

First evidence of the P-glycoprotein gene expression and multixenobiotic resistance modulation in earthworm

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Multixenobiotic resistance (MXR) is an important mechanism of cellular efflux mediated by ATP binding cassette (ABC) transporters that bind and actively remove toxic substrates from the cell. This study was the first to identify ABC transporter P-glycoprotein (P-gp/ABCB1) as a representative of the MXR phenotype in earthworm (*Eisenia fetida*). The identified partial cDNA sequence of *ABCB1* overlapped with *ABCB1* homologues of other organisms from 58.5 % to 72.5 %. We also studied the effect of five modulators (verapamil, cyclosporine A, MK571, probenecid, and orthovanadate) on the earthworm's MXR activity by measuring the accumulation of model substrates rhodamine B and rhodamine 123 in whole body tissue of the adult earthworm. MK571, orthovanadate, and verapamil significantly inhibited MXR activity, and rhodamine 123 turned out to better reflect MXR activity in that species than rhodamine B. Our results show that *E. fetida* can serve well as a test organism for environmental pollutants that inhibit MXR activity.

KEY WORDS: *ABC transport proteins; cellular detoxification; cyclosporine A; Eisenia fetida; MK571; MXR; orthovanadate; probenecid; soil organism; verapamil*

The earthworm (*Eisenia fetida*) is often used in ecotoxicological research because of its abundance in the soil and the ability to tolerate and bio-accumulate high quantities of contaminants, including man-made chemicals (1-5). Like many other organisms, it can survive in highly contaminated environments thanks to the efflux of toxicants from the cell mediated by transporter proteins and detoxification enzymes (6-7). The most studied efflux transporter proteins involved in the so-called multixenobiotic resistance (MXR) mechanism are ATP-binding cassette (ABC) transporters. These transmembrane proteins, present in all living organisms, interact with a wide number of chemicals, including xenobiotics, and pump them across the cell membrane, preventing their accumulation in the cell (8-10).

The most common MXR transmembrane proteins are P-glycoprotein (P-gp or ABCB1), multidrug resistance-associated proteins (MRPs or ABCC1-3), and breast cancer resistance protein (BCRP or ABCG2) (11-12). P-gp, the first and best-characterised ABC transporter, is essential for the efflux of substrates across the membrane (13) and highly effective in the tissues of vertebrates and invertebrates (14-19).

Since the measurement of MXR activity can tell a lot about the effects of xenobiotics on ABC transporters, MXR activity can be used as a biomarker of exposure and a tool for soil biomonitoring (10, 20). The aim of our study was 1) to identify the sequence of P-gp/*ABCB1* gene mRNA transcript in the earthworm and 2) to determine the modulation effects of MXR efflux activity in *E. fetida* by five different types of inhibitors.

MATERIALS AND METHODS

Chemicals

Verapamil hydrochloride (CAS No. 152-11-14; VER), cyclosporine A (CAS No. 59865-13-3; CA), MK-571 sodium salt hydrate (CAS No. 115103-85-0; MK571), probenecid (CAS No. 57-66-9; PROB), sodium orthovanadate (CAS No. 13721-39-6; OV), rhodamine 123 (CAS No. 62669-70-9; R123), rhodamine B (CAS No. 81-88-9; RB), ampicillin (CAS No. 7177-48-2), isopropyl β -D-1-thiogalactopyranoside (CAS No. 367-93-1; IPTG), and 5-bromo-4-chloro-3-indolyl β -D-galactoside (CAS No. 7240-90-6; X-Gal) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS: NaCl 0.138 mol L⁻¹; KCl 0.0027 mol L⁻¹; pH 7.4) and Luria Bertani medium (LB: 1.0 % tryptone, 0.5 % yeast extract, 1.0 % NaCl; pH 7.0) were also prepared with chemicals obtained from Sigma-Aldrich.

