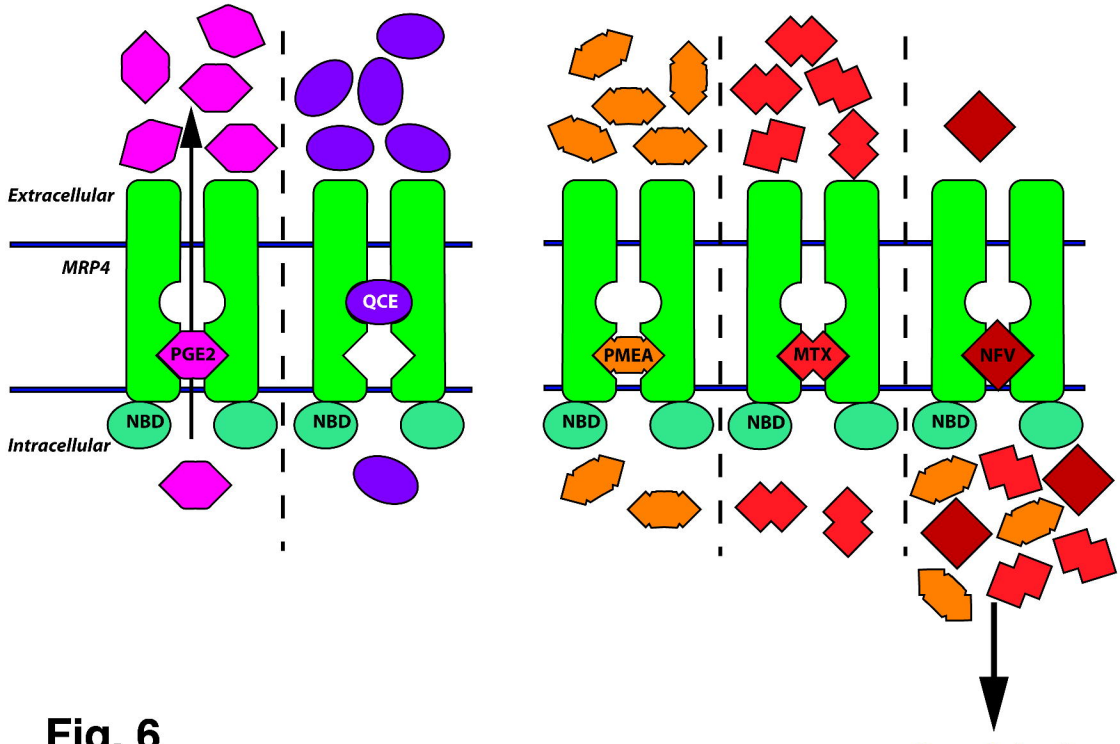

Extracellular
MRP4
Intracellular
PGE2
QCE
NBD
NBD
PMEA
MTX
NFV
NBD
NBD
NBD
Accumulation of
cytotoxic drugs

Fig. 6