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Multixenobiotic resistance efflux activity in *Daphnia magna* and *Lumbriculus variegatus*

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Abbreviations:

ABC protein  ATP-binding cassette transfer protein
Ca-AM  calcein-AM
MDR  multidrug resistance protein
MRP  multidrug resistance related protein
MXR  multixenobiotic resistance (protein)
NBD  nucleotide binding domain
P-gp  permeability glycoprotein
qRT-PCR  quantitative reverse transcription PCR
RhB  rhodamine B
Abstract

Multixenobiotic resistance is a phenomenon in which ATP-binding cassette (ABC) family proteins transfer harmful compounds out of cells. *Daphnia magna* and *Lumbriculus variegatus* are model species in aquatic ecotoxicology, but the presence and activity of ABC proteins have not been well described in these species. The aim of this work was to study the presence, activity, and inhibition of ABC transport proteins in *Daphnia magna* and *Lumbriculus variegatus*. The presence of *abcb1* and *abcc* transcripts in 8-9-day-old *D. magna* was investigated by qRT-PCR. The activity of MXR in *D. magna* and *L. variegatus* was explored by influx of the fluorescent ABC protein substrates rhodamine B and calcein-AM, with and without the model inhibitors verapamil (unspecific ABC inhibitor), reversin 205 (ABCB1 inhibitor) and MK571 (ABCC inhibitor). Juvenile *Daphnia magna* possessed all examined *abcb* and *abcc* transcripts, but only reversin 205 inhibited MXR activity. The MXR activity in *Lumbriculus variegatus* was inhibited by MK571, and to a lesser extent by verapamil, whereas reversin 205 seemed to stimulate the transport activity. Whereas calcein-AM worked better as an MXR substrate in *D. magna*, rhodamine B was a better substrate for *L. variegatus* MXR activity measurements. This is the first report on MXR activity in the order *Lumbriculida*, subclass *Oligochaeta*, and class *Clitellata*.

1 Introduction

The ATP-binding cassette (ABC) proteins are transmembrane proteins that transfer a wide range of substrates across membranes against a concentration gradient. The transport is energetically driven by the hydrolysis of ATP. The ABC proteins are present in all organisms, including plants, vertebrates, invertebrates, and microbes (Licht and Schneider, 2011).
The ABC proteins are divided into seven classes (A - G) according to their sequence homology. The cytosolic nucleotide binding domain (NBD) is highly conserved among the classes, and the membrane domain is the one that confers the substrate specificity to the protein. The ABC proteins typically involved in transferring xenobiotic compounds belong to the classes of ABCB, ABCC, and ABCG. The ones best studied are the Abcb1 protein, which is also called MDR (multidrug resistance protein), MXR (multixenobiotic resistance protein), or P-gp (permeability glycoprotein); and Abcc1, which is also called MRP (multidrug resistance related protein). Both of these proteins have wide substrate specificity, and many compounds may be substrates of both of them. The substrates are usually amphiphilic compounds with separate hydrophilic and hydrophobic moieties. The Abcb1 substrates are neutral or weakly positive lipophilic compounds whereas those of Abcc1 are often lipophilic anions, such as Phase II metabolites (Schinkel and Jonker, 2003).

Abcb1 and Abcc proteins have a role in defense against xenobiotics: As they transfer certain groups of xenobiotics out of the cell, they keep the intracellular concentration of these compounds low. Known Abcb1 or Abcc substrates include metals such as cadmium and mercury; pesticides such as dacthal and pentachlorophenol; and polycyclic aromatic hydrocarbons (Achard-Joris, et al., 2005; Campos, et al., 2014; Chao Yeh, et al., 1992; Epel, 1998). Abcb1 and Abcc proteins, and their activity, are induced by environmental chemicals in various aquatic organisms (Achard, et al., 2004; Eufemia and Epel, 1998; Ferreira, et al., 2014; Kurelec, 1997; Luckenbach, et al., 2014; Prevodnik, et al., 2007; Smital, et al., 2004). There are differences between species and strains, and between populations from clean and polluted sites, in the activity of the ABC proteins, and this correlates with sensitivity to chemicals (Kurelec, et al., 1996; Kurelec, 1997; Smital, et al., 2000; Smital, et al., 2004; Velki and Hackenberger, 2012).
Various compounds can inhibit the ABC protein transfer activity: pharmaceuticals such as verapamil, reserpine, anthracycline, and cyclosporins; synthetic musks; longchain perfluoroalkyl acids; pesticides such as endosulfan, malathion, and dichlorvos; microbial degradation products; and natural products such as algal extracts (Epel, et al., 2008; Kurelec, 1997; Smital, et al., 2004).