Test organism

For each paper contact experiment we used 75-80 adult specimens of earthworm (*Eisenia fetida* Savigny, 1826) weighing 120-250 mg, raised on a culture and kept in the dark at 20±1 °C. Before each experiment, the earthworms were removed from the culture and placed on moist filter paper in Petri dishes for 24 h (still in the dark and at 20±1 °C) to empty the gut content.

Identification of P-gp/ABCB1-related mRNA transcript

Primers P-gp-F and P-gp-R (F: 5'-GCGGCTGTGGGAAGAGCAC-3', R: 5'-TGTTGTCTCCGTAGGCAATGTT-3') for the amplification of the *ABCB1* gene were designed based on the available, highly conserved mammalian, fish, and invertebrate *ABCB1* genes (*Homo sapiens*, *Bos taurus*, *Tetraodon nigroviridis*, *Fundulus heteroclitus*, *Platichthys flesus*, and *Caenorhabditis elegans*) and were obtained from Invitrogen (Carlsbad, CA, USA). The upstream primer matches the upstream part of the Walker A module to encompass the consensus nucleotide-binding domain (NBD) sequence GXXGXGKST. This motif embodies the glycine-rich phosphate-binding loop or P-loop in the NBDs. Total RNA samples were extracted using an EZ-10 Spin Column Total RNA Mini-preps Super Kit

(Bio Basic Inc., Ontario, Canada) (21). Approximately 100 mg of earthworm tissue was homogenised in 450 μ L buffer RLT by sonication for 30 sec (Ultra-Turrax T18 homogeniser, IKA, Germany). Other steps of RNA purification were performed according to manufacturer's instructions. Quality and quantity of each RNA sample was analysed with a BioSpec-nano apparatus (Shimadzu Biotech, Kyoto, Japan). Next, 1 μ g of total RNA was reversely transcribed with the PrimeScript First Strand cDNA Synthesis Kit (TaKaRa Bio Inc, Kyoto, Japan) (21) following the manufacturer's instructions and using OligodT primer and random hexamers. The obtained cDNA was then used as a template (2 μ L per reaction) for the *ABCB1* gene fragment amplification.

Polymerase chain reaction (PCR) was performed with the following mixture: 0.5 μ mol L⁻¹ P-gp-F and P-gp-R primers, 200 μ mol L⁻¹ dNTPs, 1x Q5 reaction buffer and 0.02 U of Q5 High-Fidelity DNA Polymerase (EC 2.7.7.7) (New England BioLabs, Beverly, MA, USA) in the total volume of 50 μ L. Cycling parameters were: one cycle of denaturation at 98 °C for 30 s followed by 32 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 20 s, extension at 72 °C for 30 s, and final extension at 72 °C for 2 min. This procedure yielded a DNA fragment of ~200 bp, as confirmed by electrophoresis on 1 % agarose gel. The amplicon was excised and purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) (22).

Before TA-cloning, 10 μ L of DNA fragment was A-tailed with 1 μ L of 2 mmol L⁻¹ dATP, 1 μ L of 10x buffer, and 1 μ L (5 U) of Ex-Taq polymerase (EC 2.7.7.7), totalling 10 μ L, (TaKaRa Bio Inc, Kyoto, Japan) at 72 °C for 30 min. The DNA fragment was then TA-cloned using the pGEM-T Vector System (Promega, Madison, WI, USA) (23) according to the manufacturer's instructions. Rubidium chloride-treated *Escherichia coli* competent cells were transformed using ligation mixture (21), plated onto LB/ampicillin/IPTG/X-Gal plates, and incubated at 37 °C overnight. Positive colonies (white) were selected and grown overnight at 37 °C in 2 mL of the Luria-Bertani medium supplemented with ampicillin (100 μ g mL⁻¹). Plasmids were isolated using the QIAprep Miniprep kit (Qiagen) (21) and inserts were sequenced on an ABI PRISM® 3100-Avant Genetic Analyser (Applied Biosystem, Carlsbad, CA, USA) using the ABI PRISM BigDye Terminator v 3.1 Ready Reaction Cycle Sequencing Kit and pUC/M13 forward and reverse primers (Promega, Madison, WI, USA).

