

MRP4- and MRP 5-Mediated Efflux of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) by Microglia.

Shannon Dallas¹, Lyanne Schlichter²
and Reina Bendayan¹

¹Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada; ²Division of Cellular and Molecular Biology, Toronto Western Research Institute, University Health Network, and Department of Physiology, University of Toronto, Toronto, Ontario, Canada.

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Corresponding Author: Dr. Reina Bendayan
Associate Professor
Department of Pharmaceutical Sciences
Faculty of Pharmacy, University of Toronto
19 Russell St.
Toronto, Ontario, M5S-2S2
Canada
Tel: 416-978-6979
Fax: 416-978-8511
email: r.bendayan@utoronto.ca

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Abbreviations List: AZT-MP, azidothymidine-monophosphate; bis(POM)PMEA, bis(pivaloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine; bp, base pairs; BSO, L-buthionine-[S,R]-sulfoximine; GSH, glutathione; HIV-1, human immunodeficiency virus type 1; MRP, multidrug resistance protein; P-gp, P-glycoprotein, PMEA, 9-(2-phosphonylmethoxyethyl)adenine; rMRP, rat multidrug resistance protein; RT-PCR, reverse transcriptase-polymerase chain reaction; TLC, thin layer chromatography.

Abstract

The pathogenesis of Human immunodeficiency virus-associated dementia has been linked to microglial responses following infection. We have recently confirmed expression of several ATP-dependent efflux transporters in microglia, namely multidrug-resistance protein 1 (MRP1) and P-glycoprotein (P-gp). In the present study, we investigated whether cultured rat microglia express two additional MRP family members, rMRP4 and rMRP5. Using reverse transcriptase-polymerase chain reaction, rMRP4 and rMRP5 mRNA was detected in primary cultures of microglia and in a rat microglia cell line, MLS-9. Western blot analysis further confirmed protein expression of the two MRP isoforms in MLS-9 cells. Bis(pivaloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine [bis(POM)PMEA], a lipophilic ester pro-drug of the well-characterized MRP4 and 5 substrate 9-(2-phosphonylmethoxyethyl)adenine (PMEA), was chosen to examine transport characteristics in MLS-9. Using Thin Layer Chromatography, we verified that more than 90% of radioactivity recovered in MLS-9 loaded with 1 μ M [3 H]bis(POM)PMEA for 1 hr under ATP depleting conditions was converted to PMEA. Efflux of PMEA by MLS-9 cell monolayers was ATP-dependent, glutathione-independent and significantly inhibited by several MRP inhibitors (i.e., sulfinpyrazone, genistein, indomethacin, probenecid) as well as the antiretroviral drug azidothymidine-monophosphate. Similar results were not observed in MRP1 or P-gp overexpressing cell lines suggesting that PMEA is not a substrate for either P-gp or MRP1. These studies provide further evidence that microglia express multiple subfamilies of ATP-Binding Cassette transporters (i.e., P-gp, MRP1, MRP4, MRP5) that could restrict permeation of several different classes of antiretroviral drugs in a brain cellular target of HIV-1 infection.

The multidrug resistance proteins (MRP's) are a collection of membrane-bound ATP-dependent transporters that confer resistance to a variety of structurally unrelated compounds. All MRP isoforms utilize ATP hydrolysis to export substrates and are a part of the larger ATP binding cassette superfamily of proteins, which includes P-glycoprotein (P-gp). Nine functional MRP's have been identified in humans (MRP1-9), and range in size from 1325 to 1545 amino acids. Complementary DNA's for rat MRP isoforms of the first 6 proteins (designated rMRP1-6) have also been characterized and show 66 – 94% homology with their human counterparts. The MRPs can be divided into three different subgroups based on their membrane topologies: MRP1-3, and MRP6-7 contain three transmembrane domains and two nucleotide-binding sites. MRP4, 5, and 8 contain only two membrane-spanning domains and two nucleotide-binding sites, while MRP9 appears to be a “half-transporter”, containing only one nucleotide-binding domain. Not surprisingly, the various MRP's show remarkable differences in their tissue distribution, substrate specificities and proposed physiological functions.

MRP4, 5 and 8 are the only MRP isoforms presently identified that confer resistance to cyclic nucleotides (cAMP; cGMP), acyclic nucleoside phosphonates such as 9-(2-phosphonylmethoxyethyl)adenine (PMEA), and monophosphorylated nucleoside analogs such as azidothymidine-monophosphate (AZT-MP; 2'-azido-2',3'-dideoxythymidine, zidovudine-monophosphate), thioxanthosine monophosphate and thioinosine monophosphate (Schuetz et al., 1999; Jedlitschky et al., 2000; Wijnholds et al., 2000; Chen et al., 2001; Wielinga et al., 2002; Guo et al., 2003). Neither MRP4 nor MRP5 interacts with typical substrates of MRP1-3, including vincristine, etoposide, daunorubicin and cisplatin (McAleer et al., 1999; Lee et al., 2000), and are therefore functionally quite distinct from these proteins.

Both MRP4 and MRP5 are highly expressed in a variety of tissues, including various brain compartments. High levels of MRP5 mRNA transcripts have been identified in whole brain homogenates from both humans and rats, as well as within isolated brain segments including cerebral cortex, cerebellum and hippocampus (Belinsky et al., 1998; McAleer et al., 1999). Expression of MRP4 mRNA in whole brain appears to be only moderate (Lee et al., 1998). Interestingly, in brain microvessel endothelial cells MRP4 and MRP5 transcripts were reported to be higher than MRP1, an isoform that is highly expressed in the brain (Zhang et al., 2000). Expression patterns of the MRP's in brain parenchymal cells, such as microglia and astrocytes are only beginning to be investigated. Microglia act as a reservoir for the human immunodeficiency virus (HIV) and are implicated in the pathogenesis of a variety of neurodegenerative disorders including HIV-associated dementia, Parkinson's, and Alzheimer's disease (Nelson et al., 2002; Liu and Hong, 2003). We have recently characterized functional expression of rMRP1 in a rat microglia cell line, MLS-9 (Dallas et al., 2003). In two other studies, several other MRP mRNA's, including rMRP4, have been identified in primary cultures of rat microglia (Ballerini et al, 2002; Hirrlinger et al., 2002); however, rMRP5 was only observed by Hirrlinger et al. (2000). Neither group assessed the ability of these MRP proteins to actively extrude compounds, and thereby contribute to drug resistance. In the present study, we examined transport properties of the MRP4 and MRP5 substrate, PMEA, in a rat microglia cell line, MLS-9, and show that rMRP4 and rMR5 are not only present in these cells, but also functionally active.

Methods and Materials

Chemicals. [^3H]9-(2-phosphonylmethoxyethyl)adenine (PMEA; 26.2 Ci/mmol), and [^3H]bis(pivaloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine (bis(POM)PMEA; 2.3 Ci/mmol) were purchased from Moravek Chemicals (Brea, CA). Unlabelled bis(POM)PMEA was a generous gift of Gilead Sciences (Foster City, CA). Indomethacin, 2-deoxy-D-glucose, L-buthionine-[S,R]-sulfoximine, genistein, probenecid, sodium azide, sulfinpyrazone and azidothymidine-monophosphate were purchased from Sigma-Aldrich (Oakville, ON, Canada). PSC883 and GF120918 were kind gifts of Novartis (Basel, Switzerland) and Glaxo-Wellcome (Hertfordshire, United Kingdom), respectively. The polyclonal antibody M5I-1 was purchased from Kamiya Biomedical (Seattle, WA). The polyclonal antibody rM4-p1, was a kind gift from Dr. F. Russel (Nijmegen Center for Molecular Life Sciences, Nijmegen, The Netherlands).

Cell Culture. Cultures of a microglia cell line (MLS-9) were prepared as described previously (Cayabyab and Schlichter, 2002). Briefly, neopallia of 2- or 3-day old Wistar rat pups were dissociated enzymatically and cultured in endotoxin-free medium (Invitrogen, Burlington, ON, Canada) for 10-12 d without feeding. Following shaking of 10-12 day old flasks, essentially pure microglia cultures were obtained. The MLS-9 microglia cell line was originally produced from such cultures by treating them with colony stimulating factor-1 to increase proliferation. Microglia colonies arose after 2-6 wk, which continued to grow in the absence of added growth factors. The MLS-9 cell line was derived from one of these colonies. Whereas MLS-9 cells were negative for characteristic markers of astrocytes (glial fibrillary acidic protein) and fibroblasts (fibronectin), they stained for several microglia cell markers, including isolectin B4, OX-42 antibody and ED-1 antibody (Cayabyab and Schlichter, 2002).

MLS-9 cells also exhibit morphological similarities and phenotypical characteristics of primary microglia, such as pinocytosis of dyes (DiI-acetylated LDL, or Lucifer Yellow).

Monolayers of MLS-9 cells (passages 5-32) were cultured on 75 cm² polystyrene tissue culture flasks (Sarstedt, St.-Leonard, QC, Canada) or multiwell dishes (BD Biosciences, San Jose, CA) at 37°C, in 5% CO₂/95% air and 95% humidity. For transport experiments, cells were grown to confluency (4-6 d following seeding) in Minimum Essential Medium (pH 7.2) containing 2 mM L-glutamine, 5.55 mM D-glucose, 5% fetal bovine serum, 5% horse serum and 0.5% penicillin/streptomycin. For sub-culturing, subconfluent MLS-9 cells (~75% confluent) were released from culture flasks using a 15 mM sodium citrate solution, containing 130 mM NaCl, 10 mM glucose and 10 mM HEPES, pH 7.4.

The human MRP1 overexpressing cell line, WT-MRP1, and parental cell line, VF-HeLa, were kind gifts from Dr. S.P.C. Cole (Queen's University, Kingston, ON, Canada). Cells were maintained in Dulbecco's Modified Eagle Medium, containing 4 mM L-glutamine, 25 mM glucose, 400 µg/ml G418 with 5% fetal bovine serum, (pH 7.1), and were subcultured weekly using 0.05% trypsin. The P-gp overexpressing cell line, CH^RC5, was kindly provided by Dr. V. Ling (University of British Columbia, Vancouver, BC, Canada). Cells were maintained in Minimum Essential Medium-alpha, containing 2 mM L-glutamine, 5.55 mM D-glucose, 10% fetal bovine serum, with 0.5% penicillin/streptomycin (pH 7.2), and were subcultured weekly using 0.25% trypsin. Overexpression of P-gp in CH^RC5 cells and MRP1 in WT-MRP1 cells was previously confirmed in our laboratory (Lee et al., 2001; Dallas et al., 2003).

