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# Haemolymph as compartment for efficient and non-destructive determination of P-glycoprotein (Pgp) mediated MXR activity in bivalves

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**Abstract**

Measurement of the modulation of accumulation rate of model P-glycoprotein (Pgp) substrates has been a well established methodology for determination of the presence and activity of the multixenobiotic resistance (MXR) defence mechanism in aquatic invertebrates. Most studies have been focused on the gill tissue of various bivalves as a primary compartment for this type of measurements. In this study, we evaluated the potential of measuring the accumulation rate of a fluorescent model Pgp substrate rhodamine B (RB) in haemolymph, plasma and haemocytes of the freshwater painter's mussel (*Unio pictorum*) as additional potentially useful compartments. The obtained results demonstrated several important advantages of the determination of Pgp mediated MXR transport activity in haemolymph over determinations in gill tissue. The overall MXR response correlated well with the level of Pgp activity simultaneously determined in gills. The method is more sensitive, the procedure is easier and less laborious, and repeated use of same individuals is possible. Finally — the approach is non-destructive, offering a potentially powerful biomarker and research tool for studies directed to the evaluation of ecotoxicological importance of MXR defence and the presence of MXR inhibitors in the environment.


*Keywords:* Haemolymph; Mussel; MXR activity; P-glycoprotein; Rhodamine B

## 1. Introduction

The multixenobiotic resistance (MXR; Kurelec, 1992) mechanism in aquatic organisms represents a defence system directed against numerous xenobiotics, preventing their intracellular accumulation and potentially toxic effect. The molecular basis of MXR mechanism is similar to the well-known multidrug resistance (MDR) phenomenon involved in tumour cell lines resistant to chemotherapeutic drugs. MDR is mediated by the expression of a variety of transmembrane transport proteins. The most common among them is a 170 kDa transmembrane P-glycoprotein (P170 or Pgp) that belongs to the ABC superfamily of transport proteins. Pgp uses energy (ATP) to pump a wide variety of xenobiotics with different chemical structures and modes of action out of cells. The Pgp role in transporting xenobiotics is well studied and well documented; ranging from studies on drug resistance of tumour cells in which an acquired resistance to chemotherapeutic drugs coincided with the Pgp expression, to transfection experiments in which cells not expressing Pgp were transfected with the *MDR1* (Pgp coding) gene, resulting in acquisition of the drug resistance phenotype (reviewed in Krishna and Mayer, 2000; Dean et al., 2001). Critical evidence comes from studies on *MDR1* gene knock out mice. Although the phenotype of these mice was apparently normal, exposure to xenobiotics (such as pesticides) which have no effect on wild-type mice resulted in death of the knock out mice (Schinkel et al., 1994).

Numerous lines of drug binding, immunological cross-reactivity or functional-activity studies, along with toxicological

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evidence strongly indicate that the Pgp is frequently present in aquatic organisms (Epel, 1998; Smital and Kurelec, 1998; Bard, 2000). As in the case of toxicologically relevant mammalian tissues, the MXR defence most probably works in synergy with enzyme-mediated detoxification systems (Chan et al., 2004). By pumping many structurally different endo- and exogenous xenobiotics, parental compounds as well as phase I and/or II metabolites out of the cell, these ABC efflux transporters are integral parts of the cellular detoxification machinery, having a critical influence on uptake, bioavailability, bioconcentration and overall (eco)toxicity of environmental pollutants.

Although the Pgp activity is inducible and correlates well with the level of pollution, it is sensitive to the presence of specific chemicals termed MXR inhibitors. A significant increase of toxic effects in mussels co-exposed to known toxins and model MXR inhibitors has been shown in several studies (Kurelec et al., 1996; Britvić and Kurelec, 1999; Epel, 1998). Furthermore, high MXR inhibitory potential of various environmental samples has been demonstrated (Kurelec, 1995; Smital and Kurelec, 1998), followed by identification of potent inhibitors among both conventional and emerging environmental contaminants (Smital et al., 2004). Therefore, it is important to establish reliable methods for determination of MXR transport activity, serving as: (1) a standardized research technique for scientifically sound characterization and overall ecotoxicological evaluation of MXR phenomenon; (2) a potentially useful biomarker of exposure of aquatic environments to pollution; and (3) a powerful tool for screening of complex environmental samples or man-made chemicals for MXR inhibitory property.

