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Characterisation of the multixenobiotic resistance (MXR) mechanism in the freshwater snail Physa acuta from Patagonia (Argentina)

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RESEARCH ARTICLE

Characterisation of the multixenobiotic resistance (MXR) mechanism in the freshwater snail *Physa acuta* from Patagonia (Argentina)

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P-glycoprotein (P-gp) mediated multixenobiotic resistance (MXR) is a mechanism analogous to multidrug resistance (MDR), which has been extensively characterised in mammalian tumours. The expression and function of the MXR mechanism has been demonstrated in numerous aquatic organisms and has been proposed as a biomarker for pollution assessment. The purpose of this study was to evaluate the activity and expression of the MXR mechanism in freshwater snails (*Physa acuta*) of the Andean–Patagonian region. The accumulation and efflux rates of the model P-gp substrate Rhodamine B in snails previously exposed to pollution were measured. Results confirmed that MXR activity decreased after maintenance in clean water, and that a depuration period of 7 d was long enough to observe a significant deinduction of this system. The results demonstrate the presence of a P-gp-like transport system in the freshwater snail *P. acuta* from Patagonia, and suggest its function as a defence system. The results of this study could be used to provide information on the possible use of these snails as bioindicators in toxicological testing.

Keywords: MXR; P-glycoprotein; freshwater snails; Patagonia; bioindicators

Introduction

Aquatic organisms have developed mechanisms that protect them from environments containing multiple anthropogenic pollutants or natural product toxins (Kurelec 1992). The multixenobiotic resistance (MXR) phenotype found in aquatic organisms represents a defence system that is analogous to the well-known multidrug resistance (MDR) phenomenon characterised in tumour cells resistant to chemotherapeutic drugs. MDR is considered one of the major causes of failure in cancer chemotherapy and is often associated with the overexpression of a 170-kDa membrane P-glycoprotein (P-gp)—an adenosine triphosphate-dependent pump that transports a wide variety of structurally unrelated compounds out of cells. Consequently, P-gp mediates a reduction in intracellular drug accumulation and hence reduces its efficacy (for reviews see Borges-Walmsley et al. 2003; Loo & Clarke 2005).

Since the first observation of the environmental relevance of these transporters by Kurelec and Pivcevic (1989), functional analyses that support immunochemical and biological evidence for the presence of the P-gp-related multixenobiotic transporter were reported in various organisms including mussels, sponges, oysters and fish, among others (Kurelec 1992; Kurelec et al. 1996; Bard 2000; Smital et al. 2000). Field and laboratory xenobiotic exposures induce the expression of P-gp and MXR activity in several aquatic organisms (Minier & Moore 1996; Eufemia & Epel 1998; Smital & Kurelec 1998; Smital et al. 2003; Amé et al. 2009). Moreover, given that the activity of the MXR system is proportional to the level of pollution, it has been proposed as a biological

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marker of exposure (Kurelec et al. 1996; Epel 1998). However, this detoxification mechanism may not be a sensitive indicator of contaminant exposure in certain species (Damaré et al. 2009).

MXR emerges as a general defence system which represents the first line of defence against xenobiotics in many aquatic organisms. To our knowledge, there is no prior evidence of this mechanism in the aquatic organisms of Patagonia. In this context, this study aims to describe the MXR phenotype in the snail *Physa acuta*—an easily available, widespread species that is relatively resistant to organic pollution present in different streams and rivers of the Patagonian Andes.

A combination of functional assays that measure bioaccumulation and efflux of fluorescent substrate Rhodamine B (RB) and molecular approaches were used to examine the MXR system. We also investigated MXR activity after a laboratory depuration period to determine the background level of MXR activity in this system.