ABCB1 sequence analysis

The obtained, partial P-gp/*ABCB1* cDNA sequence was further analysed using the National Center for Biotechnology Information (NCBI) basic alignment search tools tBlastx and Blastp. Multiple sequence alignments and determinations of identity rates between deduced amino acid sequences of ABC transporters from different species were performed using BioEdit software and Clustal X version 2.0 incorporated in the MEGA 5 software (24), both set at default parameters. We used the obtained alignment for phylogenetic reconstruction relying on Neighbor-Joining (NJ) and maximum likelihood (ML) analysis. For the construction of the NJ tree we used the MEGA 5 software. Reliabilities of phylogenetic relationships were evaluated using a non-parametric bootstrap analysis with 1,000 replicates (25). Bootstrap values exceeding 70 were considered well supported. Before the construction of the ML tree, we determined LG+G (26) as the optimal model of protein evolution using the PROTTEST 2.4 server (27). The tree was constructed using PhyML 3.0 (28) and was improved with five random starting trees using both subtree pruning and regrafting and nearest neighbour interchange (29). Branches were tested with the approximate likelihood-ratio test (30).

Exposure of earthworms to model MXR inhibitors

To determine MXR activity in adult *E. fetida* we used the filter paper contact test method (5). The principle of this *in vivo* assay is to measure bioaccumulation of a model fluorescent substrate in the whole body of the earthworm. Exposure is done in the absence (control group) or presence of a model MXR inhibitor. We used two fluorescent model MXR substrates - rhodamine B (RB) and rhodamine 123 (R123) - and five MXR inhibitors - verapamil (VER), cyclosporine A (CA), MK571, probenecid (PROB), and sodium orthovanadate (OV). The following procedure was used: earthworms were placed into 90-mm Petri dishes with filter paper at the bottom, damped with 1.7 mL of tested chemicals. Five earthworms were placed in each Petri dish for each treatment group (n=5) and the measurement was done in triplicate. The final concentrations of MXR inhibitors and fluorescent substrates were as follows: VER - 10 $\mu\text{mol L}^{-1}$, CA - 10 $\mu\text{mol L}^{-1}$, MK571 - 50 $\mu\text{mol L}^{-1}$, OV - 100 $\mu\text{mol L}^{-1}$, PROB - 500 $\mu\text{mol L}^{-1}$, and RB and R123 - 10 $\mu\text{mol L}^{-1}$. Earthworms were first exposed to the inhibitor for 24 h (pre-treatment)

and then transferred to a mixture of the inhibitor and fluorescent substrate for additional 24 h or 48 h. All Petri dishes were kept in the dark at 20 ± 1 °C.

Measurement of rhodamine fluorescence

The amount of accumulated RB or R123 was measured 24 and 48 h after exposure. For sample preparation, whole earthworms were weighed and then homogenised with the Ultra-Turrax T18 homogeniser (IKA, Königswinter, Germany) in 2 mL of cold PBS and centrifuged at 9000 g for 10 min. Supernatant was transferred to black 96-well microplates (Nunc, Thermo Fisher Scientific, Hvidovre, Denmark). The amount of fluorescent dye in the supernatant was determined in triplicate using FLUOstar OPTIMA plate reader (BMG Labtech Ltd., Aylesbury, UK) with excitation at 544 nm and emission at 590 nm for RB and excitation at 490 nm and emission at 544 nm for R123.

Statistical analysis

Before the statistical analysis of accumulated fluorescence in treated tissues, each sample was normalised relative to non-treated controls (i.e. background fluorescence was subtracted from each reading). Background fluorescence in tissues with no RB did not differ from that of the phosphate buffer extraction medium ($p < 0.05$). All experiments were performed at least three times and mean values and standard deviations (SD) determined. The results for rhodamine dye accumulation were expressed as fluorescent units per mg of earthworm tissue (FU mg^{-1}) and then plotted as percent of control values for final comparison. Significant differences were determined using the unpaired Student's *t*-test. Data are presented as mean \pm SD using Daniel's XL Toolbox open-source add-in for the Microsoft® Excel® spreadsheets.