Since the discovery of the MDR/MXR phenotype in the freshwater mussel *Anodonta cygnea* (Kurelec and Pivčević, 1989), most of MXR studies have been focused on the determination of the P-glycoprotein (Pgp) expression and/or its activity in mussels gill tissue. Many studies confirmed the presence and transport activity of the Pgp in gills of several marine and freshwater bivalve species. The critical endpoint most often used in those studies has been the Pgp transport activity, determined by modulation of accumulation or efflux (or retention) rate of fluorescent model Pgp substrates (Table 1) in the gills of exposed specimens. In the absence of inhibitors, fluorescent Pgp substrates are excluded from the cell. But if inhibitors, competitive substrates or modulators of the transporter are added, substrates enter the cell. The rate or kinetics of this uptake can be easily followed by fluorescence measurements. With the use of model Pgp inhibitors as a necessary positive control for Pgp specific transport activity, these methods have been established as a relatively simple, inexpensive and reliable approach for determination of MXR activity. Nevertheless, although the obtained results were promising, the critical drawback has been the fact that obtained interindividual variations were quite high. In other words, to obtain an appropriate level of discriminating power of the method, a significant number of experimental animals have to be sacrificed.

Since mussel haemolymph can easily be withdrawn from live animals without sacrificing them, this study tried to evaluate haemolymph and its sub-compartments (plasma and haemocytes) as additional potentially useful compartments for determination of MXR activity in bivalves. Our primary intention was to find out whether the Pgp substrate accumulation rate in haemolymph correlates well with the level that can be determined in gills and whether it can be reliably determined in a non-destructive manner, serving as a better approach for MXR focused research and/or biomonitoring.

Our presumptions were based on two key indications. Firstly, the open circulatory system present in bivalve molluscs involves the movement of haemolymph not only in the heart and vessels but also seepage through tissues (Cheng, 1981). This means that the body tissues are continuously bathed in haemolymph and the amount of Pgp substrates in haemolymph might be a cumulative result of the activity of MXR transport proteins present in more tissues than the gills alone (digestive gland, mantle, gonads and muscles). Secondly, the studies done by Minier and Moore (1996, 1998) on mussel blood cells revealed the presence of Pgp, as well as characteristic pattern of rhodamine B accumulation and sensitivity to MXR inhibitors in haemocytes of marine mussel species *Mytilus edulis* and *M. galloprovincialis*. A more recent study confirmed the presence and induction of MXR activity in *M. edulis* haemocytes after in vitro exposure to doxorubicine or

Table 1
Identification of the MXR phenotype in bivalves — present situation

| Scientific name | Common name | Tissues/organs | Method | Reference |
|---|---|---|---|---|
| *Anodonta cygnea* | Swan mussel | Gills, digestive gland, mantle | TA ($^{14}$C-AAF); rhodamine B | Kurelec and Pivčević, 1989; Smital et al., 2000 |
| *Corbicula fluminea* | Asiatic clam | Gills | TA ($^{3}$H-vincristine); IM | Waldmann et al., 1995 |
| *Dreissena polymorpha* | Zebra mussel | Gills | TA (rhodamine B) | Smital and Kurelec, 1997 |
| *Crassostrea gigas* | Pacific oyster | Gills | IM; DNA–DNA hybridization | Minier et al., 1993 |
| *Crassostrea virginica* | American oyster | Gills, embryos | IM | Keppler and Ringwood, 1996 |
| *Mytilus californianus* | California mussel | Gills | TA (rhodamine B); IM | Cornwall et al., 1995 |
| *Mytilus edulis* | Blue mussel | Gills | IM; DNA–DNA hybridization; mRNA/ Multiplex PCR | Minier et al., 1993 Lüdeking and Köhler, 2002 |
| | | Haemocytes | TA (rhodamine B); IM | Minier and Moore, 1996, 1998 |
| | | Embryos | TA (rhodamine B) | McFadzen et al., 2000 |
| *Mytilus galloprovincialis* | Mediterranean blue mussel | Gills, digestive gland, mantle | TA ($^{14}$C-AAF,rhodamine B) | Kurelec and Pivčević, 1991; Smital et al., 2000 |

Abbreviations: TA, transport activity measurements (MXR-model substrates used); IM, immunoreactivity with mammalian anti-Pgp antibodies.