Materials and methods

Site description and animal collection

For this study we used *Physa acuta* freshwater snails within a size range of 8–11 mm. The specimens were collected in the Esquel stream, Futaleufú-Yelcho watershed (43°S, 73°W) between September 2011 and April 2012, from 0.2 to 0.5 m depth. The water temperature was 8–15 °C. The middle of this water system is disturbed by discharges from the city of Esquel sewage treatment plant, and is also polluted with household waste. Snails were collected at the post-urban section of the stream, which displays high levels of nutrients including phosphorus and nitrogen compounds. The mean values of nitrates and nitrites, ammonia and soluble reactive phosphate were 86, 79 and 19 times higher, respectively, than the values recorded at the pre-urban reaches as revealed by Miserendino et al. (2008). Another study has also found very high values of organic matter, biochemical oxygen demand and faecal coliforms at the post-urban section of the stream (Pizzolon & Miserendino 2001).

After collection, all of the specimens were maintained in flowing dechlorinated water at 8–12 °C in the laboratory's glass aquaria. Snails were given no food during the adaptation period and then were fed ad libitum with lettuce leaves (Núñez 2010).

Measurement of MXR activity

The in vivo measurement of the level of MXR activity was performed using the accumulation and efflux versions of the method with RB as the model P-gp substrate and with minor modifications to previously published assays (Smital & Kurelec 1997; Kurelec et al. 2000; Smital et al. 2000).

Accumulation version of the method

The principle of this assay is the measurement of the accumulation level of fluorescent dye RB in aquatic organisms after the exposure without (control) and with a model MXR inhibitor such as verapamil. Snails were placed into a light-protected beaker (five specimens/beaker) in 100 mL of dechlorinated tap water, supplemented with 1, 5 or 10 µM RB (Sigma, St. Louis, MO, USA) in the absence or presence of 30 µM verapamil (Sigma) for 4 h. Incubation of snails in a medium containing RB enabled the probe to cross the cellular membranes by passive diffusion and to accumulate within the organism.

After the exposure period, specimens were washed three times in 100 mL of dechlorinated tap water using a tea strainer. The entire body from each specimen was gently isolated without damaging the tissue, using a dental explorer probe. Each body was weighed, transferred to a flat-bottomed tube containing 0.5 mL of distilled water and homogenised for 15 s (Pro200 Homogenizer, Pro Scientific Inc., USA). Homogenates were centrifuged at 3000 × *g* for 7 min and the supernatants carefully transferred to clean tubes. The fluorescence of accumulated dyes in the entire body of each snail, for each condition, was measured immediately using a fluorometer (QuantiFluor-TM, Promega, USA). The fluorometer was calibrated with known

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solutions of RB at the beginning of each working day; thus, the fluorescence data was provided in concentration units. Data were expressed in picomoles of accumulated RB per gramme of entire snail bodyweight.

The ratio between the RB accumulation in the presence of verapamil and the accumulation in the control condition was determined, in order to quantify the experimental RB accumulation rate.

Efflux version of the method

The principle of this assay is to measure the rate of efflux of the previously accumulated RB in aquatic organisms. The RB released is measured in the medium without (control) or with verapamil.

All specimens for the experiment (10 specimens/treatment) were placed into a light-protected beaker for 4 h in 200 mL of dechlorinated tap water supplemented with 5 µM RB. After the loading period, the snails were washed three times in 100 mL of dechlorinated tap water using a tea strainer. Five specimens were then exposed to 30 mL of dechlorinated water and the others to 30 mL of dechlorinated water containing 30 µM of verapamil. One hundred µL of efflux medium from each glass was transferred to borosilicate glass cuvettes every 5 min over a period of 60 min, avoiding disturbances to the snails. The fluorescence of the RB expelled by the snails into the medium was immediately measured using a fluorometer, and the results were expressed in picomoles of released RB per gramme of entire snail bodyweight.

The difference between the total efflux and the remaining efflux following verapamil inhibition was determined in order to assess the efflux rate of RB (Smital & Kurelec 1997; Parant & Pain 2001).

Both assays were carried out within 24 h of collection, but not before the 4 h needed for the release of environmental pollutants (MXR substrates) previously bound on active sites of P-gp (Kurelec et al. 2000). During the whole procedure all material was light protected with aluminium foil to avoid the possible loss of the RB fluorescence intensity caused by direct exposure to light.