RESULTS

Identification of P-gp/ABCB1-related sequence in E. fetida

Specific pairs of primers (P-gp-F and P-gp-R) generated a fragment which after cloning and sequencing resulted in the following 154 bp partial *ABCB1* sequence: 5'-AATTGTCCAGCTA CTGGAGAGATTCTACGATATTCCTGAGG G A C A A G T G A T G G T G G A T G G T C G C G

ATGTGAAGTCATTGAACGTCGCTTG GTT
AGAGCTCAGCTGGGCATTGTCTCCCAGGAAC
CGACTCTCTTTGACTGTAGCATTCGAGAA-3'.
The amplified fragment corresponds to the Walker
A module and the translated 51 amino acids were:
I V Q L L E R F Y D I P E G Q V M V D G R
DVKSLNVAWLRAQLGIVSQEPTLFDCSIRE.
This partial PCR product showed a high degree of
identity with P-gp from various other organisms
(Figure 1), and this homology was confirmed with
NCBI Blast2 Protein Database Query (data not
shown). Multiple alignment of P-gp sequences is
shown in Figure 2(b).

Phylogenetic analysis

We used phylogenetic analysis to establish the relationship between the identified P-gp/ABCB1 transport protein sequence and other available ABC transporters. The identified sequence grouped well

within the ABCB subfamily. The NJ tree is presented in Figure 2(a). ML analysis confirmed the position of the identified *E. fetida* sequence in ABCB subfamily (data not shown).

Accumulation of rhodamine substrates in the presence of MXR inhibitors

The filter paper contact test showed significant accumulation of both R123 and RB in whole earthworm tissue (Figure 3). Control values of R123 accumulation were 1.5 and 2.5 times higher than for RB, after 24 and 48 h, respectively (data not shown). Twenty-four-hour exposure significantly increased overall accumulation of R123 when MK571 and OV were used (48 % and 95 % increase, respectively; $p<0.05$). After 48 h, significant increase was observed with MK571, VER, and OV (42 %, 34 %, and 88 %, respectively, Figure 3a). When RB was used in the same set of experiments, significant increase in dye