The human MRP4- and MRP5-overexpressing HEK293 cells were a generous gift of Dr. P. Borst (Cancer Institute, Amsterdam, The Netherlands). Cells were maintained in Dulbecco's

Modified Eagle Medium (GLUTAMAX™), containing 5.55 mM D-glucose, 5 % fetal bovine serum and were subcultured weekly using 0.25% trypsin.

RT-PCR Analysis. RT-PCR was performed as previously described by our group (Dallas et al., 2003). Total RNA was isolated using TRIZOL reagent, and then reverse transcribed at 42°C for 50 min in 20 µl of 1x RT buffer (75 mM KCl, 50 mM Tris, pH 8.3, 3 mM MgCl₂, 10 mM DTT) with 0.5 mM dNTPs, 25 µg/ml pd(N)₆, and 40 units SuperScript II reverse transcriptase. Then 2 µl of cDNA was amplified by PCR using a GeneAmp PCR2400 system (PerkinElmer, Mississauga, ON, Canada) in 20 µl of reaction mixture, containing 1 Unit Platinum Taq polymerase, 1x PCR buffer, 1.5 mM MgCl₂, 0.2 mM d(NTP)s, and 0.8 µM forward and reverse primers. The sequences of rMRP4 primers were: forward primer, 5'- GGA CAC TGA ACT AGC AGA ATC -3' and reverse primer, 5'- TGC ACT GGG CAA ACT TCT -3'. The rMRP5 primers were: forward primer, 5'- AGA ATA CAT CAT CCC CAG TCC -3' and reverse primer, 5'- GCC ACA CAT CCT CCA TTA -3'. The RT-PCR program consisted of 35 cycles of 3 phases: denaturing (20 sec at 94°C), annealing (30 sec at 55°C), and extension (30 sec at 72°C). Amplified DNA products were resolved on 1.5% agarose gels with 0.5 mg/ml ethidium bromide. Each RT-PCR product was purified and sequenced (ACGT Corp., Toronto, ON, Canada). All reagents were from Invitrogen (Burlington, ON, Canada).

Western Blotting Analysis. Crude membrane proteins were obtained as follows: cells were harvested by centrifugation (400xg), the pellet collected and cells lysed for 30 min at 4°C in a 250 mM sucrose buffer containing 1 mM EDTA and protease inhibitor cocktail (P8340; Sigma-Aldrich, Mississauga, ON, Canada). Cell lysates were homogenized (10,000 rpm for three cycles of 20 sec each) in a Dounce homogenizer and homogenates were centrifuged (3000xg) to

remove cellular debris. The supernatant was further centrifuged (100,000xg for 1 hr at 4°C) and the resulting membrane pellet was resuspended and frozen at -20°C until use.

Crude membrane samples were separated on 8.5 - 10% polyacrylamide gels using a Bio-rad minigel system (Bio-Rad, Hercules, CA), and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes. Ponceau S staining verified protein transfer and the membranes were blocked overnight (4°C) in Tris-buffered saline (15 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.05% Tween-20 (TBS-T) and 5% dry skim milk powder. The membranes were then incubated at room temperature with either the polyclonal MRP4 antibody rM4-p1 (1:500 dilution) or a polyclonal MRP5 antibody M5I-1 (1:500 dilution). These MRP4 and MRP5 antibodies recognize epitopes in the carboxy portions of the proteins (Van Aubele et al., 2002; Wijnholds et al., 2000). Following several washes, the membranes were then incubated in the presence of either anti-rabbit (1:5000) or anti-rat (1:5000) horseradish peroxidase-linked secondary antibodies (Sigma-Aldrich, Mississauga, ON, Canada). Proteins were visualized using enhanced chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL).

Transport Studies. Cells were plated onto multiwell plates at densities of ~40,000-125,000 cells/well and efflux of [³H]PMEA was measured according to the methodology of Schuetz et al. (1999), with a few modifications. Briefly, cells were loaded with 1 μM [³H]bis(POM)PMEA for 1 hr (37°C) in glucose-free Dulbecco's Modified Eagle Medium containing 10 mM sodium azide and 10 mM 2-deoxy-D-glucose (i.e., under ATP-depleting conditions). At the end of the loading period, cells were washed with ice-cold 0.16 M NaCl and incubated with complete medium (i.e., Dulbecco's Modified Eagle Medium containing 10 mM D-glucose) in the presence or absence of an inhibitor. At predetermined time intervals, supernatant was removed and the wells were

washed with ice-cold 0.16 M NaCl. Radioactivity in the medium samples was measured by a Beckman scintillation counter (Beckman Coulter; Fuller, CA) with automated quench correction. Data were standardized to the amount of cell protein (mg/ml) in each culture plate, determined by the Bradford colorimetric method, with bovine serum albumin (Sigma-Aldrich, Mississauga, ON, Canada) as the standard. Viability of the various cell cultures in the presence of sodium azide and bis(POM)PMEA was verified using the Trypan blue exclusion test. Following 1 hr incubation in the presence or absence of 10 mM sodium azide, 10 mM 2-deoxy-D-glucose, and 1 μ M bis(POM)PMEA, viable (unstained) and dead (stained) cells were counted using a hemocytometer (Neubauer chamber).

Following sodium azide and 2-deoxy-D-glucose incubation, depletion of intracellular ATP was verified in all cell lines using a modified HPLC method (Stocchi et al., 1985). Briefly, cell monolayers were solubilized in 1 M KOH, centrifuged at 5000 *g* in Amicon Ultra filters (Millipore, Billerica, MA), and the resulting cell lysate was neutralized using 1M KH_2PO_4 . Cellular extracts were then separated by gradient elution (Buffer A: 0.1 M KH_2PO_4 , pH 4; Buffer B: 0.1 M KH_2PO_4 , containing 10% methanol, (v/v), pH 4) using a Phenomenex (Torrance, CA) Synergi 4u Hydro-RP 80A column (150 x 4.60 mm) at a flow rate of 1 ml/min. The HPLC system consisted of a Shimadzu LC-6A pump, C-R3A Chromatopac integrator and a SPD-6AV UV-Vis Spectrophotometric detector. Absorbance was monitored at 254 nm. Separation of adenine ribonucleotides (ATP, ADP, AMP) was verified using stock solutions of varying concentrations. The intra- and inter-day variability of the modified HPLC assay was less than 10%.

The modulatory effects of the reduced form of glutathione (GSH) were examined by depleting intracellular GSH concentrations. GSH was depleted in MLS-9 cells using the GSH-

synthesis inhibitor, L-buthionine-[S,R]-sulfoximine (BSO), which is a standard method for depleting cellular GSH (Anderson, 1998). Alterations in cellular GSH were examined in MLS-9 cells using a HPLC assay that has been previously described and validated by our group (Stempak et al., 2001; Dallas et al., 2003). The assay intraday and interday variability is less than 10%. Incubation of MLS-9 cells with 25 μ M BSO for 24 hr decreases intracellular GSH by greater than 94 % (Dallas et al., 2003). Efflux studies were then conducted as described above.

Intracellular Conversion of [3 H]bis(POM)PMEA to PMEA. Intracellular conversion of [3 H]bis(POM)PMEA to [3 H]PMEA within the various cell lines was confirmed using thin layer chromatography (TLC). Cells were incubated for 1 hr (37°C) with DMEM containing 10 mM sodium azide, 10 mM 2-deoxy-D-glucose and 1 μ M [3 H]bis(POM)PMEA. Following the incubation period, cells were washed with ice-cold 0.16 M NaCl and solubilized in 1N NaOH. 20 μ l aliquots of the cell lysate were spotted onto 250- μ m thick silica-gel coated TLC plates (Sigma-Aldrich, Mississauga, ON, Canada) and allowed to air dry. Cellular extracts and radioactive standards of PMEA and bis(POM)PMEA were separated using a solvent system consisting of isopropanol:ammonia:water (7.5:1:1.5 v/v/v). At the end of the development period, TLC plates were allowed to air dry for 2 hr, and then sprayed with EN³HANCE™ autoradiography spray (PerkinElmer, Boston, MA). Autoradiography was performed by exposing the chromatogram to X-ray film at -80°C. Following film development, zones corresponding to PMEA and bis(POM)PMEA (i.e., zones co-eluting with the standards of each compound) were scraped and radioactivity was determined by liquid scintillation counting. Total radioactivity present on the TLC plate was calculated by adding the PMEA and bis(POM)PMEA values to the radioactivity present in scrapings from the remainder of the TLC

plate. Results are expressed as percentage of total intracellular radioactivity recovered (mean \pm SD).

Data Analysis. Concentration of released [^3H]PMEA is expressed as pmol/mg protein and is presented as mean \pm S.D. from a minimum of 3 separate experiments. In an individual experiment, each data point represents a minimum of quadruplicate trials. Statistical analysis for unpaired experimental data was carried out using the unpaired Student's *t* test. For multiple comparisons, the test of repeated measures analysis of variance (ANOVA) and/or the post hoc multiple-comparison Bonferroni *t* test was used. A value of $p < 0.05$ was considered statistically significant.

Results

MRP4 and MRP5 mRNA and Protein Expression in Cultured Microglia Cells.

RT-PCR, conducted on primary rat microglia, the microglia cell line (MLS-9) and positive control cells (rat kidney) from four separate batches of cells showed the presence of mRNA for both rMRP4 (Fig. 1A) and rMRP5 (Fig. 1B). The RT-PCR product sizes were correct; i.e., ~180 bp for MRP4 and ~390 bp for MRP5. Sequencing, alignment in Clustal W and comparison with the Genbank sequences (AF376781; AB020209) showed that rMRP4 from the different tissues was >96% identical to rMRP4, and rMRP5 was >94% identical to rMRP5. Western blot analysis using MRP4 (rM4-p1) and MRP5 (M5I-1) antibodies directed against an epitope in the carboxy portion of the proteins (Wijnholds et al., 2000; Van Aubel et al., 2002), detected bands of the expected size in MLS-9 cells, i.e., at ~220 kDa for both rMRP4 (Fig. 2A) and rMRP5 (Fig. 2B), not surprisingly, at lower levels than in the stably overexpressing hMRP4- and hMRP5-HEK293 cell lines.

Conversion of [^3H]bis(POM)PMEA to PMEA in cell cultures.

