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Inhibition of multidrug/xenobiotic resistance transporter by MK571 improves dye (Fura 2) accumulation in crustacean tissues from lobster, shrimp, and isopod

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ABSTRACT

Multidrug/xenobiotic resistance transporters are present in living organisms as a first line defence system against small, potentially harmful molecules from the environment or from internal metabolic reactions. Multidrug resistance associated proteins (MRP) are one type of ATP-Binding-Cassette (ABC) transporters, which also transport dyes such as Fura 2, a calcium chelating fluorescence indicator. The specific MRP inhibitor MK571 was used to investigate the fluorescence intensity of cells in tissues of the brain and the midgut gland of the crustaceans *Homarus gammarus* (lobster), *Crangon crangon* (brown shrimp) and *Idotea emarginata* (isopod) during incubation with Fura 2AM (1 µM). In the presence of the inhibitor MK571 (50 µM), the fluorescence of brain tissue significantly increased in all of the three species. The midgut gland of *H. gammarus* showed a significant increase of fluorescence, whereas there was no effect in the midgut glands of *C. crangon* and *I. baltica*. The half maximal concentration of MK571 was 50 µM as measured in the midgut gland of *H. gammarus*. In conclusion, MRP transporters are present in the three investigated crustacean nervous systems. Using the midgut glands of the three species, only in *H. gammarus* MK571 inhibited dye extrusion, indicating species-specific differences of transporter systems, their specificity, or tissue specific expression.

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1. Introduction

Xenobiotic/multidrug resistance transporters belong to the large protein family of the ATP-Binding-Cassette transporters (ABC) which transfer small molecules across cellular membranes (reviewed by Borst and Elferink, 2002). The ABC-transporters are a first line defence against xenobiotics as well as anti-cancer drugs, extruding upon activation not only the activating agents but also several other molecules. The medical term 'multidrug resistance transporter' (MDR) was established as it became evident that tumor cells developed resistance against several types of cytostatics. In aquatic organisms the term "xenobiotic resistance transporter" is commonly used instead. These ABC-transporters are, however, similar to those in mammals (reviewed by Kurelec, 1992). The best investigated and ubiquitously found xenobiotic transporter is the p-glycoprotein or MDR1. It is present in e.g. crustaceans (Koehler et al., 1998), bivalves (Luedeking and Köhler, 2004), echiurids (Kurelec, 1992), and fishes (Bard, 2000).

Multi drug resistance associated proteins (MRP) were found in several mammalian tissues (reviewed by Higgins, 2007), their orthologous genes were found in fish and echinoderms (Miller et al., 2002; Cai and Gros, 2003; Sauerborn et al., 2004; Zaja et al., 2007) and recently also shown to be present in marine diatoms (Scherer et al., 2008) and in bivalves (Luckenbach et al., 2008) by use of a transporter specific inhibitor, MK571 (Leier et al., 1994; Gekeler et al., 1995).

Especially in aquatic organisms this first line defence should be of considerable importance because the whole organism may be exposed to bioactive molecules additionally to ingested compounds.

We selected three crustacean species from the North Sea and studied the presence of MRP transporters in two tissues: the midgut gland as the major digestive organ and the nervous system as especially vulnerable target for bioactive molecules. The experimental approach was based on the property of the calcium chelating fluorescence dye Fura 2 to act as a substrate for MRP transporters in vertebrate nervous system (Manzini et al., 2008) and in small marine algae (Scherer et al., 2008). Accordingly, the accumulation of this dye in living cells after inhibition of extrusion served as a measure for the presence of MRP transporters. Preliminary data were recently published as a conference abstract (Bickmeyer et al., 2008a).

2. Materials and methods

2.1. Animals

Lobsters (*Homarus gammarus*) and isopods (*Idotea emarginata*) were taken from the rearing facilities of the Biologische Anstalt Helgoland (Franke and Janke, 1998; Walter, 2005; Mehrtens et al., 2005). Only juvenile lobsters (stage 4; 1st juvenile stage) were used for the experiments. Brown shrimp, *Crangon crangon*, were captured

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in the North Sea by trawling with the research vessel FK Uthörn. The animals were first cooled for 10–20 min at −20 °C in the freezer and then killed under a binocular by dissecting the brains and the midgut glands as indicated in Fig. 1.