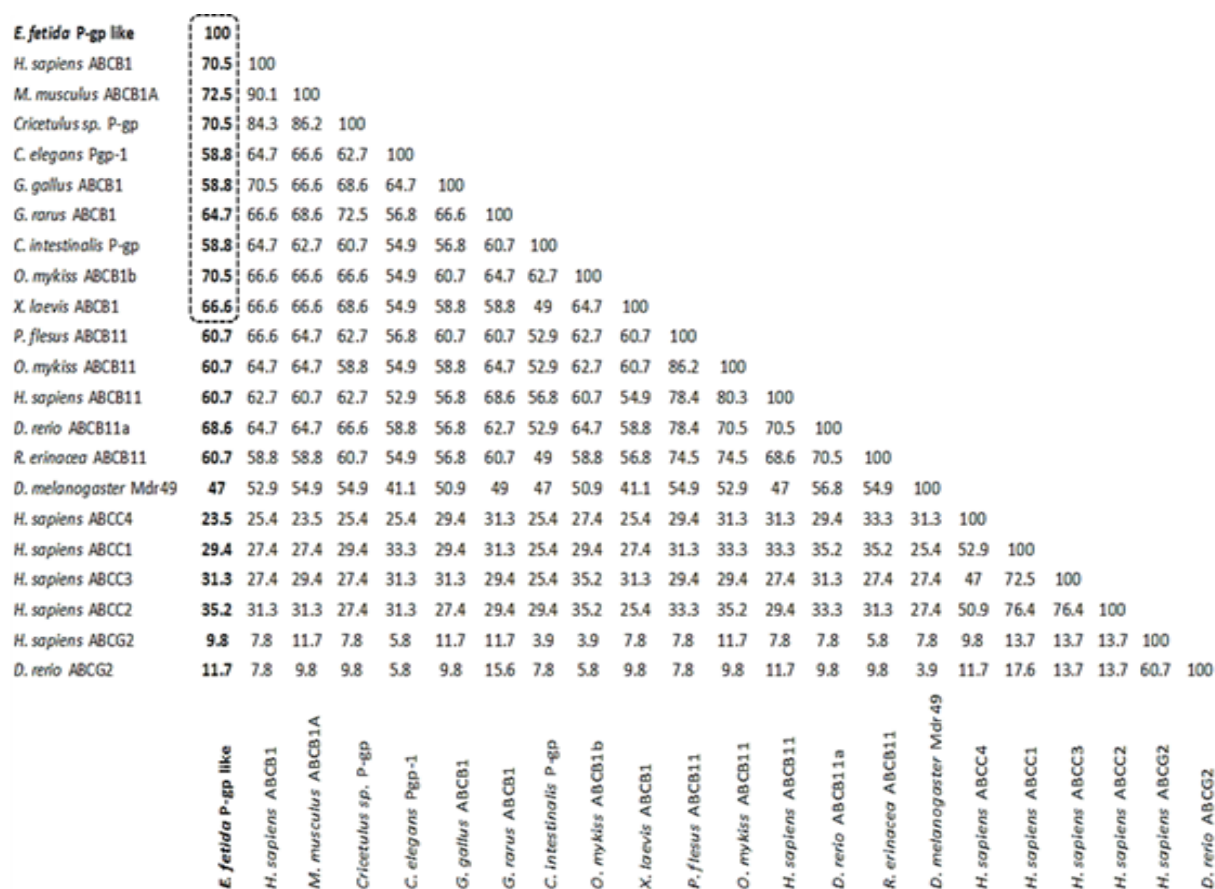


Figure 1 Percentage of identity between *E. fetida* P-gp like fragment protein sequence and other ABC transporters (based on ClustalW alignment, BioEdit)