Laboratory depuration

Since the efflux method is non-invasive and does not require the sacrifice of organisms, it is possible to use the same individuals collected that day for the depuration assays. Thus, after MXR evaluation on the initial experiment day, *P. acuta* snails were kept in dechlorinated clean water at 8–12 °C for 7 d to determine the basal level of MXR activity. For the accumulation version of the method (which requires the sacrifice of organisms), after capture a group of specimens was separated for laboratory depuration and then analysed as described above. In both cases the medium was changed every day and snails were fed ad libitum with lettuce leaves. At the time of the experiment the snails were still in good condition; this was determined by mobility and bodyweight maintenance relative to the collection day, assuring that the MXR level measured was not an artefact due to decreased body fitness.

Western blot analysis

This sensitive method is used to determine the expression and the relative level of a specific protein in the given sample of tissue protein extract. The western blot technique can be applied to the detection of the MXR transport system since commercial antibodies against human P-gp cross-react with this protein in a wide range of aquatic organisms (Kurelec et al. 2000).

The bodies from five *P. acuta* snails were gently isolated, washed twice in dechlorinated tap water, transferred to flat-bottom tubes in 0.5 mL of lysis buffer (250 mM NaCl, 50 mM HEPES, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail; Sigma), and homogenised for 15 s on ice (Pro200 Homogenizer, Pro Scientific Inc., USA) to prepare total protein extracts. Homogenates were then centrifuged at 6000 × *g* for 10 min at 4 °C and the supernatants carefully transferred to clean tubes. Total protein in each sample was quantified according to the Bradford method and stored at −20 °C until use. Total protein extract from a cell line which overexpresses P-gp (vincristine-resistant K562 human leukemic cells) was run on all gels as a positive control.

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For western blot analyses, samples were dissolved in loading buffer (50 mM Tris, pH 6.8, 2% [w/v] SDS, 10% [v/v] glycerol, 100 mM DTT, and 0.2 mg/mL bromophenol blue). The heating step was omitted to minimise membrane protein aggregation. Then, 100 µg of total protein was separated by 8% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membrane (Hybond ECL, GE Healthcare, Amersham, Buckinghamshire, UK). Five µL of molecular weight marker (Promega) was also migrated. The membrane was then blocked with 10% dry milk in Tris-saline buffer containing 0.05% Tween 20 (T-TBS) and then incubated with either P-gp-specific C219 monoclonal antibody (Calbiochem, USA) diluted 1:2500 for 3 h at room temperature, or α-tubulin (Santa Cruz Biotechnology, Inc., CA, USA) diluted 1:5000 for 1 h at room temperature. We determined optimal working dilutions and found that the chosen incubation conditions gave the clearest results.

The membranes were washed five times with T-TBS and then incubated with horse anti-mouse Immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) diluted 1:5000 for 1 h at room temperature. After washing five times with TBS, positive reactivity was detected using the ECL Western Blotting Analysis System (GE Healthcare). The chemiluminescence reaction was visualised on AGFA Medical X-Ray films (Agfa-Gevaert S.A., Argentina). The relative level of each protein was obtained using the ImageJ 1.37v densitometric software (National Institutes of Health, USA).

Statistical analysis

All data are given as mean ± standard deviation (SD). Mean values were calculated from the results obtained for at least three groups of five snails, and *n* indicates the number of groups used in each experiment. A paired or unpaired Student's t-test was used for comparison between two groups. One-way analysis of variance (ANOVA) was performed to determine if significant differences between groups existed, except for the data shown in Figure 2A where two-way ANOVA was performed to analyse the effect of depuration and verapamil inhibition together. Differences were considered significant when $P < 0.05$.

Results

MXR activity in freshwater snails Physa acuta

We tested the activity of the MXR system in freshwater snails *P. acuta* collected from a contaminated urban stream by way of the bioaccumulation version of the method, using RB. We incubated the snails in the medium with increasing concentrations of RB, ranging from 1 to 10 µM. Figure 1A shows that the MXR transporter was effective at exporting all tested concentrations of RB, and the accumulation of RB within the organisms increased with higher concentrations of the dye in the loading medium. In all cases, snails incubated in the presence of high concentrations of the MXR inhibitor (30 µM verapamil) accumulated much more dye than in RB alone ($P < 0.05$, $n = 5$). However, the RB accumulation rate was significantly lower for 1 µM than the other concentrations tested (Fig. 1B). Since there was no difference in the RB accumulation rate with either 5 or 10 µM, we decided to use the lowest concentration (5 µM) as the loading medium for the subsequent experiments.