Intracellular metabolism of the lipophilic pro-drug bis(POM)PMEA to PMEA was verified using TLC. Figure 3 shows a representative autoradiograph of metabolites recovered intracellularly from MLS-9 cells incubated with 1 μM [^3H]bis(POM)PMEA for 1 hr at 37°C, in the presence of 10 mM sodium azide. Standards of radiolabeled PMEA (*lane 1*) and bis(POM)PMEA (*lane 2*) were used to identify metabolites generated in MLS-9. At time zero, 98% of the radioactivity recovered in the supernatant was associated with bis(POM)PMEA (*lane 3*). Negligible amounts of radioactivity were present intracellularly (*Lane 4*). Following 1 hr of incubation with 1 μM [^3H]bis(POM)PMEA under ATP-depleting conditions, less than 6% of the radioactivity remaining in the supernatant was PMEA with >92% identified as the ester,

bis(POM)PMEA (*lane 5*). Conversely, PMEA and bis(POM)PMEA accounted for 91% and 1%, respectively, of total radioactivity recovered intracellularly from MLS-9 cells after 1 hr (*lane 6*). The remaining 8% of intracellular radioactivity likely represents a combination of mono(POM)PMEA (an intermediate compound formed during the conversion of bis(POM)PMEA to PMEA) and several phosphorylated metabolites of PMEA (i.e., PMEA monophosphate and PMEA diphosphate). Low concentrations of these metabolites have been detected following bis(POM)PMEA administration in various human lymphoid cell lines (Srinivas et al., 1993; Robbins et al., 1995). Conversion of bis(POM)PMEA to PMEA in the remaining cell lines used in the study (hMRP1 and P-gp overexpressing cells), were similar to that observed in MLS-9, i.e., >88%. In addition, > 95% of the radioactivity recovered in the medium after 1 hr of efflux in complete medium was associated with PMEA in all the cell lines tested.

Efflux Studies in MLS-9 Cell Monolayers.

To determine whether MRP4/5 isozymes present in MLS-9 cells were functionally active, transport studies were conducted using the MRP4/5 substrate, PMEA. Following a 1 hr loading of 1 μ M [3 H]bis(POM)PMEA under ATP-depleting conditions (10 mM sodium azide; 10 mM 2-deoxy-D-glucose), PMEA efflux was measured from MLS-9 cells by determining the concentration of PMEA released into the medium. Addition of 10 mM sodium azide and 2-deoxy-D-glucose to MLS-9 cell monolayers decreased intracellular ATP by >90% in the first 5 min of incubation and did not affect viability of the cells, as determined by the Trypan blue exclusion test. There was a time-dependent increase in PMEA in the medium surrounding MLS-9 cell monolayers, with a plateau beginning to be reached by 90 min (Fig. 4). This efflux was significantly decreased ($p<0.001$) in the presence of 2 mM of the antihyperuricemic agent, and

known MRP inhibitor sulfinpyrazone (i.e., by 51% at 1 hr). PMEA efflux was also inhibited by several other well-established MRP inhibitors including the flavanoid genistein, the anti-inflammatory drug indomethacin, and the antihyperuricemic agent probenecid (Fig. 5). Compared to untreated control cells (90 ± 2 pmol/mg protein), genistein (200 μ M), indomethacin (100 μ M), and probenecid (100 μ M) decreased PMEA efflux to 34 ± 3 , 45 ± 3 , and 76 ± 3 pmol/mg protein, respectively, as did the antiretroviral compound, AZT-MP (100 μ M; 57 ± 2 pmol/mg protein). Both sulfinpyrazone (Fig. 6A) and AZT-MP (Fig. 6B) inhibited PMEA efflux from MLS-9 cells in a dose-dependent manner.

All members of the MRP family of transporters require ATP to facilitate substrate transport (Borst et al., 1999). To confirm that efflux of PMEA by MLS-9 monolayers was ATP-dependent, effects of the ATPase inhibitor, sodium azide, on PMEA efflux were assessed (Fig. 7). At three different time points, 45, 60 and 90 min, presence of 10 mM sodium azide significantly decreased PMEA efflux from MLS-9 cells, compared to control cells ($p < 0.001$).

Transport of some MRP substrates (e.g., vincristine transport via MRP1) requires physiological concentrations (1–5 mM) of intracellular GSH (Loe et al., 1996). Thus, lowering intracellular GSH concentrations decreases drug efflux, whereas increasing GSH increases efflux. We have previously determined that a 24-hr incubation with 25 μ M BSO results in greater than 94% GSH depletion in MLS-9 cells, as determined by a highly specific and sensitive HPLC method previously validated by our group (Dallas et al., 2003; Stempak et al., 2001). Following depletion of intracellular GSH with 25 μ M BSO, efflux of PMEA in MLS-9 was unchanged at several time points (Fig 8).

Efflux of PMEA from Cell Lines Over-expressing hMRP1 or P-gp.

In addition to MRP4 and 5, MLS-9 cells also express significant levels of MRP1 and P-gp (Lee et al., 2001; Dallas et al., 2003). To verify that alterations in drug efflux were not due to these transporters, efflux of PMEA was examined in human MRP1 and P-gp overexpressing cell lines applying the same transport experiments used for MLS-9 cells. Metabolism of [³H]bis(POM)PMEA to [³H]PMEA (>88 %) was verified using TLC. ATP depletion by 10 mM sodium azide in each of the cell lines was confirmed as >90 % after 5 min. In the presence of 2 mM sulfinpyrazone, PMEA efflux was unaltered in the hMRP1 overexpressing cell line, WT-MRP1, at 30 and 60 min and this efflux did not differ significantly from that observed in the parental, VF-HeLa cells (Fig. 9A). Similarly, none of the inhibitors, AZT-MP, probenecid, indomethacin or genistein altered PMEA efflux from WT-MRP1 cells, compared to control (Fig. 9B). In a P-gp overexpressing cell line, CH^RC5, PMEA efflux was unchanged in the presence of several well-established and potent P-gp inhibitors, including the immunosuppressant cyclosporin A (10 μM), the cyclosporine A analog PSC833 (1 μM), or the acridone carboxamide derivative, GF120918 (1 μM) (*data not shown*). Taken together, these results confirm that PMEA is not a substrate for MRP1 or P-gp and that the observed PMEA efflux is most likely mediated by rMRP4 and rMRP5.

PMEA Efflux by hMRP4- or hMRP5-Overexpressing HEK293 Cells. Inhibition of PMEA in the presence of various MRP inhibitors was verified in hMRP4- or hMRP5-overexpressing HEK293 cells. MRP4- and MRP5-overexpression and transport properties of PMEA are well-characterized in these two cell lines (Reid et al., 2003; Wielinga et al., 2003). PMEA efflux by hMRP4-HEK293 cells was decreased significantly in the presence of 100 μM AZT-MP (13%), 200 μM genistein (46%), 100 μM indomethacin (53%) and 2 mM sulfinpyrazone (41%), but was unaffected by 100 μM probenecid (Fig. 10A). Efflux of PMEA

by hMRP5-HEK293 cells (Fig. 10B) was significantly decreased by all inhibitors tested i.e., 100 μ M AZT-MP (20%), 200 μ M genistein (30%), 100 μ M indomethacin (39%), 100 μ M probenecid (14%) and 2 mM sulfinpyrazone (41%).

Discussion

Effective treatment of HIV-associated neurodegenerative disorders requires sufficient accumulation of antiretroviral agents into the brain. Many of the currently used antiretroviral drugs show poor penetration across brain barriers, possibly due to expression of efflux transporters within various brain compartments (Taylor, 2002). The pathogenesis of HIV-associated dementia has been primarily linked to microglial response after viral infection (Diesing et al., 2002). Therefore, presence of drug transporters within these important HIV cellular targets may contribute to therapeutic failure. In the present study we extensively examined functional expression of two MRP isoforms, MRP4 and MRP5 in a microglia cell line, MLS-9, using the acyclic nucleoside phosphonate and antiviral compound PMEA. We found both mRNA and protein for MRP4 and MRP5 in MLS-9 cells, consistent with earlier reports of MRP4 (Ballerini et al., 2002; Hirrlinger et al., 2002) and MRP5 (Hirrlinger et al., 2002) mRNA. Ours is the first study of MRP4 and MRP5 function in microglia using PMEA.

To examine the combined functional expression of MRP4 and 5 in microglia, PMEA was chosen as our sample substrate. PMEA has been confirmed to be a MRP4 and 5 substrate in a variety of different cell systems, and efflux of PMEA from hMRP4 or hMRP5-transfected cells occurs at comparable rates (Reid et al., 2003). PMEA does not appear to be a substrate for the ABC transporters, P-gp or the breast cancer resistance protein (Reid et al., 2003), nor does it interact with typical substrates for MRP1-3 (McAleer et al., 1999; Lee et al., 2000). Cellular accumulation of PMEA into intact cells is slow and inefficient and requires an endocytosis-like process to cross membrane barriers (Palú et al, 1991). On the other hand, the PMEA ester prodrug, bis(POM)PMEA, enters cells rapidly via passive diffusion (Hatse et al., 1998). In K562 erythroleukemia cells, cellular uptake of bis(POM)PMEA was 100 times more efficient, than

PMEA uptake (Hatse et al., 1998). Once inside, bis(POM)PMEA is converted to PMEA in a two-step process: bis(POM)PMEA is first spontaneously hydrolyzed to mono(POM)PMEA (Hatse et al., 1998), which is then converted to PMEA by cellular esterases (Srinivas et al., 1993). PMEA is further metabolized to mono- and diphosphorylated derivatives by the action of several enzymes, including adenylate kinase (Robbins et al., 1995b) and/or 5-phosphoribosyl-1-pyrophosphate synthetase (Balzarini et al., 1991). In the present study, following 1 hr incubation with 1 μ M of [3 H]bis(POM)PMEA, greater than 88% of intracellular radioactivity was associated with the cleaved parent compound PMEA, in all cell lines tested, which is in good agreement with previous studies. Intracellular conversion efficiency of bis(POM)PMEA *in vitro* to PMEA is on the order of 80-90% for initial concentrations of between 0.125 and 1 μ M of bis(POM)PMEA (Robbins et al., 1995a; Hatse et al., 1998; Wijnholds et al., 2000).