2.2. Chemicals

Fura-2-acetoxymethylester (Fura 2 AM, Sigma-Aldrich F0888) (Grynkiewicz et al., 1985) was used in the final concentration of 1 µM (1 mM stock solution in DMSO), which facilitated the measurement of dye loading in the cytosol compared to higher concentrations. MK571 (Biomol, RA109) is a *(E)*-3-[[[3-[2-(7-Chloro-2-quinolinyl)ethenyl]phenyl]-[[3-dimethylamino)-3-oxopropyl]thio] methyl]thio]-propanoic acid is described to be a competitive antagonist of leukotriene D4 (LTD4) receptor and a specific inhibitor of MRP transporters (Leier et al., 1994; Gekeler et al., 1995). MK571was used in several final concentrations (1 µM–200 µM) as indicated (20 mM stock solution in water) (Fig. 2).

2.3. Fluorometric measurements

Fluorescence was monitored with an imaging system (Visitron) and a CCD camera mounted on an inverted microscope (Zeiss Axiovert 100) as fluorescence intensity units (without dimension). The microscope was equipped with an UV objective (Zeiss NeoFluar 20×). For optical excitation a monochromator (Visichrome, Visitron Systems) was used. Ten to twenty regions of the studied tissue were selected using "the region of interest"-function of the software (Metafluor, Meta Imaging Series).

2.4. Statistics

The data of all measured regions of one individual animal were pooled and averaged and treated as $N=1$. Results are presented as means ± SD. Statistics and calculations were performed using computer software GraphPad Prism and Igor (WaveMetrics) using unpaired and two tailed *t*-test. Results of each experimental group were calculated from $N=10$ individuals. One group (*I. emarginata* midgut gland, without MK571) consists of $N=9$ animals.

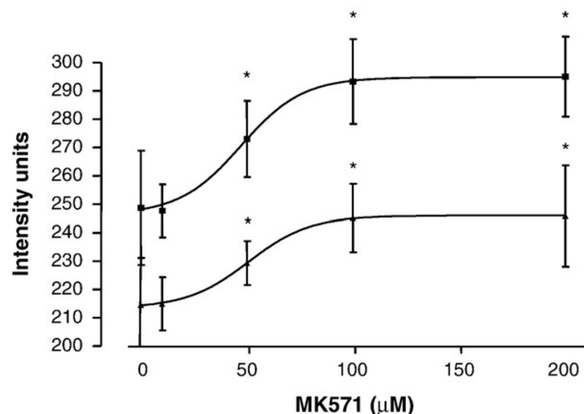

Fig. 2. Concentration–effect relationship of dye loading with Fura 2AM (1 µM, 30 min) in the midgut gland of *H. gammarus* using different concentrations of MK571. Upper curve: Fluorescence at 380 nm excitation. Lower curve: Fluorescence at 350 nm. Asterisks indicate significant differences compared to controls in both curves (* = $p<0.05$; $N=3$–10; means ± SD).

3. Results

In the initial experiment we established a concentration–effect relationship of MK571 and dye loading in the midgut gland of *H. gammarus*. This experiment showed that Fura 2 accumulated in the tissue of *H. gammarus* in the presence of MK571. The dye loading was most distinct at concentrations of 100 µM and higher (Fig. 2). However, to avoid non-specific side effects of MK571 we used in all following experiments the half maximal concentration of 50 µM. Concentrations of around 50 µM proved suitable in previous studies as well (Manzini and Schild, 2003; Scherer et al., 2008). The concentrations of Fura 2 AM was chosen to be 1 µM, because higher concentrations revealed very fast dye uptake, which made it difficult

