A New Type of Hazardous Chemical: The Chemosensitizers of Multixenobiotic Resistance

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The purpose of this overview is to introduce the property of a new class of hazardous chemicals—the inhibitors of multixenobiotic resistance (MXR) in aquatic organisms, referred to as chemosensitizers. Aquatic organisms possess MXR, a mechanism similar to the well-known P-glycoprotein extrusion pump in multidrug resistant (MDR) tumor cells. MXR in aquatic organism moves from cells and organisms both endogenous chemicals and xenobiotics, including also some man-made chemicals. MXR in aquatic organisms represents a general biological first-line defense mechanism for protection against environmental toxins. Many chemical agents, the chemosensitizers, may alter the function of this fragile mechanism. It is this new, MXR-inhibiting property, unrecognized as yet, that classifies these chemicals among top-rank hazardous water pollutants. The knowledge that the presence of one xenobiotic may block the pumping out of other xenobiotic(s), and hence accelerate their accumulation, may have important implications on environmental parameters like exposure, uptake, bioaccumulation, and toxicity. In this overview we present the evidence for the expression of MXR-phenotype in aquatic organisms, the demonstration of toxic consequences caused by MXR inhibitors, and the description of methods for measurement of concentration of MXR inhibitors in environmental samples. — *Environ Health Perspect* 105(Suppl 4):855–860 (1997)

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Introduction

The phenotype of multixenobiotic resistance (MXR) system found in aquatic organisms (*1*) is similar to the well-known multidrug resistance (MDR) phenomenon involved in tumor cell lines resistant to chemotherapeutic drugs (*2*). MXR mechanism in aquatic organism pumps out of cells and organisms both endogenous chemicals and xenobiotics, including also some man-made chemicals, preventing their accumulation and toxic effect. MXR in aquatic organisms represents a general biological firstline defense mechanism for protection against environmental toxins. Many chemical agents, the chemosensitizers, may alter the function of this fragile mechanism. The knowledge that the presence of one xenobiotic may block the pumping out of other xenobiotic(s), and hence accelerate their accumulation, may have important implications for ecotoxicology. Such property classifies these xenobiotics among top-rank hazardous water pollutants. In this overview we present the evidence for the expression of MXR-phenotype in aquatic organisms, the use of induction of MXR as a biomarker of exposure, the description of methods for measurement of concentration of MXR-inhibitors in environmental samples, and the demonstration of toxic consequences caused by MXR-inhibitors.

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Abbreviations used: MDR, multidrug resistance; MXR, multixenobiotic resistance; P170, membrane 170 kD P-glycoprotein.

MDR Phenotype in Tumor Cell Line

In MDR-positive tumor cells, a major determinant of reduced drug accumulation and a dominant feature in a model of classical multidrug resistance is the 170-kD membrane glycoprotein (P170) (*3*). P170 binds a cytotoxic drug and facilitates its efflux in an energy-dependent manner (*4*). Consequently, P170 mediates a reduction of drug accumulation and causes drug resistance. The gene coding for glycoprotein P170, *mdr*1, has been cloned (*5*), and its amplification and overexpression were found to be proportional to the degree of resistance in resistant cell lines (*6,7*). Some drugs, like verapamil, bind to the active site of glycoprotein P170, causing an inhibition of efflux of cytotoxic drugs and hence restoring the previous sensitivity to the cytotoxic agent (*8*). In addition, P170-transporting function can be modulated by phosphorylation (*9*). This posttranslational modification is catalyzed by protein kinase C (PKC); its activators, like phorbol-12-myristate-13-acetate (*10*), or its inhibitors, like staurosporine (*11*), stimulate or inhibit the efflux of drugs out of the cell.