The rapid uptake and conversion of bis(POM)PMEA to PMEA by a variety of different cell types makes the pro-drug an attractive candidate to study cellular PMEA efflux. Following loading with bis(POM)PMEA, cells overexpressing hMRP4 or hMRP5 extrude PMEA predominately in an unmodified form, i.e., >98% of the recovered product is PMEA (Wijnholds et al., 2000; Reid et al., 2003). We observed similar results with MLS-9 cells. However, transepithelial flux of bis(POM)PMEA across Caco-2 cell monolayers is inhibited by verapamil, suggesting involvement of P-gp in translocating the pro-drug across polarized membranes (Annaert et al., 1998). In the present study bis(POM)PMEA loading was done in the presence of 10 mM sodium azide, an oxidative phosphorylation inhibitor that rapidly reduces intracellular ATP (i.e., > 90% in 5 min), which prevented the ester pro-drug from being extruded from the cells during the loading period.

In the present study, efflux of PMEA from MLS-9 was decreased significantly in the presence of 2 mM sulfinpyrazone over several time points. Sulfinpyrazone is a strong inhibitor of PMEA efflux in hMRP4- ($IC_{50} = 420 \mu M$) or hMRP5- ($IC_{50} = 300 \mu M$) overexpressing HEK 293 cells (Reid et al., 2003). We verified these findings using the same cell systems. PMEA efflux was also decreased by 16-61% in the presence of well-established MRP inhibitors genistein (200 μM), indomethacin (100 μM) or probenecid (100 μM) and by 37% in the presence of the antiretroviral drug AZT-MP (100 μM). Similar to sulfinpyrazone, genistein was a strong inhibitor of PMEA efflux in our microglia cell system. In PMEA-selected human erythroleukemia (K562) and murine leukemia (L1210) cells, PMEA resistance was unaltered by genistein (Hatse et al., 1998). However, in that study, effects of genistein on PMEA efflux were not examined directly, nor was expression of hMRP4 and 5 verified. Our studies in hMRP4- and hMRP5-overexpressing cells confirmed genistein to be a good inhibitor of MRP4, and MRP5-mediated transport of PMEA, respectively.

Probenecid (100 μM) was the weakest inhibitor of PMEA efflux by MLS-9 cells. In hMRP4- and hMRP5-HEK 293 overexpressing cells, probenecid altered MRP4-mediated transport of PMEA considerably less than MRP5-mediated transport i.e., $IC_{50} = 2300 \mu M$ versus 200 μM , respectively. Accumulation of PMEA in hMRP4-transfected breast cancer cells (MCF7) was also unaffected by 100 μM probenecid, whereas 100 μM indomethacin increased accumulation 6 fold (Adachi et al., 2002). Not surprisingly, PMEA efflux from the MRP4-HEK 293 cells used in our study was also uninhibited by 100 μM probenecid. A modest decrease in PMEA efflux by 100 μM probenecid (i.e. 14 %) was observed in the MRP5-HEK293 cells. On the other hand, indomethacin (100 μM) decreased PMEA considerably in the three cell lines tested (i.e., MLS-9, hMRP4-HEK293 and hMRP5-HEK293). The monophosphorylated form of

the antiretroviral and nucleoside analog drug azidothymidine, also showed a modest decrease in PMEA efflux by MLS-9, hMRP4- and hMRP5-HEK293 cells at a concentration of 100 μ M.

Recently, Guo et al., (2003) reported that MRP8, a new member of the MRP family cloned in humans that closely resembles MRP4 and MRP5, confers resistance to PMEA in hMRP8-transfected LLC-PK1 cells. Efflux of PMEA and cAMP was also significantly higher in the hMRP8-transfected cells, compared with non-hMRP8 expressing controls. MRP8 transcript has been identified in a variety of tissues including the brain. Although MRP8 has yet to be cloned in the rat, we cannot rule out the possibility that this isoform, or another currently unidentified efflux transporter, in addition to MRP4 and MRP5, may contribute to the alterations observed in PMEA transport in microglia in the presence of the various MRP inhibitors tested.

All members of the ABC protein family require ATP in order to translocate substrates (Leslie et al., 2001). The ATP-dependence of PMEA efflux from MLS-9 cells was verified following incubation with the metabolic inhibitor sodium azide (10 mM). Sodium azide significantly decreased PMEA efflux from MLS-9 cells. Efficient transport of some substrates by several of the MRP protein family members also requires physiological concentrations of the antioxidant, GSH (Leslie et al., 2001). We have previously demonstrated GSH-dependent transport of vincristine in MLS-9 cells, using the glutathione-depleting agent, BSO (Dallas et al., 2003). We were therefore interested in the effect of glutathione depletion on PMEA-mediated efflux. Despite decreasing intracellular GSH concentrations by greater than 90 % in MLS-9, no differences were observed in PMEA efflux in BSO-treated (25 μ M) versus untreated cells in the present study. Therefore, in our cell system glutathione does not appear to play a significant role in PMEA-mediated efflux. Similarly, in hMRP4- and 5-overexpressing HEK293 cells, GSH depletion did not alter the efflux of the cyclic nucleotides, cGMP or cAMP, nor did GSH

depletion interfere with MRP4 or MRP5-mediated resistance to PMEA (Wielinga et al., 2003). In contrast, Lai and Tan (2002) have reported that MRP4-transfected HepG2 cells treated with very high concentrations of BSO (500 μ M) show altered drug resistance in the presence of 200-400 μ M PMEA and that BSO alters the transport of cyclic nucleotides, such as cAMP. The reasons for the discrepancies in effects of GSH are not clear.

We have previously confirmed the expression of several drug transporters in microglia, including a sodium-dependent nucleoside uptake transporter, an organic cation-like uptake transporter, and two ATP-dependent efflux transporters, MRP1 and P-gp (Hong et al., 2000; Hong et al., 2001; Lee et al., 2001; Dallas et al., 2003). However, we now show that none of the efflux transporters can account for PMEA efflux from MLS-9 cells, as follows. To verify that MRP1 did not contribute to altered of PMEA efflux in MLS-9 cells we examined PMEA transport in a hMRP1-overexpressing cell line, WT-MRP1. We recently characterized functional expression of MRP1 in MLS-9 and WT-MRP1 cells, and found that vincristine transport was inhibited by indomethacin (50 μ M), genistein (200 μ M), probenecid (100 μ M), and sulfinpyrazone (2mM) (Dallas et al., 2003). No significant differences were observed in PMEA efflux by WT-MRP1 cell monolayers in the presence of the MRP inhibitors, genistein (200 μ M), indomethacin (50 μ M) or probenecid (100 μ M), or the antiretroviral drug AZT-MP (100 μ M). In addition, 2 mM sulfinpyrazone produced no difference in the amount of PMEA released from WT-MRP1 cells versus the parent (non-hMRP1 expressing) cell line VF-HeLa. Since P-gp is a second transporter positively identified in MLS-9 cells (Lee et al., 2001), it is notable that we confirmed results by Annaert et al., (1998) and Reid et al., (2003) that PMEA is not a substrate of P-gp. Finally, neither of the potent P-gp inhibitors, PSC833 (1 μ M) or GF129018 (1 μ M) affected PMEA efflux from the P-gp overexpressing cell line CH^RC5. Taken together, these

results verify that PMEA is not a substrate for either P-gp or MRP1. Thus, the simultaneous presence of both of these transporters in MLS-9 does not contribute to alterations observed in PMEA extrusion.

Combined with our previous reports of functional expression of P-gp and MRP1, our current results show that microglia express multiple subfamilies of ABC transporters (i.e., P-gp and MRP) and multiple members of these subfamilies (i.e., MRP1, MRP4, MRP5). Considering the differing substrate specificities of each of these transport proteins, our results imply that the overall distribution and accumulation of several classes of antiretroviral drugs including reverse transcriptase inhibitors and protease inhibitors can be highly regulated in cellular targets of HIV infection in the brain.

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Footnotes

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Reprint Requests:

Dr. Reina Bendayan
Associate Professor
Department of Pharmaceutical Sciences
Faculty of Pharmacy, University of Toronto
19 Russell St.
Toronto, Ontario, M5S-2S2
Canada
Tel: 416-978-6979
Fax: 416-978-8511
e-mail: r.bendayan@utoronto.ca

Figure Legends

Fig.1. RT-PCR analysis of MRP4 (A) and MRP5 (B). *Lane 1*, size marker (1 kb Plus DNA ladder); *lane 2*, primary microglia; *Lane 3*, MLS-9 microglia cell line; *lane 4*, positive control, rat kidney; *lane 5*, negative control (no RT).

Fig.2. Western Blot analysis of MRP4 (A) in crude membrane fractions were prepared from cell lysates and then were separated on a 8.5% polyacrylamide gel containing 0.1% SDS. Proteins were transferred to PVDF membrane electrophoretically. Blots were incubated with the MRP4 antibody rM4-p1; *Lane 1*, 1 μ g MRP4-overexpressing HEK293 cells; *Lane 2*, 50 μ g MLS-9 microglia cell line. Similar conditions were used for MRP5 (B) protein detection using the MRP5 antibody M5I-1 and a 10% polyacrylamide gel. *Lane 1*, 1 μ g MRP5-overexpressing HEK293 cells; *Lane 2*, 50 μ g MLS-9 microglia cell line.

Fig.3. Autoradiogram demonstrating TLC separation of PMEA and bis(POM)PMEA. MLS-9 cells were loaded with 1 μ M [3 H]bis(POM)PMEA and radioactivity present in the supernatant or cellular extracts at specified times was quantified (see Methods). *Lane 1*, PMEA standard (1 μ M); *lane 2*, bis(POM)PMEA standard (1 μ M); *Lane 3*, MLS-9 cells, supernatant at time zero; *lane 4*, MLS-9 cells, intracellular at time zero; *lane 5*, MLS-9 cells, supernatant after 1 hr; *lane 6*, MLS-9 cells, intracellular after 1 hr.

Fig.4. Efflux of [3 H]PMEA from MLS-9 cells as a function of time, in the presence of sulfapyrazone. Monolayers of MLS-9 cells were incubated with 1 μ M [3 H]bis(POM)PMEA

under ATP-depleting conditions (10 mM sodium azide; 10 mM 2-deoxy-D-glucose) for 1 hr at 37°C. Complete medium was added, with or without 2 mM sulfinpyrazone, and efflux was determined at various times between 0 and 120 min. Results are expressed as mean \pm S.D. of 3 separate experiments. *** p <0.001, significantly different from control.

Fig.5. Efflux of [3 H]PMEA from MLS-9 cells in the presence of various MRP inhibitors.