We also evaluated the in vivo transporter activity using the efflux assay of RB, and the test results were similar to those obtained with the bioaccumulation assay. *P. acuta* snails showed activity in the MXR system, which was clearly demonstrated by the decreased efflux in the presence of 30 µM verapamil of previously accumulated RB (Fig. 1C). The mean level of released RB measured between 50 and 60 min for the control condition was 145.3 ± 10.9 and 65.2 ± 3.5 pmol RB/g in the presence of verapamil in the medium ($P < 0.05$, $n = 4$). The calculated efflux rate of RB was 79.0 ± 6.4 pmol RB/g.

MXR laboratory depuration

Following a depuration period of 7 d, the accumulation of RB in control conditions was increased by 2.6 ± 0.6 fold, relative to the values obtained

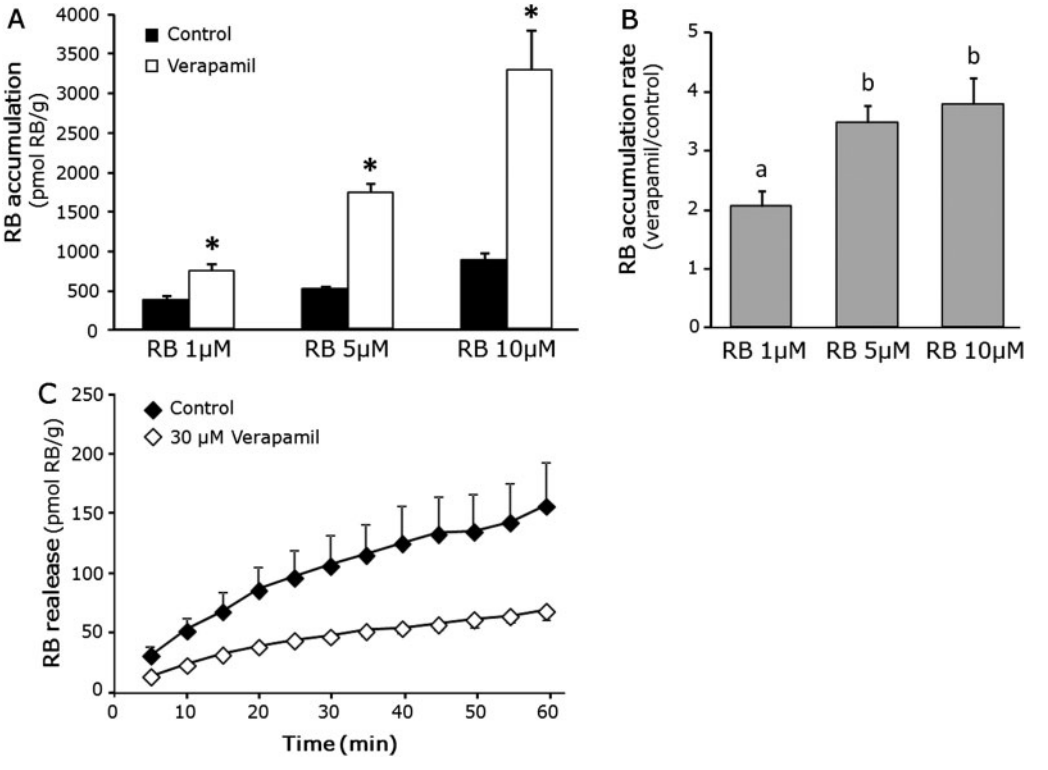

Figure 1 Measurement of MXR activity in *Physa acuta* snails from Patagonia tested within 24 h of collection. **A**, The accumulation of RB has been assessed in control snails and after inhibition with 30 µM verapamil at different concentrations of RB in the loading solution. Bars represent standard deviation of the mean ($n = 7$). The symbol (*) indicates a significant difference ($P < 0.05$) compared to the control. **B**, RB accumulation rate (verapamil/control) for the three tested concentrations of RB in the loading solution ($n = 7$). Bars with the same letter are not significantly different from each other. **C**, Efflux of RB was performed either in dechlorinated water or in 30 µM verapamil after a loading period of 4 h. Bars represent standard deviation of the mean ($n = 4$). Data are expressed in picomoles of accumulated/released RB per gramme of entire snail bodyweight.