If the activity of the ABC proteins that keep xenobiotics out of cells is inhibited, the intracellular concentration of those compounds increases. This may lead to toxic effects at environmental concentrations that are not normally toxic to the organism (Anselmo, et al., 2012; Epel, et al., 2008; Faria, et al., 2011; Smital, et al., 2004; Waldmann, et al., 1995). It has been speculated that this may be one of the mechanisms behind mixture effects (Anselmo, et al., 2012; Epel, et al., 2008; Faria, et al., 2011).

*Daphnia magna* and *Lumbricus variegatus* are model species in aquatic ecotoxicology, and they are widely used for ecotoxicological testing of compounds and environmental samples. The presence and activity of ABC proteins have not been well described in these species. The genes of the ABC transporter family members have been characterized in *Daphnia pulex* (Sturm, et al., 2009), and partial sequences have been cloned in the genome of *D. magna* (NCBI), but no gene sequences are available for *L. variegatus*. ABC transporter activity in *D. magna* has been characterized in one study (Campos, et al., 2014), but no published studies exist for *L. variegatus* MXR activity. In the phylum *Annelida* MXR activity has only been characterized in the echiuroid *Urechis caupo*, and in the earthworms *Eisenia andrei*, *Eisenia fetida*, *Lumbricus rubellus*, *Octolasion lacteum* and *Dendrobaena octaedra* (Bošnjak, et al., 2014; Hackenberger, et al., 2012; Toomey and Epel, 1993; Velki and Hackenberger, 2012; Velki, et al., 2013). These organisms possess verapamil-sensitive MXR system that uses rhodamine B as a substrate.
The activity of the MXR system can be examined with the help of fluorescent substrates. Rhodamine B (RhB) is a fluorescent substrate of the MXR system that readily crosses cell membranes. If the MXR system is inhibited, the concentration of the substrate in the cells increases, and this can be seen as a rise in fluorescence. Calcein-AM (Ca-AM) is a cell-permeable substance that is transformed to fluorescent calcein by esterases inside the cells. The ABC proteins transfer Ca-AM out of cells before the esterase action takes place, and thus, the more ABC protein activity there is, the less fluorescence is produced. When ABC proteins are inhibited, Ca-AM reaches the cytoplasm, gets transformed to calcein, and there is a rise in fluorescence.

The aim of this work was to study the presence, activity, and inhibition of ABC transport proteins in *Daphnia magna* and *Lumbriculus variegatus*. The presence of *abcb1* and *abcc* transcripts in *D. magna* was investigated by qRT-PCR. The activity of MXR in *D. magna* and *L. variegatus* was explored by influx of RhB and Ca-AM with and without the model inhibitors verapamil (unspecific ABC inhibitor), reversin 205 (ABCB1 inhibitor) and MK571 (ABCC inhibitor).

**2 Materials and methods**

**2.1 Daphnia culture**

*Daphnia* were cultured in Elendt M7 medium at the density of 50 animals per liter in constant temperature of 20 ± 1 °C and a photoperiod of 18 h light/6 h darkness. They were daily fed with *Scenedesmus* green alga (about $1.2 \times 10^5$ cells/mL).

The daphnids used for the RhB and Ca-AM influx assays were 6 to 8 days old, and qPCR was conducted on newborn (<24 h), juvenile (6 - 8 d), and adult (28 d) daphnids.
2.2 Lumbriculus culture

The *Lumbriculus* were cultured in ISO test water (ISO, 2012) the total hardness of which was 1 mM, in constant temperature of 20 ± 1 °C and a photoperiod of 18 h light/6 h darkness. The bottom of the aquarium was covered with strips of paper towels, and the animals were fed with fish food flakes (Sera mikropan). The mean wet weight (SD) of the organisms used in the study was 4.6 (0.8) mg.