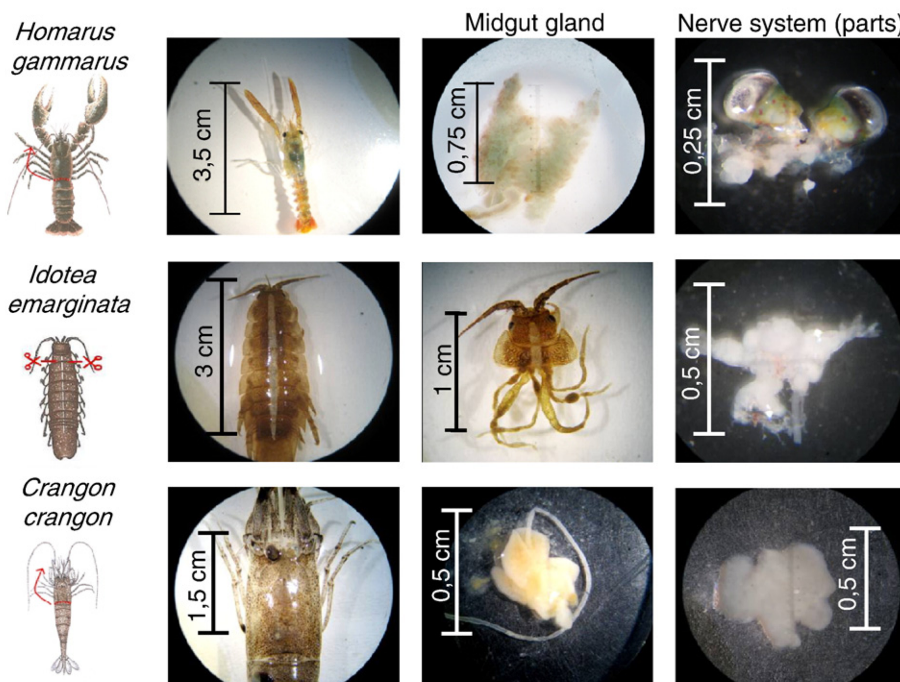

Fig. 1. Preparation of the midgut gland and the supraesophagyal ganglion in lobster (*Homarus gammarus*), shrimp (*Crangon crangon*) and isopod (*Idotea emarginata*) as indicated. The red line at the left sketch demonstrates line of cutting. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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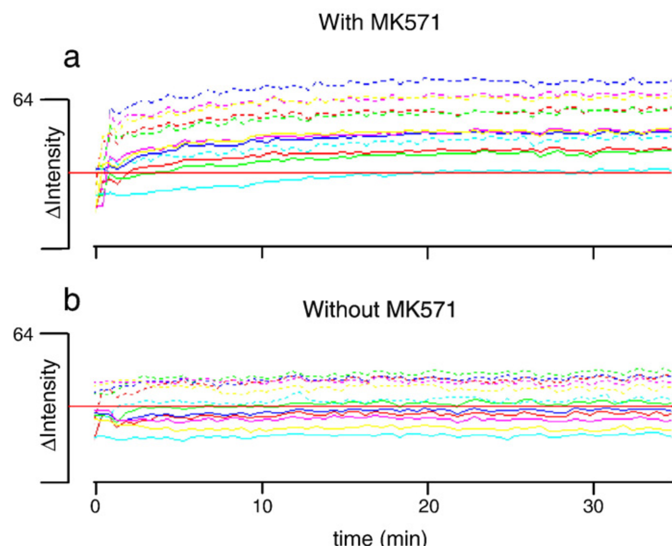

Fig. 3. Incubation of midgut gland tissue of *H. gammarus* with Fura 2AM (1 µM) for 35 min in the presence (a) and in the absence (b) and of 50 µM MK571 (2 images/min, each curve represents one region of interest). The *y*-axis shows the increase of fluorescence in Δ intensity units.

to report the increase of dye concentration during the time of incubation.

Fig. 3 shows the details of a single experiment using 1 µM Fura 2 and 50 µM MK571 in comparison to a control without MK571 (*H. gammarus*, midgut gland). There is a distinct increase of fluorescence in the presence of MK571. The increase is strongest during the first minutes but continues for about another 30 min. UV-excitation was at 350 nm and 380 nm, the calcium sensitive wavelengths during

Table 1
Summary of differences of Fura 2AM loading experiments with and without MK571.

	Nerve system		Midgut gland	
	λ350	λ380	λ350	λ380
Homarus gammarus	$p=0.04$	$p=0.004$	$p=0.021$	$p=0.005$
Crangon crangon	$p=0.037$	$p=0.044$	$p=0.21$ n.s.	$p=0.13$ n.s.
Idotea emarginata	$p=0.0007$	$p=0.0003$	$p=0.31$ n.s.	$p=0.18$ n.s.

ratiometric calcium measurements. The emission was measured at 510 nm. To avoid effects of calcium binding on fluorescence measurements at a single excitation wavelength (increase at 350 nm, decrease at 380 nm during Ca^{2+}-chelation), we recorded fluorescence at both excitation wavelengths, thus demonstrating the increase of dye concentration. There was a significant increase of dye loading in the nervous systems of all three crustacean species (*H. gammarus*, *C. crangon*, *I. emarginata*) in the presence of MK571 (Fig. 4).

In midgut glands, increased dye accumulation was only recorded in *H. gammarus* (Fig. 4) but not in the midgut glands of *C. crangon* and *I. emarginata*. The results are summarized in Table 1 including *p* values.