Monolayers of MLS-9 cells were incubated with 1 μ M [3 H]bis(POM)PMEA under ATP-depleting conditions (10 mM sodium azide; 10 mM 2-deoxy-D-glucose) for 1 hr at 37°C. Complete medium was added with or without azidothymidine-monophosphate (AZT-MP; 100 μ M), genistein (GEN; 200 μ M), indomethacin (INDO; 100 μ M), probenecid (PROB; 100 μ M), or sulfinpyrazone (SULF; 2 mM). Efflux of [3 H]PMEA was determined after 1 hr. Results are expressed as mean \pm S.D. of 3 separate experiments. *** p <0.001, significantly different from control.

Fig.6. Dose-dependent effect of sulfinpyrazone (A) and azidothymidine-monophosphate (B) on the efflux of [3 H]PMEA by MLS-9 cells. Monolayers of MLS-9 cells were incubated with 1 μ M [3 H]bis(POM)PMEA under ATP-depleting conditions (10 mM sodium azide; 10 mM 2-deoxy-D-glucose) for 1 hr at 37°C. Complete medium was added with or without an inhibitor and medium was removed after 1 hr. Results are expressed as mean \pm S.D. of 4 separate experiments.

** p <0.01; *** p <0.001, significantly different from control.

Fig.7. Effect of sodium azide on [3 H]PMEA efflux from MLS-9 cells. Monolayers of MLS-9 cells were incubated with 1 μ M [3 H]bis(POM)PMEA under ATP-depleting conditions (10 mM

sodium azide; 10 mM 2-deoxy-D-glucose) for 1 hr at 37°C. Complete medium or medium in the presence and absence of 10 mM sodium azide and 10 mM 2-deoxy-D-glucose was added and efflux of [³H]PMEA was determined at 45, 60 and 90 min. The results are expressed as mean ± S.D. of 3 separate experiments. ****p*<0.001, significantly different from control.

Fig.8. [³H]PMEA efflux from MLS-9 cells following depletion of intracellular glutathione.

MLS-9 cell monolayers were incubated with 25 μM BSO for 24 hr at 37°C. Cells were then loaded with 1 μM [³H]bis(POM)PMEA under ATP-depleting conditions (10 mM sodium azide; 10 mM 2-deoxy-D-glucose) for 1 hr. Complete medium was added and efflux of [³H]PMEA was determined at 30, 60 and 90 min. Results are expressed as mean ± S.D. of 3 separate experiments.

Fig.9. Efflux of [³H]PMEA from human MRP1-overexpressing cells. Cell monolayers were loaded with 1 μM [³H]bis(POM)PMEA under ATP-depleting conditions (10 mM sodium azide; 10 mM 2-deoxy-D-glucose) for 1 hr. **(A)** Following loading, efflux of [³H]PMEA was determined in WT-MRP1 and VF-HeLa at 30 and 60 min in the presence of 2 mM sulfinpyrazone. **(B)** Following loading, PMEA efflux from WT-MRP1 cells at 1 hr was determined in the presence or absence of the MRP inhibitors, genistein (GEN; 200 μM), indomethacin (INDO; 100 μM), or probenecid (PROB; 100 μM) or the antiretroviral drug, azidothymidine-monophosphate (AZT-MP; 100 μM). The results are expressed as mean ± S.D. of 4 separate experiments, with each data point in an individual experiment representing quadruplicate measurements.

Fig.10. Efflux of [3 H]PMEA from hMRP4 (**A**) or hMRP5 (**B**)-overexpressing HEK293 cells in the presence of various MRP inhibitors. Cell monolayers were incubated with 1 μ M [3 H]bis(POM)PMEA under ATP-depleting conditions (10 mM sodium azide; 10 mM 2-deoxy-D-glucose) for 1 hr at 37°C. Complete medium was added with or without azidothymidine-monophosphate (AZT-MP; 100 μ M), genistein (GEN; 200 μ M), indomethacin (INDO; 100 μ M), probenecid (PROB; 100 μ M), or sulfinpyrazone (SULF; 2 mM). Efflux of [3 H]PMEA was determined after 1 hr. Results are expressed as mean \pm S.D. of 3 separate experiments.

*** p <0.001, significantly different from control.

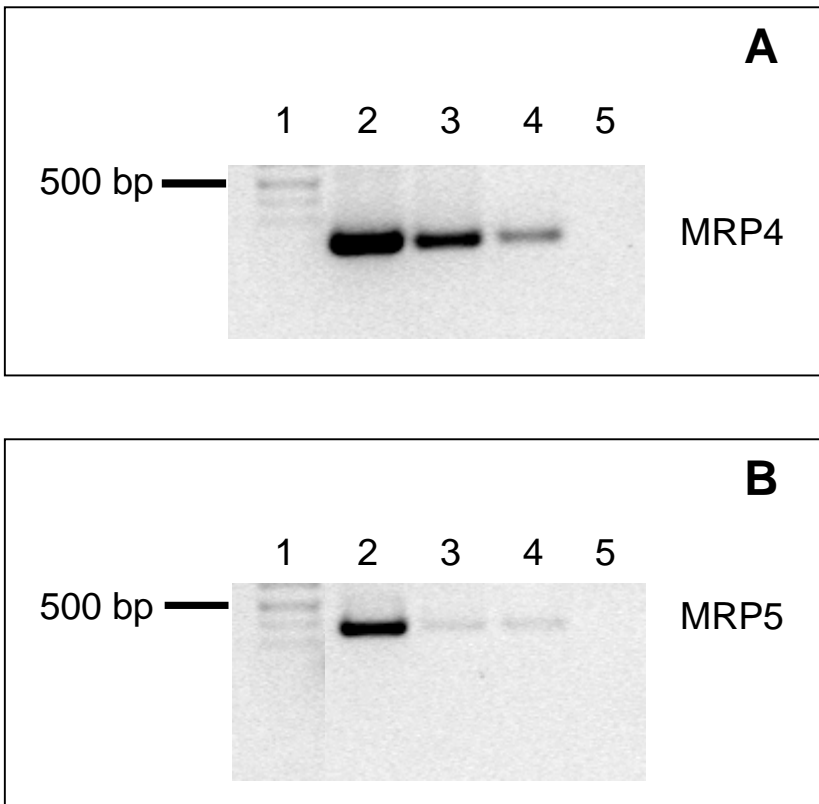


Fig.1

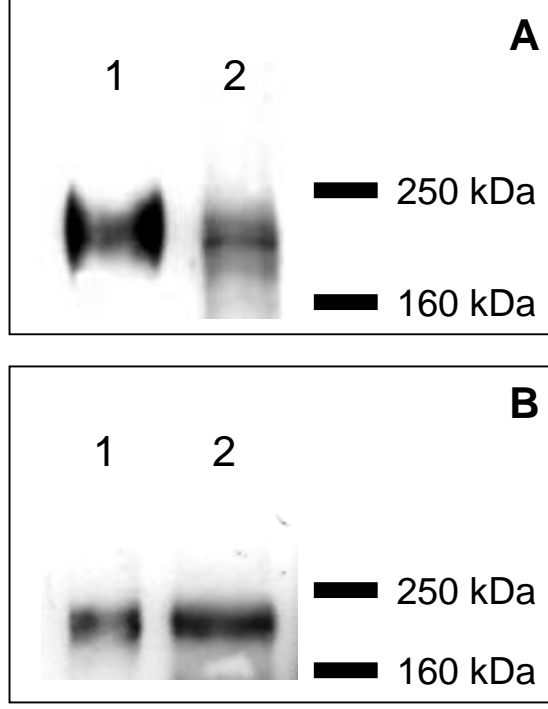


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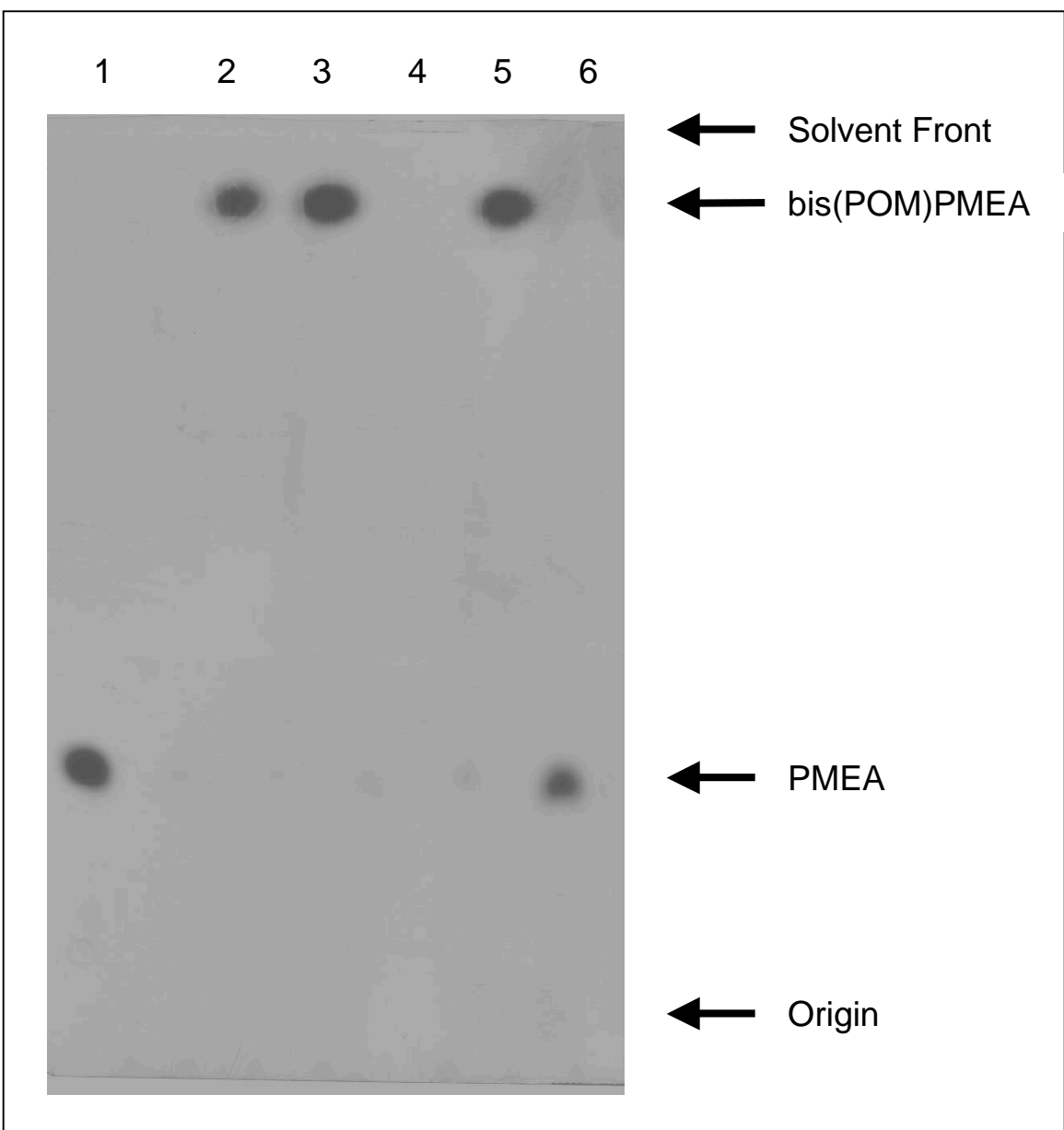