2.3 Quantitative reverse transcription PCR (qRT-PCR)

The primers for alpha-tubulin were adopted from (Heckmann, et al., 2006) and abcc1/3, abcc4 and abcc5 from (Campos, et al., 2014). The primers for glyceraldehyde-3-phosphate dehydrogenase and p-glycoprotein (ABCB/mdr) were designed with Primer3 (version 4.0.0. at http://primer3.wi.mit.edu/), and checked for specificity with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The features of the primers are presented in Table 1.

Total RNA was extracted from pools of ca. 70 newborn (<24 h), or 8-10 juvenile (6-8 d) or adult (28 d) *D. magna* using Tri reagent (Molecular Research Center) following manufacturer’s instructions. Extractions were performed on 3 independent biological replicates. NanoDrop 1000 (Thermo Fisher Scientific) was used to estimate the RNA concentration and purity, and Agilent 2100 BioAnalyzer (Agilent) to assess RNA integrity, using Eukaryote total RNA 6000 nano kit (Agilent).

After DNase treatment (DNase I, Thermo), 1 µg total RNA was reverse transcribed to cDNA (iScript cDNA Synthesis Kit, Bio-Rad, USA) and diluted 1+9 with nuclease-free water. One 25 µl qPCR reaction
7

consisted of 5 µl of the diluted cDNA, 0.75 µl each of forward and reverse primers (final concentration 300 nM), 6 µl sterile H2O and 12.5 µl of iQ SYBR Green Supermix (Bio-Rad). The reactions were run in triplicates on clear 96-well PCR plates (Bio-Rad). The qPCR was run on a CFX96 Real-Time PCR cycler (Bio-Rad). The protocol was 3 min at 95 °C; 40 cycles (10 s at 95 °C, 30 s at 58 °C; 10 s at 95 °C and a melt curve from 55 °C to 95 °C). The Ct values of no template controls (water instead of cDNA) were always over 38. Melt curves showed a single peak, confirming formation of only one PCR product.

2.4 ABC protein activity assays - dye uptake

The dye influx assays were modified from (Smital and Kurelec, 1997). Daphnids were exposed to 1 µM RhB or 0.5 µM Ca-AM in ISO test water (ISO 6341, 2012), with or without specific inhibitors at various concentrations for two hours in the dark. The substrate concentrations were chosen after conducting preliminary exposures in which several concentrations based on literature values were tested. The model inhibitors were verapamil (unspecific ABC inhibitor), reversin 205 (ABCB1 inhibitor) and MK571 (ABCC inhibitor), and all the inhibitors were used at concentrations of 1, 5, and 10 µM, and verapamil and MK571 also at concentration of 20 µM. Treatments were replicated three times, and each replicate consisted of eight daphnids in 8 ml of the exposure solution. After the exposure, the daphnids were examined for mortality, sieved, blotted dry, and weighed to the nearest µg. The eight daphnids of each vial were homogenized in a microcentrifuge vial in 200 µl distilled water with a plastic homogenizing rod. The homogenate was centrifuged (13 000 x g, 5 min), and 150 µl of the supernatant was taken for fluorescence measurement (ex 584 nm, em 612 nm for RB and ex 485 nm, em 538 nm for Ca-AM; Fluoroskan Ascent fluorometer, Labsystems). The fluorescence readings were proportionated to the fresh weights of the organisms (yielding fluorescence unit / mg fresh weight), and these values were normalized to control (daphnids...
exposed to 1 µM RhB or 0.5 µM Ca-AM). The experiments were conducted twice, each with a
different batch of organisms, yielding six replicates for fluorescence measurements altogether.

The *Lumbriculus* assays were conducted similarly, but four organisms were used per 8 ml of
exposure solution, and the total hardness of the artificial fresh water used in the assays was 1 mM.
The total number of samples for fluorescence measurements was six.

2.6 Statistics

Kolmogorov-Smirnov test was used to test if the data was normally distributed, and Levene’s test to
test if the variances were equal. For those compounds that met the prerequisites of ANOVA, one-
way ANOVA with Tukey’s test was used to test if the fluorescence of tissue homogenate differed
between inhibitor concentrations. Independent samples Kruskal-Wallis test was used for those
compounds that were not normally distributed, and Dunnett’s T3 test was used for the pairwise
comparisons if variances of the groups were unequal. IBM SPSS Statistics 20 was used for the
statistical analyses.