4. Discussion

Multi drug resistance transporters display a group of ATP-binding cassette transporters which are known in aquatic organisms as multi xenobiotic resistance transporters. According to current knowledge they share functions and orthologous genes. We used a functional approach to identify the MRP transporters by their substrate specificities and the specific inhibitor MK571 (Gekeler et al., 1995). In different vertebrates and in marine algae, the calcium chelating dye Fura 2 was proven to be a suitable substrate for MRP transporters (Manzini et al., 2008; Scherer et al., 2008). In sea urchin eggs MK571

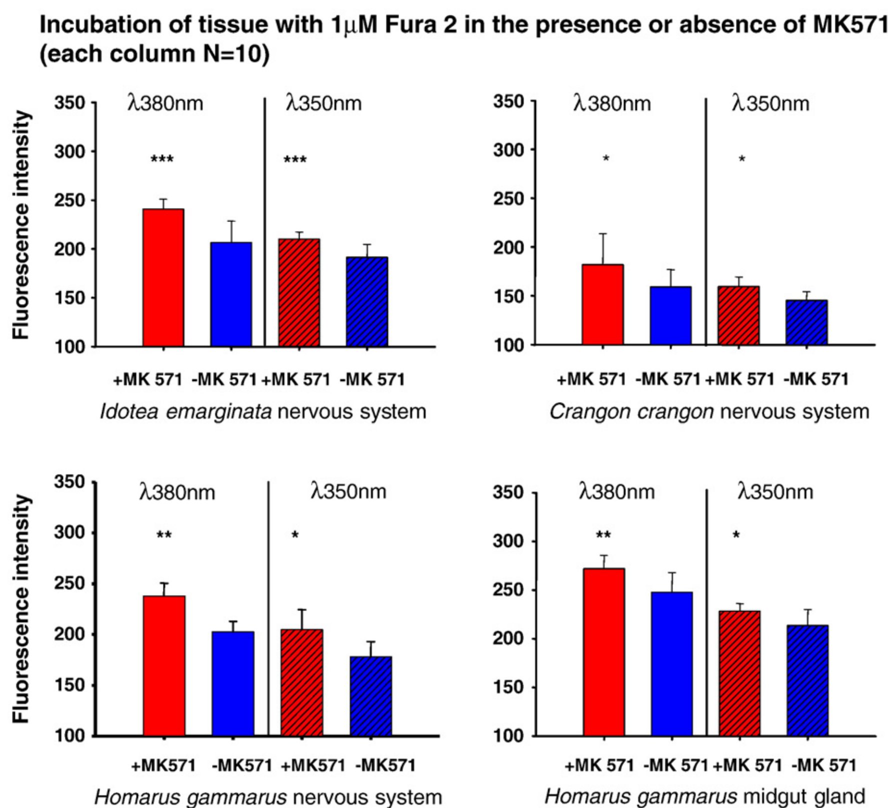

Fig. 4. Fluorescence intensities (510 nm) at two excitation wavelength (350 nm and 380 nm) of controls and with 50 µM MK571 after incubation with 1 µM Fura 2 AM (30 min). Species and tissues as indicated in the figures. All related columns show significant differences as indicated by asterisk and *p* values (*$p<0.05$, **$p<0.005$, ***$p<0.001$).

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improved dye loading with Fura 2, which facilitated calcium measurements during fertilization (Schäfer et al., 2009). Our results provide indirect evidence for the existence of MRP transporters in the crustacean brain where it may serve as an important defence system especially against toxic lipophilic and amphiphilic small compounds. These small compounds in prey and food may be secondary metabolites produced in algae or sponges which show in part feeding deterrence (Pawlik et al., 1995; Assmann et al., 2000) as well as disturbance of neuronal and hormonal signalling in mammalian endocrine cells (Bickmeyer et al., 2004; Bickmeyer, 2005; Hassenklöver et al., 2006). Some of these natural products seem to be not expelled from living tissues and cells and are therefore under suspicion to inhibit—beside other effects—xenobiotic transporters (Bickmeyer et al., 2008b).

In the investigated midgut glands only *H. gammarus* showed increased dye accumulation in the presence of MK571. This result may have different reasons: *C. crangon* and *I. emarginata* may lack MRP transporters in the midgut gland or this protein shows a different pharmacological profile and specificity due to structural alterations. In the nervous system of both crustaceans MK571 is effective, which gives a hint to the absence of MRP in the midgut glands of *C. crangon* and *I. emarginata* and not necessarily structural differences. Which kinds of transporters were present is not known and may be a target of future investigations. As we applied only the concentration of 50 µM in *C. crangon* and *I. emarginata*, it is still possible, that higher concentration could induce an inhibition of transport mechanism. To avoid unspecific side effects we omitted higher concentrations for the investigation of substrate transport inhibition. It is unlikely that a difference in dye uptake into the cytosol is responsible for the reported results as Fura 2AM is membrane permeable and trapped in the cytosol by elimination of the ester. Fura 2 was actually taken up by the midgut glands as indicated by the fluorescence. It has not been reported that MK571 has any effect on esterases.

In conclusion, MRP transporters were present in the three investigated crustacean nervous systems. In the midgut glands, only in *H. gammarus* MK571 displayed an inhibiting of dye extrusion, giving evidence to species-specific differences in transporter systems or transporter specificity or its functional expression in different tissues.

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