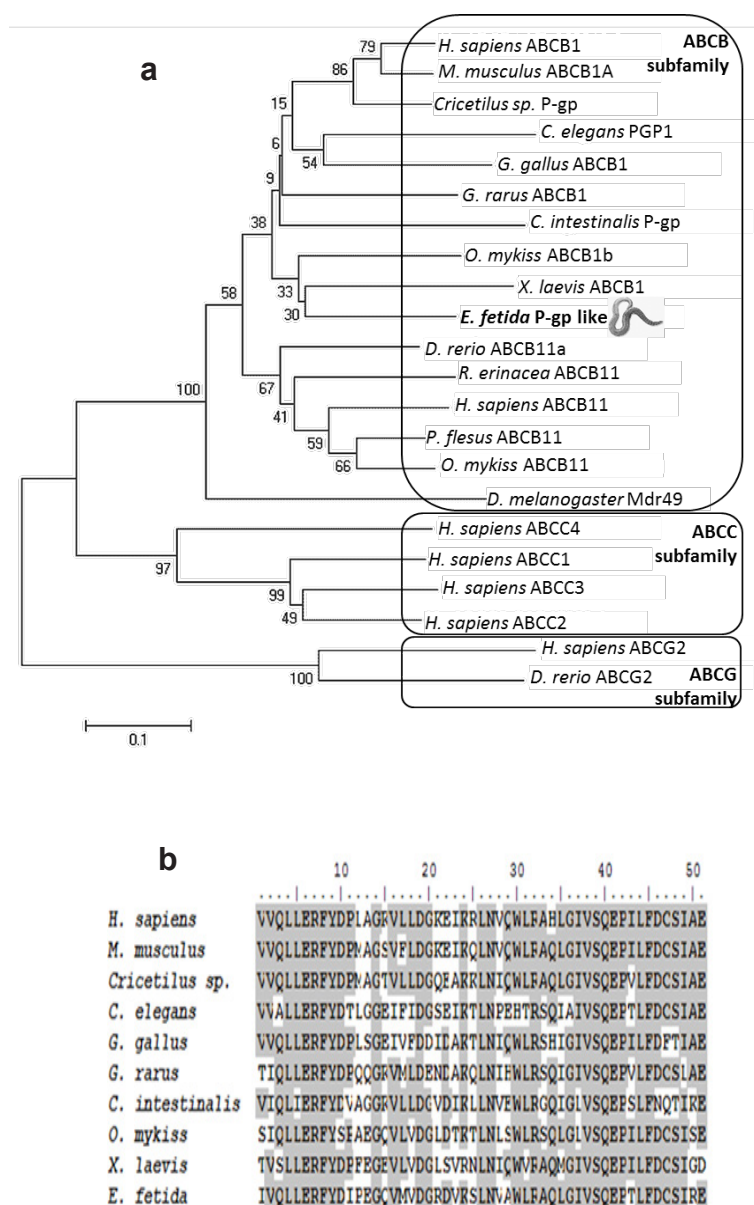


Figure 2 (a) Neighbor-Joining tree showing the relation between *E. fetida* P-gp like protein and different ABC transporter subtype sequences from various vertebrates and invertebrates. Bootstrap support values are shown at the nodes (1000 replicates). The accession numbers of ABCB transporters are: NP_502413.1, XP_002122143.1, AAA53440.1, XP_001337724.3, NP_523724.2, NP_990225.1, AFK33289.1, NP_000918.2, NP_003733.2, NP_035206.2, ADO95108.1, NP_001118128.1, CAC86593.1, AAK52958.1, NP_001081394.1; ABCC transporters: ABN79590.1, CAB45309.1, O15438.3, NP_005836.1; ABCG transporters: NP_001036240.1, NP_005836.1. **(b)** Multiple sequence alignment of *E. fetida* P-gp/ABCB1 and other P-gp/ABCB1-transporter sequences

accumulation was observed with MK571 and OV after 24 h (81 % and 78 %, respectively), and only with MK571 after 48 h (88 %, Figure 3b).

DISCUSSION

This study is the first to have identified ABC efflux transporters in *E. fetida* and has shown that MXR

efflux protein activity can be modulated by competitive and non-competitive inhibitors.

We obtained somewhat lower bootstrap support within the ABCB1 (P-gp) clade, presumably because we used a short sequence for alignment (51 amino acids). Therefore, for detailed determination of the phylogenetic position of *E. fetida* P-gp it will be necessary to obtain a longer sequence. However, this is the first ABCB sequence for the clade *Annelida* and

it is long enough for future expression studies of the *ABCB1* gene using long-term exposure filter paper contact tests.

We optimised the test for *E. fetida* by modifying the procedure for *E. andrei* (10). Like Hackenberger et al. (10), we have established that it is important to pre-treat earthworms with inhibitors for at least 24 h before adding substrate to the filter paper, as it turns out that pre-treatment helps inhibitors to enter earthworm tissue.

However, we obtained different fluorescence accumulation for RB and R123. R123 showed significant MXR inhibition with MK571, VER, and OV, and RB with to MK571 and OV. In addition, RB

accumulation was 1.5 times (24 h) and 2.5 times (48 h) lower than that of R123 in controls (data not shown). This suggests that R123 is the dye of choice for research with earthworms as model organisms. In our earlier study on several marine and freshwater invertebrates (16), the accumulation of these two substrates also differed, but that time in favour of RB.

Using different types of MXR inhibitors we were able to determine the dominant type of ABC transporter in *E. fetida*. OV inhibits a number of ATPases. VER and CA are known to inhibit P-gp/ABCB1 efflux pump, whereas MK571 and PROB inhibit MRP/ABCC-like activity (32-33). *E. fetida* seems to combine P-gp/ABCB1 and MRP/ABCC-like