on the collection day ($P < 0.05$, $n = 7$), indicating a reduction in activity of the MXR system (Fig. 2A).

This figure also shows the accumulated RB in the absence or presence of verapamil. For depurated organisms, we found significant differences in RB accumulation values in the absence or presence of verapamil; 1068.5 ± 396.4 and 1725.8 ± 277.0 pmol RB/g, respectively ($n = 7$). The accumulation of RB in the presence of verapamil after depuration was similar to that obtained immediately after collection ($n = 7$). No interaction between inhibitor treatment and collection/depuration status was revealed by two-way ANOVA ($P = 0.4184$). In addition, we evaluated the bioaccumulation of RB after different time-lengths of laboratory depuration. Figure 2B shows higher levels of accumulated RB with increasing depuration time ($n = 3$). To test if the possible presence of secondary metabolites in lettuce leaves can interfere with MXR transporters, we compared the accumulation of RB in snails depurated without feeding, with those maintained in the presence of lettuce leaves. Following a depuration period of 7 d we found no statistically significant differences between the two conditions. Figure 2C shows that the RB accumulation in the absence or presence

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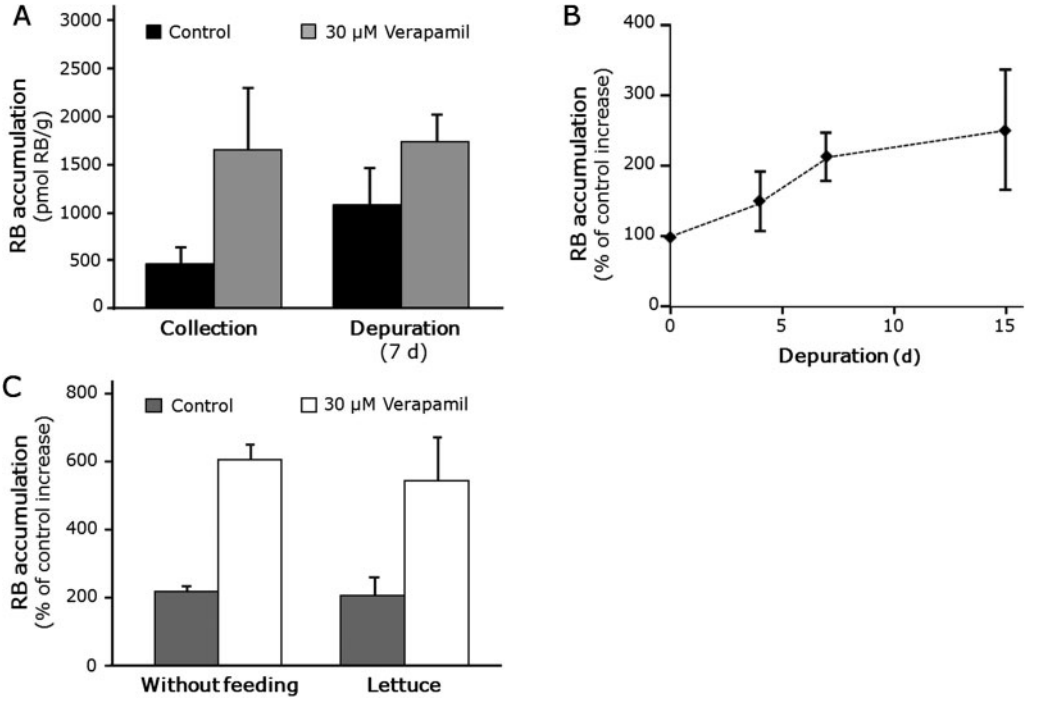