3 Results

3.1 *Daphnia magna* possesses active ABCB1

*abc/mdr* transcripts were present in *Daphnia magna* of all ages (Figure 1). Using RhB as the
fluorescent substrate, it could be seen that verapamil (ANOVA p=0.439) did not have an effect on
the amount of fluorescence in the daphnids (Fig. 2 a). Reversin 20S clearly caused an effect on
fluorescence (ANOVA p=0.006), 10 µM increasing the fluorescence compared to 0 µM (Dunnett T3,
p=0.008) and 1 µM (Dunnett T3, p=0.013) (Fig. 2b). MK571 did not affect RhB accumulation (ANOVA p=0.382) (Fig. 2c).

Similar results were obtained with Ca-AM as the substrate: The fluorescence in daphnids treated with verapamil (Kruskal-Wallis, p=0.255) did not differ from that of controls (Fig. 3a). The daphnids treated with reversin 205 possessed more fluorescence than controls (Kruskal-Wallis, p<0.001), and some dose-response could be seen, as both 5 and 10 µM reversin 205 increased fluorescence compared to 0 and 1 µM (Dunnett T3, p=0.001 for all), and daphnids exposed to 10 µM reversin 205 had higher fluorescence than those exposed to 5 µM (Dunnett T3 p=0.05) (Fig. 3b). MK571 had no effect on fluorescence (Kruskal-Wallis, p=0.378) (Fig. 3c).

3.2 ABCB1 and ABCC inhibitors affect *Lumbriculus variegatus* ABC transporter activity

Verapamil had an effect on RhB fluorescence (Kruskal-Wallis, p=0.002) in *Lumbriculus variegatus*, and the fluorescence in organisms treated with 10 µM verapamil was higher than in 0 (Dunnett T3, p=0.006) and 1 µM (Dunnett T3, p=0.009) (Fig. 4a). Reversin 205 reduced RhB fluorescence (ANOVA p=0.004), with 5 and 10 µM being significantly different from control (Tukey, p=0.020 and p=0.004, respectively) (Fig. 4b). Rhodamine B fluorescence in *L. variegatus* increased upon MK571 exposure (Kruskal-Wallis p<0.001), and 10 and 20 µM MK571 caused a statistically significant increase (Dunnett T3, p=0.002 and p=0.023, respectively) (Fig. 4c).

Contrary to the results obtained with RhB, verapamil did not increase Ca-AM fluorescence in *L. variegatus*. Though the ANOVA showed that there were differences between treatments (p=0.018), none of the treatments differed from control, and the post-hoc test confirmed significant difference
only between 5 and 10 µM verapamil (Tukey p=0.011) (Fig. 5a). Reversin 205 reduced Ca-AM fluorescence (ANOVA p=0.011), 5 and 10 µM having significantly lower fluorescence than controls (Tukey, p=0.024 and p=0.015 for 5 and 10 µM, respectively) (Fig. 5b). MK571 had no effect (Kruskal-Wallis p=0.325) on fluorescence (Fig. 5c).

4 Discussion

4.1 mRNA levels and activity of ABCB and ABCC proteins in Daphnia magna

This work shows that abcb1, abcc1/3, abcc4, and abcc5 transcripts are present in Daphnia magna neonates, 7-8-day-old juveniles, and 28-day-old adults. The work confirms and adds to the findings of Campos et al., who showed that eggs, embryos, neonates, and 5-day-old juveniles possess these transcripts (Campos, et al., 2014). There is a difference in the transcript profile between the studies in the common life stage examined, the neonates. In the study of Campos et al. the transcript levels of all abcs were approximately similar to each other, whereas in the present study the neonates possessed much lower transcript levels of abcc5 than other abcs (Campos, et al., 2014). There may be either a genetic difference between the populations, or some environmental factor may have caused the difference.