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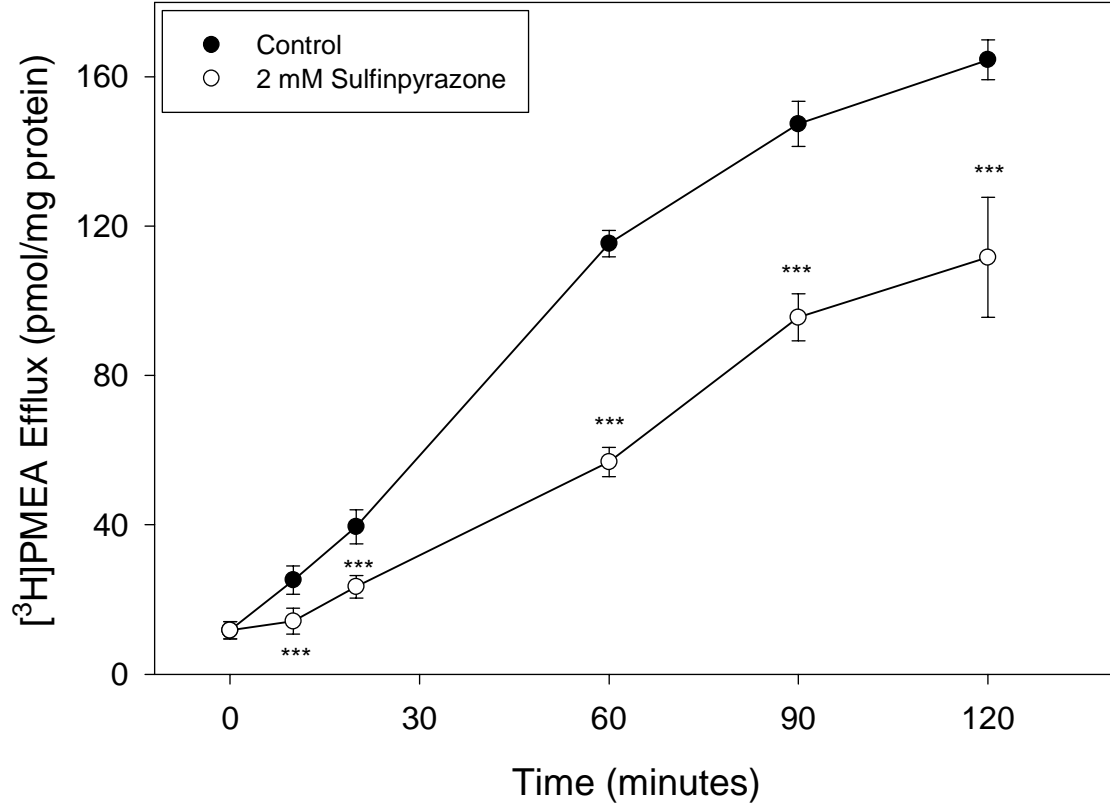


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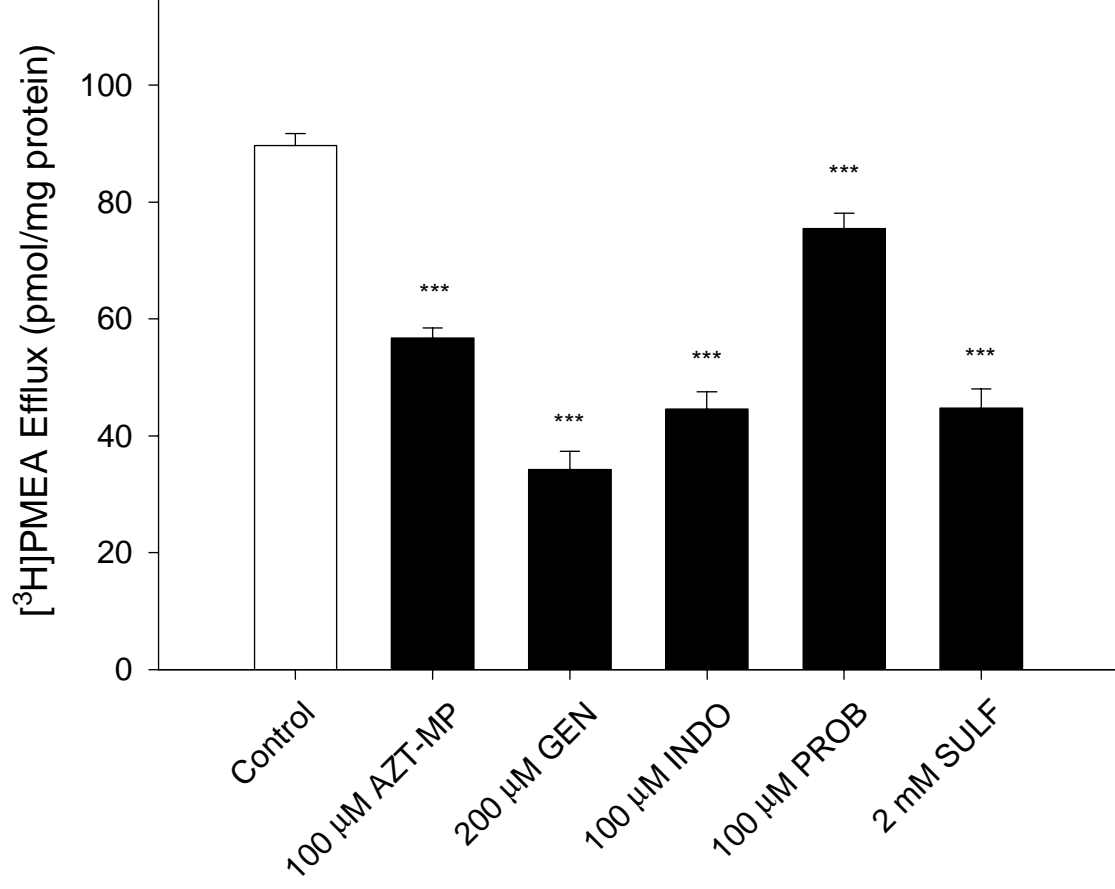


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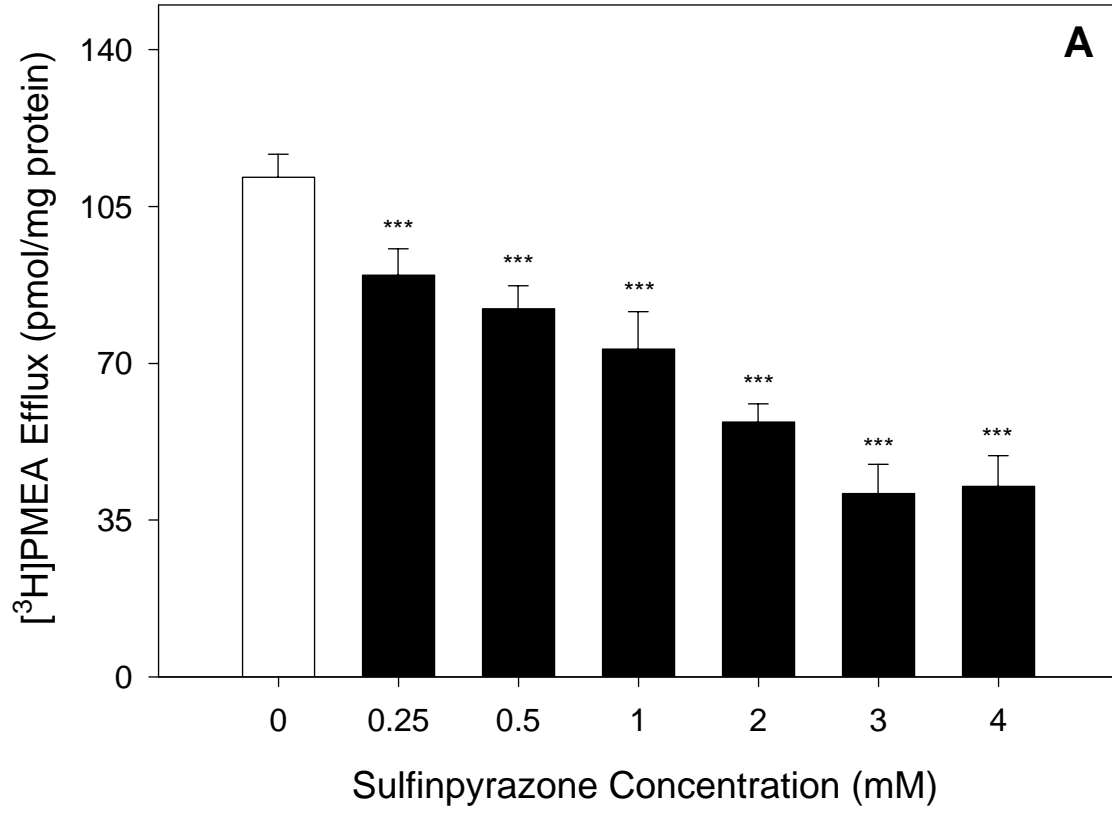