Figure 2 Activity of the MXR system in snails after a 7 d period of laboratory depuration. **A**, Data represent the accumulated RB (picomoles per gramme of entire bodyweight) in control snails and after inhibition with 30 µM verapamil (n = 7) at the indicated treatment. **B**, Time course response for the activity of the MXR system after laboratory depuration. The accumulation of RB was determined following a period of 4, 7 and 15 d in snails exposed to clean water. The data are expressed as a percentage of the increase in RB accumulation in comparison to the control group measured immediately after collection, and are representative of those obtained in three other experiments. **C**, The accumulation of RB has been assessed in snails depurated without feeding or maintained in the presence of lettuce leaves for 7 d. RB accumulation was determined in the absence or presence of verapamil and data are expressed as percentage of control on collection day ($P > 0.05$, n = 5).

of verapamil, expressed as percentage of control on collection day, was similar for both groups of animals (n = 5).

We also performed efflux assays in depurated organisms. Since this variant of the method is non-invasive, we used the same organisms tested on the initial experimental day. After a 7 d depuration period, the efflux of RB was reduced to a mean of 95.4 ± 5.8 pmol RB/g released after 50–60 min in control conditions (Fig. 3A). The RB related efflux rate in depurated organisms was 26.9 ± 4.8 pmol RB/g; 3.0 ± 0.5 fold lower than values obtained on the initial experimental day (Fig. 3B) ($P < 0.05$).

Immunochemical detection of P-gp

To assess the expression of P-gp in *P. acuta*, western blot analysis was conducted in total snail tissue homogenates using the monoclonal antibody (MAb) C219. Figure 4A shows the presence of a diffuse immunoreactive band at c. 170 kDa. The MAb C219 recognised a protein at similar molecular weight in total protein extracts from K562 vincristine-resistant cells (which are known as a rich source of P-gp), and was used as a positive control (Assef et al. 2009). The antibody also recognised a major reactive band that appears at 200–240 kDa. Following the depuration period of 7 d, the expression of P-gp was reduced. Figure 4B

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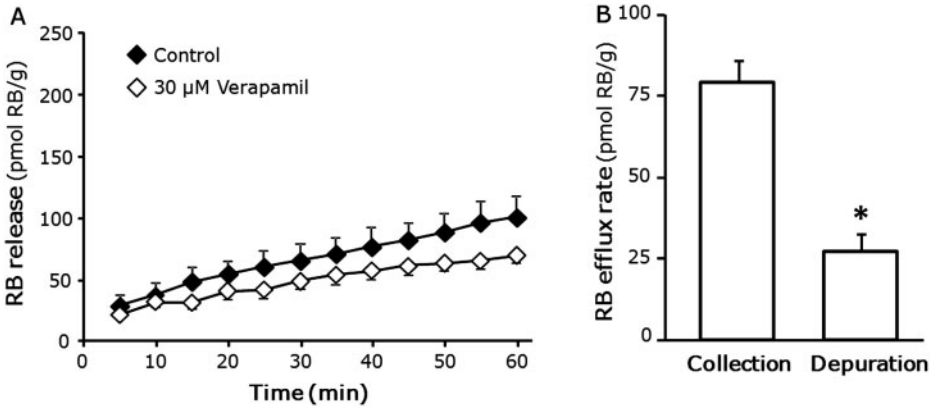

Figure 3 The activity of the MXR system in snails after a 7 d period of laboratory depuration. **A**, Efflux of RB assay was performed either in dechlorinated water or in 30 µM verapamil after a depuration period of 7 d. Bars represent standard deviation of the mean (n = 4). **B**, The MXR-related efflux rate of RB was determined in organisms tested on collection day and following laboratory depuration (7 d). Data represent the difference between the total efflux and the remaining efflux following verapamil inhibition for both conditions.