Surprisingly, the classical inhibitor verapamil increased neither RhB nor Ca-AM influx in daphnids. Similar results have been obtained in western mosquitofish and bluegill sunfish, where no inhibition with 10 µM verapamil could be observed (Damare, Kaddoumi et al. 2009). Thus it seems that even though verapamil is a good inhibitor of the MXR system in many organisms, it is not a universal inhibitor, and the lack of inhibition by verapamil in a species cannot be taken as a proof of that the organism does not possess MXR transfer activity.
In addition to \textit{abcb1} mRNA, \textit{D. magna} possesses ABCB1 protein activity, as exposure to the specific inhibitor of ABCB1, reversin 205, lead to increased fluorescence of RhB and Ca-AM. The effect was more pronounced with Ca-AM, which may thus be considered a better substrate for \textit{D. magna} ABCB1 than RhB. The rise in fluorescence in this work was higher than in the work of Campos \textit{et al.} probably due to differences in the exposure. Whereas in this work the daphnids were exposed \textit{in vivo} for two hours, Campos \textit{et al.} exposed their organisms \textit{ex vivo} for one hour to overcome the toxic effects of the inhibitors on filtrating activity (Campos, \textit{et al.}, 2014). In our work, no toxic effects of the inhibitors were seen.

Exposure to the ABCC inhibitor MK571 resulted in no effect on fluorescence in 7 - 8-day-old juvenile daphnids. This result is unexpected taken that these daphnids possess \textit{abcc1/3}, \textit{abcc4}, and \textit{abcc5} transcripts, and that Campos \textit{et al.} reported small but significant inhibition of MXR transport activity by MK571 in 4 - 5-day-old juvenile daphnids (Campos, \textit{et al.}, 2014). One possible explanation for the observation is that the activity of ABCB1 is so high in the daphnids of the present study that the fluorescent substrates are transferred out of the cells even when the ABCC activity is inhibited. RhB and Ca-AM are substrates of both ABCB1 and ABCC, and therefore both are transferred by both proteins.

4.2 ABC transport protein activity in \textit{Lumbriculus variegatus}

The study clearly shows that \textit{L. variegatus} has an active ABC transport protein system. To our knowledge this is the first report on MXR activity in the order \textit{Lumbriculida}, subclass \textit{Oligochaeta}, or class \textit{Clitellata}. The presence of an ABC transporter protein (gi|149912747) has been detected in the spionid polychaete \textit{Pseudopolydora vexillosa} (Chandramouli, \textit{et al.}, 2011), but this protein is 100%
similar to bacterial ABC transporter nucleotide-binding domains. As it has been noticed in the oligochaete *Olavius algarvensis* that its bacterial symbionts have very strong expression of ABC transporters for the uptake of nutrients (Kleiner, et al., 2012), it could be assumed that the protein detected in *P. vexillosa* is synthetized by symbiotic bacteria of the polychaete and not the polychaete itself. When comparing the MXR activity of *L. variegatus* to that of the other members of the phylum *Annelida*, inhibition of the MXR by verapamil seems to be more pronounced in *U. caupo* and *E. andreii* than *L. variegatus*. The maximal increase in fluorescence caused by verapamil was approximately 400 % in *U. caupo* (22 µM ver) and 80 % in *E. andreii* (10 µM ver) (Hackenberger, et al., 2012; Toomey and Epel, 1993), whereas in this study 20 µM ver caused only a 20 % rise in fluorescence compared to control in *L. variegatus*.

If reversin 205 had inhibited the MXR system in *L. variegatus*, the RhB and Ca-AM content in the tissue would have increased, and an increase in fluorescence would have occurred. What was seen was the opposite: there was a drop in fluorescence with both substrates. There are two possible explanations to this observation. First, reversin 205 could be toxic to *L. variegatus*. This toxicity would be seen in Ca-AM exposures as a drop in calcein fluorescence as esterase activity would be inhibited. As the membrane integrity would be compromised, the RhB content in the cells would diminish. However, acute toxicity tests (72 h) confirmed that reversin 205 at the tested concentrations (together with the substrates) was not toxic to *L. variegatus* (Vehniäinen, unpublished). The other explanation to the decreased accumulation of substrates is that in *L. variegatus* reversin 205 stimulates the activity of the MXR system. Some ABC proteins possess multiple binding sites with different substrate specificities, and binding of one substrate at one binding site may stimulate the transfer of the other substrate (Shapiro and Ling, 1997). Whereas it is possible that the same compound acts as a substrate and an inhibitor, and even as an inhibitor and an inducer of MXR activity (Srivalli and Lakshmi, 2012; Velki and Hackenberger, 2013), we are not
aware of other systems in which reversin 205 stimulates ABC protein transfer activity. As the gene
and protein sequences of *L. variegatus* ABC proteins remain unresolved, the structure of the active
site of them cannot be modeled.