Fig.6A

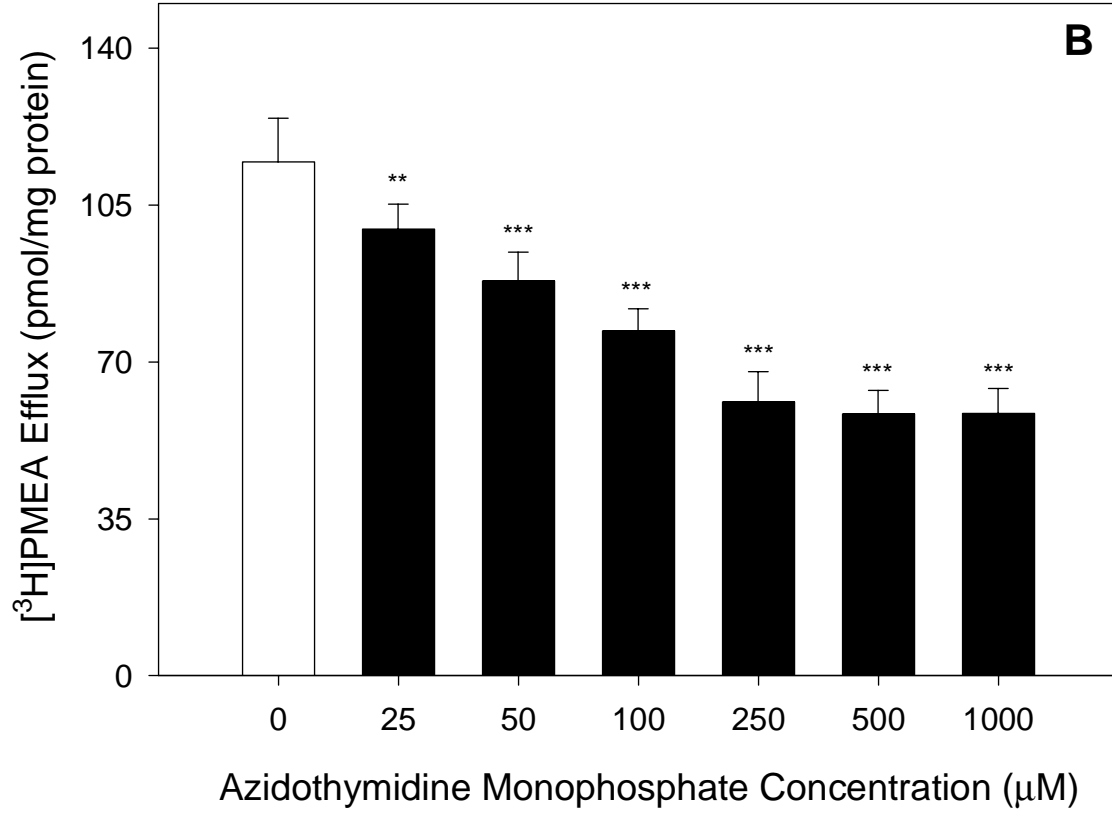


Fig.6B

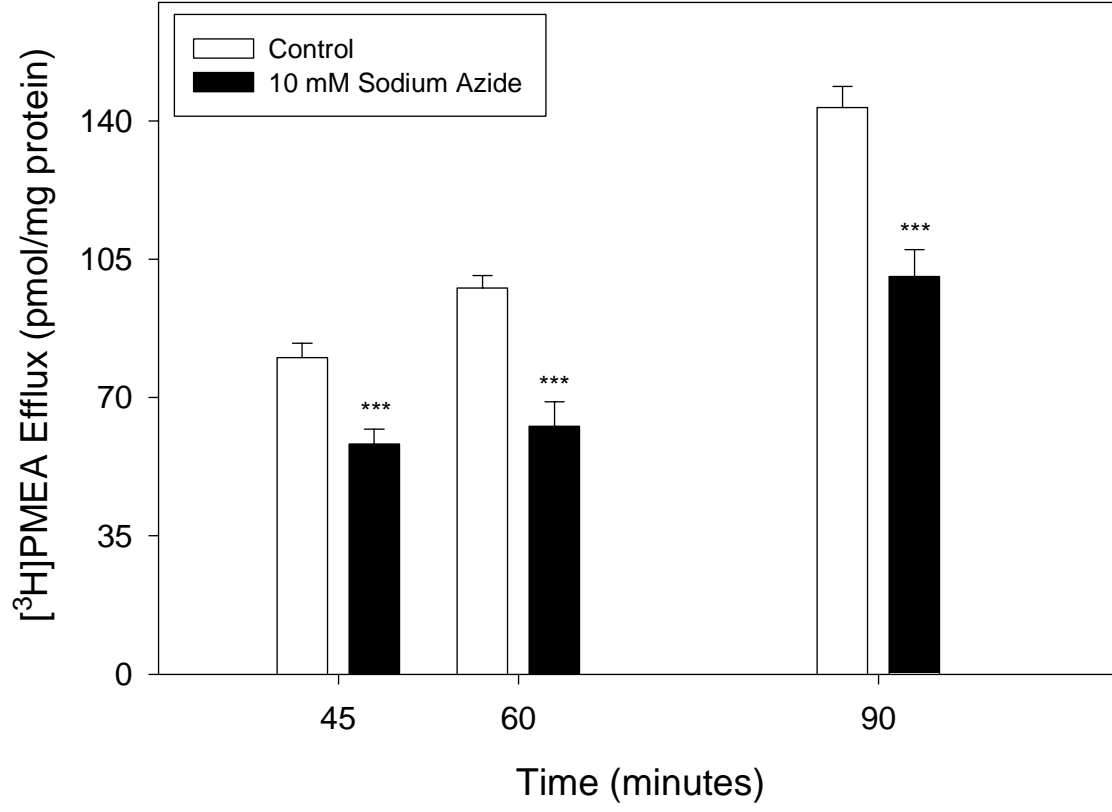


Fig.7

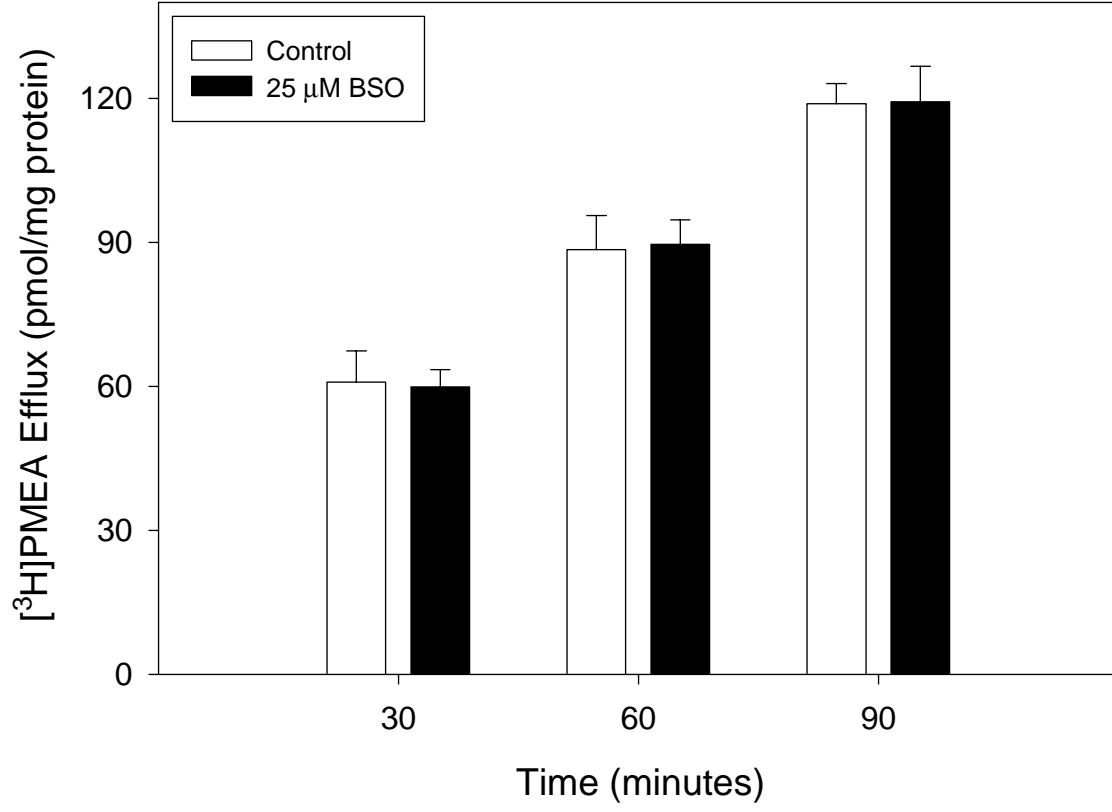


Fig.8

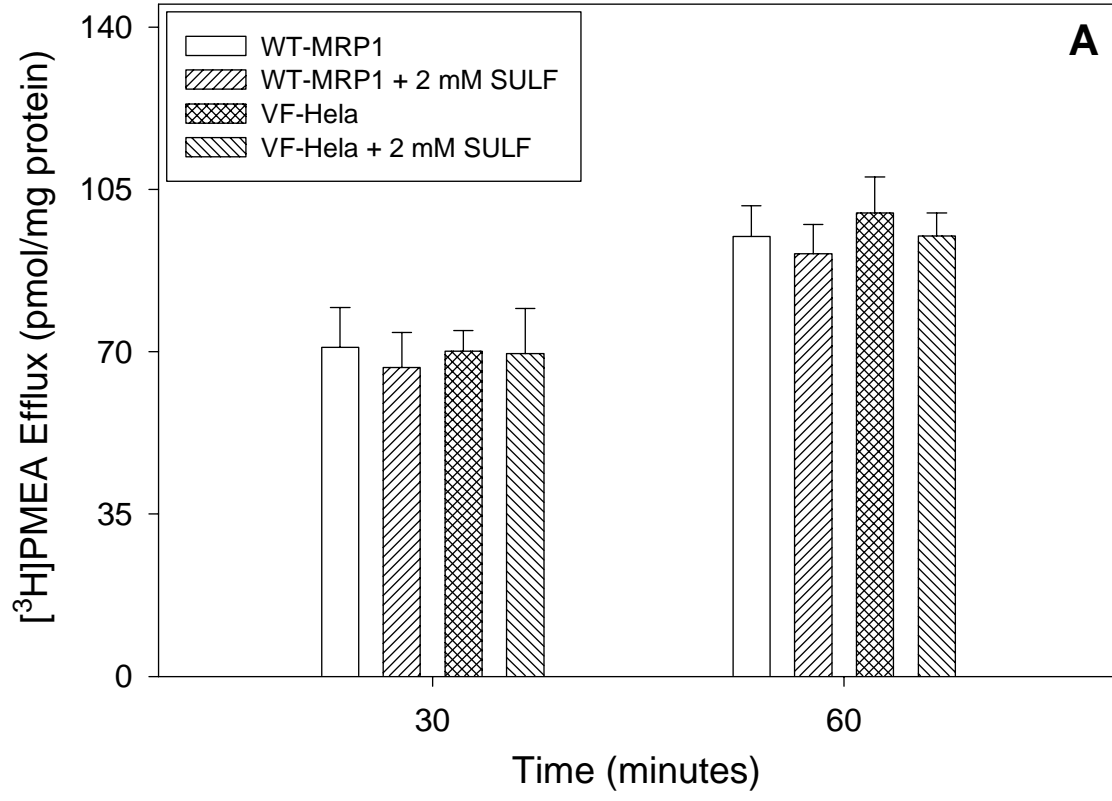