MXR in Aquatic Organisms

Membrane vesicles isolated from the freshwater mussel [*Anodonta cygnea* (*12*)], from the clam [*Corbicula fluminea* (*13*)], from the marine mussel [*Mytilus galloprovincialis* (*14*)], or from the sponges [*Tethya lyncurium* (*15*), *Suberites domuncula* (*16*), *Geodia cydonium*, and *Verongia aerophoba* (*17*)], possess a verapamil-sensitive potential to bind xenobiotics like 2-acetylaminofluorene or vincristine (VCR) in a similar manner to that measured with membrane vesicles isolated from male bovine adrenal cortex cells. Western blot studies with *G. cydonium* and *V. aerophoba* revealed that polyclonal antibodies raised against hamster P170 cross-react with the sponge protein of M_r 125,000 kD. Immunohistochemical confocal laser scanning microscopy showed that this P125 is a cell membrane-bound protein. The presence of a protein immunologically related to the mammalian MDR protein was identified also in *C. fluminea* (*13*), in embryos of a marine worm [*Urechis caupo* (*18*)], in oyster (*Crassostrea gigas*), and marine mussel [*Mytilus edulis* (*19*)] in the biliary spaces from *dab* and in phagocytic blood cells in mussels (*20*). In addition, exposure of sponges, marine mussel, freshwater clam, or marine worm to 2-acetylaminofluorene, benzo[*a*]pyrene, daunomycine, VCR, calcein acetoxy methyl ester (calcein AM), or rhodamine B showed enhanced accumulation of these compounds in the presence of verapamil (*13,14,16–18*). Finally, the addition of verapamil or staurosporine drastically

enhanced the induction of adducts and single-strand breaks in the DNA isolated from fish, sponges, and clam exposed to 2-aminoanthracene or 2-acetylaminofluorene (*1,13*). These observations are taken as an indication that a MDR-like system, termed multixenobiotic resistance (MXR), might function in these organisms also *in vivo*. All these indicators were found in specimens collected from pristine areas, defined by six biological and chemical parameters (*21*), i.e., in specimens that have not experienced exposure to pollutants. This argues strongly that the MDR-like mechanism is inherent in these species and that its expression does not require induction.

Taxonomical Distribution of MDR-like Phenotype

Multidrug resistancelike genes and/or MDR-like phenotypes have been identified in drug-resistant parasitic protozoa [*Plasmodium falciparum* (*22,23*) and *Leishmania donovani* (*24*)], in yeast [*Sacharomyces cerevisiae* (*25,26*)], in bacteria [*Bacillus subtilis* (*27*) *Staphylococcus aureus* (*28*), and *Salmonella typhimurium* (*29*)], in archaebacterium [*Haloferax volcanii* (*30*)], in the insect [*Drosophila melanogaster* (*31*)], in the soil nematode [*Caenorhabditis elegans* (*32*)] and in the genome of a marine fish, winter flounder [*Pseudopleuronectes americanus* (*33*)].

Physiological Functions of the Multixenobiotic Resistance Mechanism

Several recent studies indicate the widespread *de novo* expression of *mdr*1 gene also in human normal, healthy kidney, liver, intestine, adrenal, pancreas, placenta, pregnant uterus and blood–brain barrier sites (*34,35*). In these tissues and organisms P170 is characteristically confined to the membranes of the luminal surfaces in secreting, absorbing, or barrier tissues, reflecting their possible physiological transport or barrier function, perhaps to protect from—and excrete—toxic natural products present in the diet, or unknown endogenous metabolites (*36*) or to secrete cortisol, aldosterone, progesterone and other steroids (*37*).

In addition to these functions, we suggested, based on experiments with mussels and sponges exposed to vincristine or aminoanthracene (*12,15–17*), the function of pumping out "new," man-made toxic chemicals in aquatic organisms exposed to polluted environment (*1*). Thus, one could postulate that through the course of evolution the cell has developed a means of protecting itself from environmental insults by exporting toxins before they can exert their effect. One likely factor in the development of such systems was the need to protect the cell from low-molecular-weight toxins found throughout the environment, especially in foods. Thus, it is obvious that P-glycoprotein may have developed not to counteract clinically useful antineoplastics, like vincristine and vinblastine, but rather as a general, taxonomically broadly distributed, biological defense mechanism for protection of organisms from endogenous or environmental toxins. Because the same xenobiotics may induce simultaneously the expression of MDR genes (*38*), drug-metabolizing genes (*39–41*), glutathione *S*-transferase gene (*42*), and heat-shock proteins (*43,44*), i.e., a series of mechanisms belonging to general biologic defense system, support the conclusion that P-glycoprotein has a physiological function in the protection of cells from environmental stress. This was directly demonstrated in soil nematode (*Caenorhabditis elegans*): nematode strains with deleted P-glycoprotein genes, generated by transposon-mediated deletion mutagenesis, become sensitive to xenobiotics, which suggests the function of P-glycoprotein is to protect a nematode against toxic compounds made by plants and microbes in the rhizosphere (*32*). Based on this, it would be rational and justified to name this phenotype as multixenobiotic defense (MXD) mechanism.