Whereas the inhibitory effect of verapamil in *L. variegatus* was rather weak, a strong inhibition was
observed with the ABCC1 inhibitor MK571. This suggests that the ABC transport protein activity in *L.
variegatus* is mainly of ABCC-type. Neither verapamil nor MK571 caused a rise in Ca-AM
fluorescence, which could mean that Ca-AM is not as good a substrate as RhB for *L. variegatus* ABC
transport proteins. The autofluorescence of *L. variegatus* tissues at the wavelengths used to quantify
Ca-AM may have reduced the sensitivity of the assay, but the rise in RhB fluorescence upon MK571
exposure was so substantial (13 x) that it should have been seen also with Ca-AM, had it worked
similarly well as a substrate.

This study shows that the two invertebrate model species in ecotoxicology, *Daphnia magna* and
*Lumbriculus variegatus*, possess MXR activity, but the substrate and inhibitor specificity seems to
differ greatly between these species from different phyla. It is likely that also xenobiotics in nature
will act differently as substrates and inhibitors of the MXR system in these species. As MXR activity
affects both bioaccumulation and toxicity of chemicals, modulation of the activity may have
ecotoxicological consequences. MXR activity measurements in *D. magna* and *L. variegatus* may
provide valuable information about the effects of aquatic contaminants and contaminant mixtures in
these organisms.

Acknowledgement
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Table 1. Primers used in the qPCR.

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Figure captions

Figure 1. Daphnids of all ages possess *abcb1*, *abcc1/3*, *abcc4*, and *abcc5* mRNA. *abc* mRNA expression was studied in newborn (< 24 h), juvenile (7 - 8 d) and adult (28 d) daphnids, using *gadph* and *α-tubulin* as reference genes. Data represent mean ± SE of the mean of three replicates, each pooled of ca. 70 (< 24 h) or 8 - 10 (7 - 8 d and 28 d) organisms.

Figure 2. ABCB1 but not ABCC1 transporter inhibition increases rhodamine B accumulation in *Daphnia magna* tissues. *Daphnia magna* juveniles were exposed to 1 µM rhodamine B with or without verapamil (a), reversin 205 (b) or MK571 (c). Data represent mean ± standard deviation of the mean of two independent experiments performed in triplicate. *P < 0.05* when compared to control (one-way ANOVA, followed by Dunnett’s T3 test).

Figure 3. ABCB1 but not ABCC1 transporter inhibition increases calcein-AM accumulation in *Daphnia magna* tissues. *Daphnia magna* juveniles were exposed to 0.5 µM calcein-AM with or without verapamil (a), reversin 205 (b) or MK571 (c). Data represent mean ± standard deviation of the mean of two independent experiments performed in triplicate. ***P < 0.001* when compared to control (independent samples Kruskal-Wallis test, followed by Dunnett’s T3 test).

Figure 4. ABCB1 and ABCC1 transporter inhibition affects rhodamine B accumulation in *Lumbriculus variegatus* tissues. *Lumbriculus variegatus* were exposed to 1 µM rhodamine B with or without verapamil (a), reversin 205 (b) or MK571 (c). Data represent mean ± standard deviation of the mean of two independent experiments performed in triplicate. *P < 0.05* and **P < 0.01* when compared to control.

Figure 5. ABCB1 but not ABCC1 transporter inhibition affects calcein-AM accumulation in *Lumbriculus variegatus* tissues. *Lumbriculus variegatus* were exposed to 0.5 µM calcein-AM with or without verapamil (a), reversin 205 (b) or MK571 (c). Data represent mean ± standard deviation of the mean of two independent experiments performed in triplicate. *P < 0.05* when compared to control (one-way ANOVA followed by Tukey’s test).
Figure 1.

(a) 

Normalized expression

(b) 

FU/mg normalized to control

verapamil (µmol/L)

reversin 205 (µmol/L)
Figure 2.
Figure 3.
Figure 4.
Figure 5.

b

FU/mg normalized to control

reversin 205 (µmol/l)

0 1 5 10

-0.2 0.2 0.6 1.0 1.4 1.8

MK571 (µmol/l)

0 1 5 10 20

0 1 2 3 4

FU/mg normalized to control