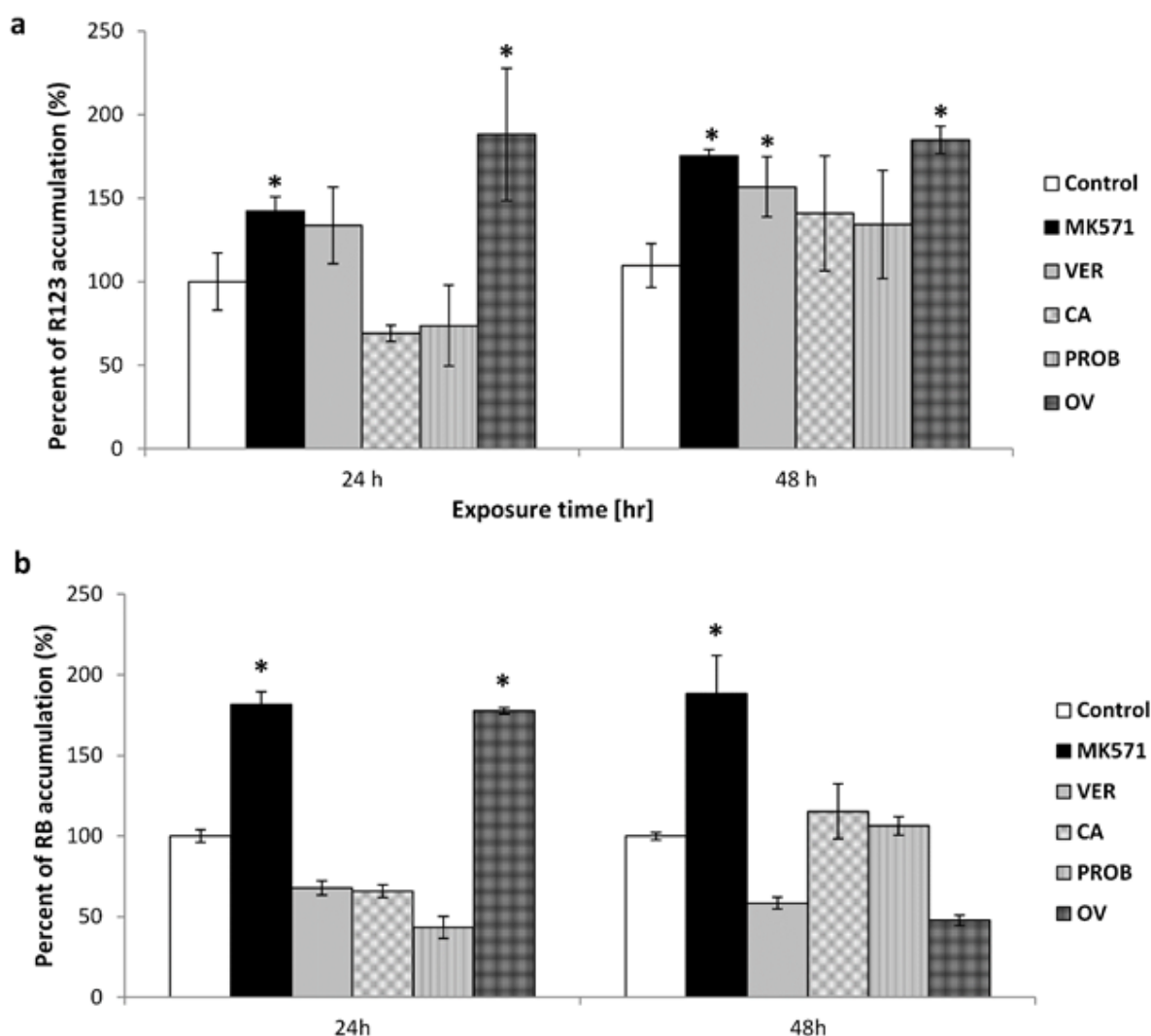


Figure 3 Accumulation of rhodamine dye in earthworm *E. fetida* after pre-treatment with different MXR inhibitors ($50 \mu\text{mol L}^{-1}$ MK571, $10 \mu\text{mol L}^{-1}$ VER, $10 \mu\text{mol L}^{-1}$ CA, $500 \mu\text{mol L}^{-1}$ PROB, $100 \mu\text{mol L}^{-1}$ OV) followed by exposure to rhodamine 123 (R123) (a) or rhodamine B (RB) (b) for 24 or 48 h. Data are expressed as the mean of triplicate \pm SD. * $p < 0.05$, t -test, $n = 5$

activity. Although we detected a P-gp sequence, MK571 showed higher inhibition than VER and CA, which suggests that MRP proteins dominate over P-gp. Unfortunately, we could not identify any MRP-related genes.