Expression and Induction of MXR in Aquatic Organisms

MXR mechanism is inherently present in all aquatic organisms investigated so far. At present we know that there are differences between species in the level of MDR expression. However, we do not know the range of differences in MDR expression on interindividual and interpopulation level. Both these parameters have been shown to be important in predictions and strategies in combating resistance in pest control (*45*). The experience from pest-resistance control demonstrates the importance of measurement of natural variation in the level of MXR expression between individuals as well as between different populations of the same species. Such information would represent the basic requirement in the assessment of overexpression of MXR in populations exposed to pollution. The basic question concerning such induced MXR is if and how it can be used as biomarker. Would it be the biomarker of exposure, or the biomarker of effect, or both? Since such enhanced expression of MXR gene product in aquatic organisms can be induced by pollution, it certainly may serve as a biomarker of biologically relevant exposure to pollution. However, if overexpression of MXR was induced by mutagenic and/or carcinogenic xenobiotics, resulting in the preferential resistance to the selective agent (*47*), then it may serve as a biomarker of effect.

Expression of MXR in Aquatic Organisms

To explore MXR expression in the populations of aquatic organisms, we compared the characteristics of MXR expression in the population of a marine snail (*Monodonta turbinata*) living at an unaffected site with the characteristics of a population inhabiting a site affected by cannery wastewaters. Snails from the unaffected site accumulated 281% more ^{3}H-VCR than those from a polluted site. It is obvious that the population of snails from the polluted area is much better protected from xenobiotics than the population of snails from the unpolluted site. The results of these experiments indicate how differences in the activity of MXR may critically influence the susceptibility of populations to the same concentration of xenobiotics. Furthermore, the accumulation of vincristine in *M. turbinata* collected at a less polluted site and exposed for 48 hr at a site heavily affected by the cannery waters was significantly lower than in control, unexposed specimens, reflecting probably the induction of P170 pumping activity. The 48 hr period of exposure to the mixture of xenobiotics present at the polluted site induced the activity of this defense mechanism to the level that decreased the accumulation of vincristine for 33%, in comparison to the accumulation measured in uninduced specimens (*46*).

Similarly, the state of induction of MXR in the gills of mussel (*Mytilus galloprovinciallis*) from the same scale of pollution was proportional to the level of pollution: gills from mussels living at polluted sites accumulate less vincristine, the vincristine accumulation is less sensitive to verapamil, and in most cases expresses higher levels of P-glycoprotein (*47*). Mussels transplanted from a unpolluted site to a polluted site for 3 days induce their MXR and behave like mussels living at a polluted site (*48*).

Similar induction of MXR was found in gills of a freshwater clam (*Corbicula fluminea*): induced clams, i.e., clams freshly

collected at a polluted Rhein River site, or control clams exposed for 3 days either to water experimentally polluted with diesel-2 oil or to Rhein River sediments, accumulated significantly less vincristine than control clams, i.e., clams held in aquaria for 6 weeks. Similarly, the number of single strand breaks (SSB) in gill DNA after exposure to acetylaminofluorene was significantly lower in induced clams than were SSB found in control clams (*49*).

Thus, all these examples illustrate how aquatic organisms may protect themselves from toxic xenobiotics. This defense mechanism is inducible: it enhances its activity in polluted waters. However, it is fragile: its protective role in all examples mentioned above was annuled in the presence of chemosenziters.