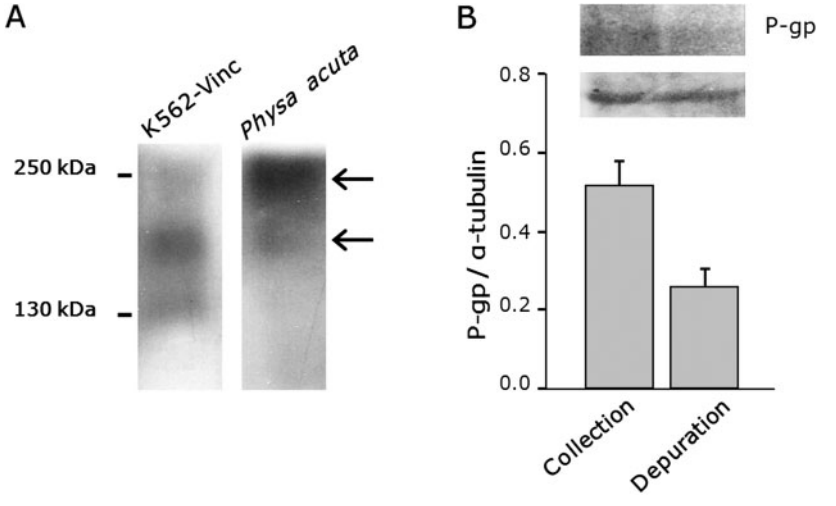

Figure 4 Detection of P-gp in *Physa acuta* snail homogenates. **A**, Western blot was probed with anti-P-gp C219 antibody which reacted with a diffuse band at c. 170 kDa and also at > 200 kDa (arrows). Vincristine-resistant K562 cell lysates were used as a positive control. Positions of molecular weight markers in kiloDaltons (kDa) are indicated on the left. **B**, Expression of c. 170 kDa band of P-gp was analysed on collection day and following a depuration period of 7 d. The relative level of P-gp using α-tubulin as the internal control is represented in the bar graph. Data represent mean ± SD from three separate experiments.

shows the mean relative levels of the specific band at c. 170 kDa using α-tubulin as the internal control obtained from three separate experiments.

Discussion

The multixenobiotic resistance mechanisms via, for example, P-gp, have been considered a general

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biological defence of many marine and freshwater aquatic organisms, and have been proposed as a biomarker for contaminant exposure (Kurelec 1997; Epel et al. 2008). In order to obtain evidence that the MXR system is functionally expressed in aquatic organisms from the Patagonian Andes, functional studies using fluorescent P-gp substrates and inhibitors were performed in the freshwater snails *P. acuta*. We observed a low accumulation of RB dye immediately after collection that was significantly increased when a transport inhibitor was added. It has been observed by other researchers that the transport system saturates at high concentration of the dye and a non-modulation by competing substrates will be seen in these cases (Toomey & Epel 1993; Galgani et al. 1996). The MXR system in *P. acuta* did not show saturation up to 10 μM of RB. The results of the testing of this freshwater species by efflux assay were similar to those obtained with the bioaccumulation assay.

Thus, the ratio of substrate accumulation/efflux in the absence and presence of the P-gp inhibitor provides evidence of MXR transport activity in those organisms inhabiting a polluted environment. Moreover, *P. acuta* collected from a relatively unpolluted site showed an increase of c. 3.7 times in the accumulation of RB under control conditions (data not shown). Although a MXR activity was still detected in these snails, this observation suggests an induction of the MXR transport system in animals inhabiting a more contaminated environment. These results are in accordance with several studies describing an induction of the MXR mechanism in mussels previously exposed to pollution (Smital et al. 2003; Pain & Parant 2007; Luckenbach & Epel 2008).