Fig.9A

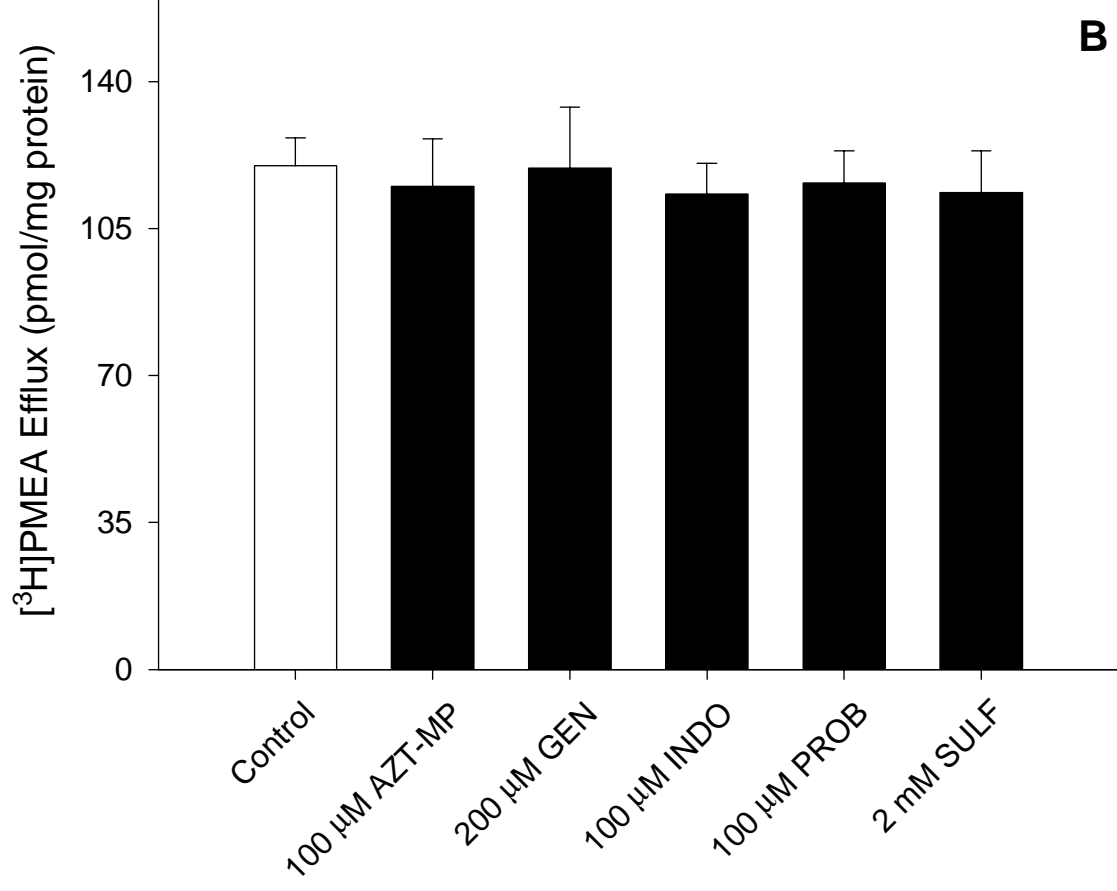


Fig.9B

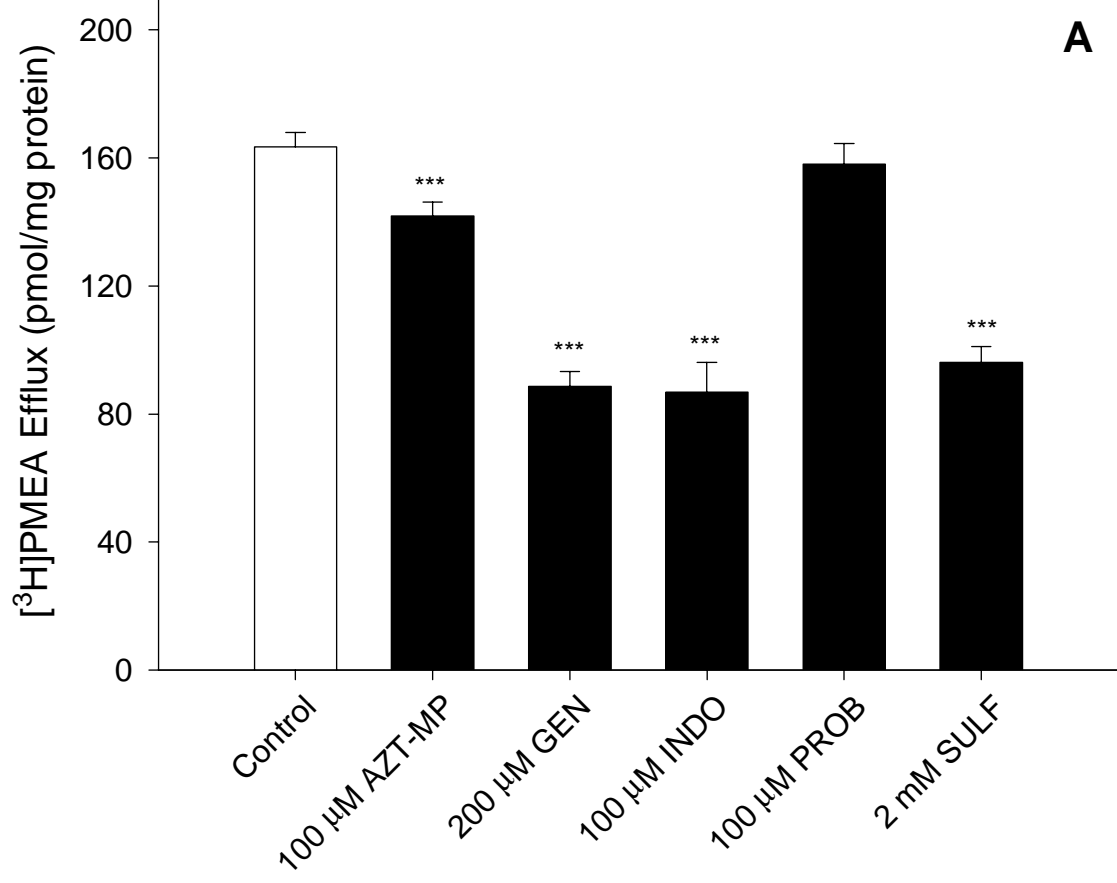


Fig.10A

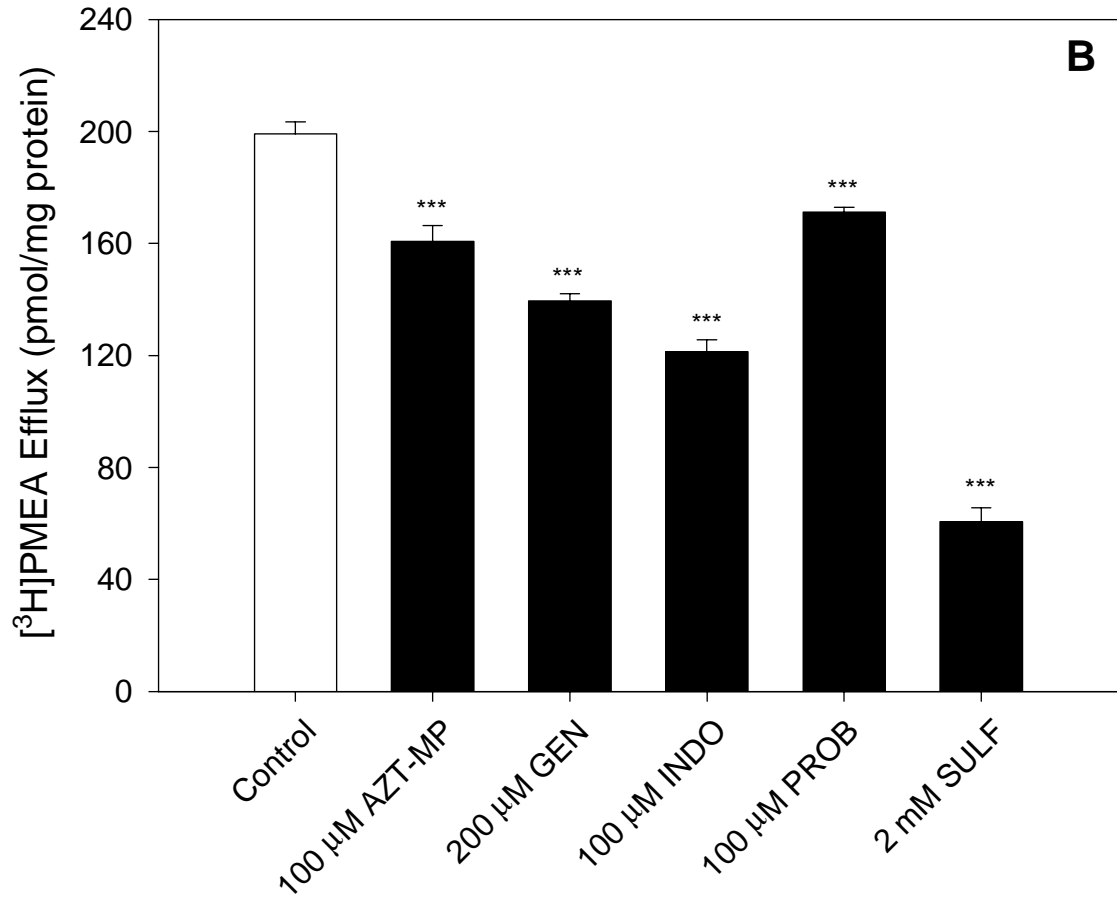


Fig.10B