MXR-reversing Agents and Their Measurement

Recognition that the presence of one xenobiotic that is a good substrate for P170 pump may inhibit or block the pumping out of other xenobiotic(s), hence reducing accumulation of the first, and unusually increasing accumulation of the second or others, may help us to understand and interpret data on bioaccumulation, bioavailability, metabolism, toxicity, dose–effect relationships, exposure experiments, and other related parameters. For example, the effect of the addition of one nontoxic compound that is a good substrate of P170, to one already polluted ecosystem may cause a toxic effect in a variety of species. Such toxic effects would be unexpected and unexplainable by the levels of toxic substances well below the established toxic thresholds. Another group of interesting speculations could be drawn from the possible consequences of blocking the physiological function of P170-pump in extrusion of endogenous toxic substances: exposure to a nontoxic "chemosensitizer" may well cause something like a "self-intoxication" in an organism with its own endogenous products. For example, the findings of tissue- or species-specific profiles of indigenous DNA adducts (I-spots) induced by estrogens, chow diet, vitamin E, caloric restriction, or aging in mammals (*50*), or I-spots induced in fish and marine invertebrates during the spawning time, or after the exposure to xenobiotics (*51–53*), may well be explained by the inhibitory effect of hormones, nonnutrient natural products, vitamin E, or xenobiotics on physiological function of P-glycoprotein to pump out the endogenous DNA-reactive electrophilic metabolites. A wide variety of compounds have now been shown to reverse MDR *in vitro*, including calcium channel antagonists (verapamil, dihydropyridines, and derivatives), calmodulin antagonists (trifluoroperazine and analogues), antihypertensive agents (reserpine), noncytotoxic analogues of cytotoxic agents (anthracyclines and vinca-alkaloids), steroids (progesterone), antiarythmics (amiodarone, quinidine), antiparasitic agents (quinacrine, quinine), immunosuppressants (cyclosporins), monoclonal antibodies against P170, and recently, a novel triazinoaminopiperidine derivative, Servier 9788 (*21,54–56*). Because of the clinical importance of acquired MDR, a great deal of effort has been focused on the discovery of novel agents that inhibit P-glycoprotein-mediated efflux of cytotoxicity drugs. These efforts have focused on either the development of analogues of known resistance modifiers (*57*), identification of novel reversing agents by screening (*58*), or through structure-based selection (*59*). MDR-inhibiting properties of substances were frequently discovered in a programs initiated to identify MDR-circumventing agents among, for example, many different strains of blue-green algae, or thousands of fungi and *Actinomycetes*, or a variety of marine species, like *tolyporphin* (*60*), or two naphto-*g*-pyrones (*61*), or patellamide (*62*), respectively. Most of these compounds act by increasing the intracellular concentration of cytotoxic drugs probably through direct interaction with the P-glycoprotein. Some of them have shown activity in *in vivo* models of MDR (*63*) and verapamil has been extensively tested as a modulator in the clinic (*64*). However, nontoxic lipophilic agents, natural or manmade, may also be recognized and processed by this molecular mechanism, and, at high concentrations, they might consequentially saturate the system and thereby reverse multidrug resistance (*65*). In addition to such nontoxic substrates of P170, there are agents that may alter the regulation of this fragile MDR mechanism, like activators and inhibitors of protein kinase C. This new class of compounds, referred to as "chemosensitizers," deserves a top rank among environmentally hazardous chemicals, as they may block the basic biologic defense mechanism and revert natural resistance to a pathobiologic sensitivity.

The development of methods to screen for such a MXR-reverting potential of xenobiotics should therefore be a rational approach for the assessment of risks from chemicals in the environment. Recently we found, using the method of Yoshimura et al. (*66*) with measurement of rhodamine 6G- or 3H-vincristine accumulation in a confluent monolayer of mouse sarcoma cells (S180) and S180 cells selected for resistance to doxorubycin (S180dox) in 96-wells microplate, that concentrates of polluted Sava River waters, or its sediments, contained about 3 times more MXR-inhibiting substances, expressed as verapamil-equivalents, than verapamil-equivalents of MXR-reversing xenobiotics present in water or sediment concentrates from a less polluted Korana River (both in Croatia) (*49*). This method has been considerably improved by use of standardized NIH 3T3 mouse fibroblasts stably expressing the human multidrug transporter (MDR-1 transfected NIH-3T3 cells) in combination with the measurement of accumulation of a calcein acetoxymethyl ester (calcein AM) (*67*), an advantageous functional assay of the multidrug transporter (*68*). In addition, environmental samples expressing anti-*MDR* potential were characterized for the nature of their interaction with the P-glycoprotein using a relatively simple, sensitive, and short-term assay described by Sarkadi et al. (*69,70*): This assay measures the property of sample to stimulate or inhibit the vanadate sensitive MDR1-ATPase activity in isolated membranes of Sf9 cells infected with a recombinant baculovirus containing an MDR1 cDNA. The results of these determinations were well correlated with results obtained by methods described earlier, i.e., both with *in vitro*, indirect, "binding" method and with the *in vivo* "accumulation" method. The latter represents the best *in vivo* confirmation of determinations obtained by cell culture technique and, together with methods demonstrating the toxication effects, illustrates the ecological significance of chemosenziters. Thus, methods needed to screen and control these hazardous chemicals are available.