Based on in vitro studies of the biosynthesis, processing and half-life of P-gp (Yoshimura et al. 1989; Zhang & Ling 2000), as well as the results of studies on the MXR mechanism in aquatic organisms, a depuration period of 5–7 d has often been used to achieve the baseline level of an MXR system (Smital et al. 2000; Bodin et al. 2004; Pain & Parant 2007). We found that following the depuration period, both accumulation and efflux assays showed a reduction in MXR activity. According to previous reports, our results suggest that in unpolluted laboratory conditions *P. acuta* snails will not maintain a high level of the MXR defence system if it is not necessary (Smital et al. 2003; Pain & Parant 2007). We found no differences in bioaccumulation of RB assays performed in depurated snails maintained without feeding or in the presence of lettuce leaves. These experiments rule out a possible induction of the MXR system by lettuce since plants contain hydrophobic natural products that are thought to interact with the transporter responsible for resistance (Gottesman & Pastan 1993).

The depuration period reported in the literature is highly variable, from several days to several months depending on the species studied (Smital & Kurelec 1997; Bard et al. 2002). The time–response experiments suggest that, although a 7 d depuration period was long enough to observe a significant deinduction of this system, some remaining MXR activity was still detected in *P. acuta* snails, even when they are kept in clean water. These results allow us to speculate that there might be further declines in MXR activity after this time. However, this observation is also in line with the concept of MXR as the first line of defence system, prepared to act against incoming toxicants, and it may increase in response to higher concentrations of xenobiotics (Epel 1998). The presence of the MXR phenotype may at least partially explain the association of *P. acuta* with developed catchments and higher levels of nutrients (Quinn & Hickey 1990).

Several studies performed on aquatic organisms have demonstrated the presence of protein using antibodies directed against conserved P-gp epitopes (an antigenic determinant of known structure of the protein), resembling the mammalian 170-kDa P-gp. Moreover, this P-gp expression is strongly associated with tissue pollutant concentrations (Minier et al. 1993, 2006; Smital et al. 2003). Our observation in *P. acuta* snails is in full agreement with previous results showing the presence of protein at different molecular weights ranging from 80 to 240 kDa using the C-219 antibody (Bard 2000; Tutundjian et al. 2002; Tutundjian & Minier 2007; Amé et al. 2009). Western blot analysis conducted in total snail tissue revealed the

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presence of two immunoreactive bands (c. 170 kDa and > 200 kDa), possibly representing differentially glycosylated isoforms of P-gp or the presence of multiple proteins in *P. acuta* homogenates (Schinkel et al. 1993; Whalen et al. 2010).

An unresolved question is whether the larger molecular weight bands are indeed transport molecules (Galgani et al. 1996). It has been reported that C219 antibodies cross-react with a c. 200 kDa band corresponding to the heavy chain of muscle myosin (Thiebaut et al. 1989). For this reason we performed the densitometry measurements on the c. 170 kDa protein, the homologues of the mammalian transporter responsible for the observed transport activity. After the depuration period in clean water we observed a decrease in P-gp level, in accordance with functional assays.

Even though using the C219 antibody can yield basic information about the expression of P-gp in snails, these results should be interpreted with caution since the exact specificity of these antibodies in aquatic organisms is not known (Smital et al. 2000; Sturm et al. 2001; Zaja et al. 2008). Further research needs to be conducted on the effects of seasonal natural variables on P-gp expression and MXR activity as demonstrated in other aquatic organisms (Keppler & Ringwood 2001; Pain et al. 2007).

In conclusion, this study demonstrates the presence of the MXR mechanism in *P. acuta* snails exposed to environmental contaminants. After a period of depuration in the laboratory, the decreased P-gp level corresponds to increased dye accumulation, indicating a deinduction of the MXR system in animals maintained in clean water. Our results indicate that MXR transporter activity may play an important role in tolerance to environmental contaminants in *P. acuta* snails from Patagonia.

The presence of pharmaceuticals in the environment due to human use has been extensively demonstrated (Andreozzi et al. 2003; Kümmerer 2009). Regarding our findings, the study of toxicological responses in this experimental model also represents a promising approach to assessing the potential risk of exposure to different compounds and new emerging contaminants. In this sense, these snails will potentially be useful for the study of compounds that have the ability to modulate the MXR system in our ecosystems, defined as 'chemosensitisers'. These compounds could potentially alter toxicity in aquatic organisms by decreasing their natural resistance (Smital & Kurelec 1997; Smital et al. 2004).

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