One of the interesting findings was lower fluorescence in earthworms exposed to CA and PROB than in control, regardless of the dye. Lower fluorescence was more pronounced with RB than with R123 and could be explained by varying expression of MXR efflux pumps. Additional experiments are needed for a more definitive conclusion.

Chemicals that are capable of inhibiting the MXR mechanism should rank high among environmentally hazardous chemicals because they threaten the basic biological defence and can revert natural resistance to disease (7, 9, 12, 16, 17). In addition, environmental samples expressing reverse MXR potential should be monitored and controlled. Our results show that *E. fetida* can serve well as a test organism for environmental pollutants with the potential to inhibit MXR activity.

CONCLUSION

This study has demonstrated that: a) the MXR defence system in earthworm *E. fetida* is at least partly mediated by the P-gp efflux transporter; b) the identified sequence is closest to the ABCB subfamily; c) MXR activity can be decreased by specific inhibitors (VER, MK571, OV), resulting in significant R123/RB accumulation in earthworm tissue; and d) R123 is the substrate of choice in *E. fetida* as model organism. These new findings should help to better characterise and identify all ABC proteins that may be involved in MXR in *E. fetida*.

In addition, optimised filter paper contact test could be a valuable tool for the toxicological assessment of polluted soil samples that contain MXR inhibitors, while the expression of the P-gp gene (up or down regulation) has a great potential to become a biomarker of pollution response.

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Sažetak

Prvi dokaz ekspresije gena za P-glikoprotein (P-gp/ABCB1) i djelovanja inhibitora na multiksenobiotičku otpornost u gujavice

Mehanizam multiksenobiotičke otpornosti (MXR) prisutan je u mnogim organizmima kao važan stanični detoksikacijski mehanizam. Posredovan je aktivnošću ABC prijenosnika koji vežu i aktivno izbacuju različite toksične tvari iz stanice. U ovom radu dani su podaci o molekularnoj identifikaciji ABC prijenosnika (eksportera) - P-glikoproteina (P-gp/Abcb1), kao jednog od predstavnika MXR fenotipa, u gujavici *Eisenia fetida*. Određen je djelomični slijed identificiranoga gena *Abcb1*, njegov predviđeni aminokiselinski slijed uspoređen je s ABCB1 homologima iz drugih organizama i utvrđena je identičnost od 58,5 do 72,5 %. Uz to, istraživali smo učinak pet modulatora (verapamil, ciklosporin, MK571, probenecid, ortovanadat) na aktivnost MXR mehanizma tih gujavica. Kako bismo potvrdili modulirajuće djelovanje istraživanih modelnih inhibitora na MXR mehanizam u *E. fetida*, mjerili smo akumulaciju modelnih supstrata rodamina B (RB) i rodamina 123 (R123) u tijelu spolno zrelih jedinki gujavica testom izlaganja na filter papiru. Rezultati su pokazali da svi istraživani modulatori značajno inhibiraju MXR transportnu aktivnost. Naši podaci prvi upućuju na prisutnost P-gp/*Abcb1* srodnih gena u gujavici *E. fetida*. Osim toga, ukazali smo na veliku važnost MXR-a kao specifičnog detoksikacijskog mehanizma koji omogućuje preživljavanje gujavica u onečišćenom okolišu.

KLJUČNE RIJEČI: ABC prijenosni proteini; ciklosporin A; *Eisenia fetida*; MK571; organizam iz tla; ortovanadat; probenecid; stanični mehanizam detoksikacije; verapamil

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