MXR in Aquatic Organisms: Implications for Ecotoxicology

There is no doubt that the discovery of the presence and operation of MXR mechanism in aquatic organisms should have important implications in environmental xenobiotic-risk assessment studies. Apparently, this paradigm plays a central role among the phenomena most often used in both the assessment of the impact of pollution and in serving as a basis for

legislative regulation, like uptake, bioavailabilty, toxicity, bioaccumulation, and exposure. Therefore, it is reasonable to raise the question of how to capitalize on *a*) the implementation of this new knowledge to our present concepts in ecotoxicology, *b*) the potential use of measurements of the activity of MXR and exploitation of inhibition or inducibility of MXR as a biomarker of pollution, and *c*) the measurement of concentration of MXR-reversing substances in polluted aquatic environments.

To demonstrate how xenobiotics that are good substrates of P170 may competitively inhibit the pumping out of other xenobiotics, we exposed *M. galloprovincialis* to (G-3H) vincristine in the presence of diesel-2 oil. The presence of this conventional pollutant enhanced the accumulation of the radioactivity by 3-fold, or to the level equivalent to enhanced accumulation caused by 8.5 µM verapamil (*48*).

The second demonstration was done by an indirect "chemosensitizer," the PKC inhibitor staurosporine. Staurosporine (0.5 mM) reversed the MXR in a fresh-water clam *Corbicula fluminea* and switched the no observed effect concentrations (NOEC) (*71*) of aminoanthracene (0.01 µM), as measured by alkaline filter elution detection of single strand breaks, to the observed effect concentrations (OEC) equivalent to that caused by an order of magnitude higher (0.10 µM) concentration of acetylaminofluorene (*13*).

The third demonstration of the toxicating effect of an MXR-inhibitor was done by a direct "chemosensitizer," verapamil. The time needed for the induction of mixed-function oxidase activity (EROD and benzo[*a*]pyrene monoxygenase) in the livers of carp exposed to a low concentration of diesel-2 oil was shortened in the presence of 2 µM verapamil to 2 days, i.e., to the period reached otherwise after exposure to five times higher concentration of diesel-2 oil, demonstrating how the inhibition of P170 glycoprotein enhances the internal dosing of diesel-2 oil (*72*).

Finally, we demonstrated that environmental samples, like water concentrates and sediment extracts, enhanced the accumulation of rhodamine123 or calceine AM *in vivo* in clams *Dreissena* and *Corbicula*. However, even the xenobiotics present in native polluted river (Rhein River) water enhanced the accumulation of these dyes, in comparison with unpolluted (Morgenbach) waters. Similarly, waters collected from beads of *Caulerpa taxifolia*, a rapidly expanding marine seaweed introduced into Mediterrannean, contain agents that reverse MXR in *M. galloprovincialis* (enhancement of R123 accumulation) (*73*). A lipophilic extract from *C. taxifolia* contains a strong anti-MDR agent. It belongs to a cyclosporinelike inhibitors, since it inhibits the MDR-protein ATPase (*74*).

Multixenobiotic resistance phenotype expressed in aquatic organisms serves as a defense mechanism that protects organisms by the mechanism that pumps out of the cell many structurally diverse lipophilic xenobiotics. The exposure to polluted water induces the expression of MXR. Thus, measurement of the level of MXR-expression can be used as a biomarker of exposure. Many classes of chemicals are capable of inhibiting the MXR mechanism. This new class of compounds, referred to as "chemosensitizers," deserves a top rank among environmentally hazardous chemicals, since it may block the basic biologic defense mechanism and revert natural resistance to pathobiologic sensitivity. Therefore the detection and control of MXR inhibitors deserves the highest priority in ecological risk assessment studies. Methods for measuring the concentration of such MXR chemosensetizers in environmental samples, or for measurement of the MXR-inhibiting property of chemicals, are available.

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