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Abbreviations

FSW: filtered seawater, K_{ow} : octanol-water coefficient, MXR: Multixenobiotic resistance, PPCPs: pharmaceutical and personal care products, MK: musk ketone, MX: musk xylene, for abbreviations of other compounds see Table 1

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Abstract

Synthetic musk compounds, widely used as fragrances in consumer products, have been detected in human tissue and, surprisingly, in aquatic organisms such as fish and mollusks. Although their persistence and potential to bio-accumulate are of concern, the toxicity and environmental risks of these chemicals are generally regarded as low. Here, however, we show that nitromusks and polycyclic musks inhibit the activity of multi-drug efflux transporters responsible for multi-xenobiotic resistance (MXR) in gills of the marine mussel *Mytilus californianus*. The IC₁₀ values for the different classes of musks were in the range from 0.09 to 0.39 μ M and IC₅₀ values were between 0.74 and 2.56 μ M. The immediate consequence of inhibition of efflux transporters is that normally excluded xenobiotics will now be able to enter the cell. Remarkably, the inhibitory effects of a brief two-hour exposure to musks were only partially reversed after a 24 to 48-hour recovery period in clean seawater. This unexpected consequence of synthetic musks — a long-term loss of efflux transport activity — will result in continued accumulation of normally excluded toxicants even after direct exposure to the musk has ended. These findings additionally point to the need to determine whether other environmental chemicals have similar long-term effects on these transporters. The results are relevant to human health, as they raise the possibility that exposure to common xenobiotics and pharmaceuticals could cause similar long-term inhibition of these transporters and lead to increased exposure to normally excluded toxicants.

1. Introduction

Artificial musk compounds find broad use as inexpensive fragrances and fixatives in personal care products including detergents, cleaning agents, air fresheners and cosmetic products (for names and structures see Table 1). The world-wide production of musks increased from about 7000 to 8000 t/year between 1987 and 1996, with a concurrent production shift from nitromusks to polycyclic musks (Rimkus 1999). The most widely used polycyclic musk is Galaxolide (HHCb), followed by Tonalide (AHTN) (Rimkus 1999), and production of these two polycyclics was about 1800 t in 2000 in Europe, whereas production of other polycyclic musks was below 20 t (Kupper et al. 2004). Musk xylene is the most common nitromusk, but use was discontinued in Japan and a voluntary ban is in force in Germany (Käfferlein et al. 1998). Use is still high in the US, although it is banned on products with a risk of oral uptake (e.g. lipsticks). Toxicological data suggest no severe health risks associated with artificial musks, although long-term carcinogenic effects cannot be ruled out (Api et al. 2004; Frosch et al. 1995; Käfferlein and Angerer 2001; Schmeiser et al. 2001), and there is also concern about accumulation in adipose tissue, blood plasma and breast milk (Käfferlein and Angerer 2001; Liebl and Ehrenstorfer 1993; Ott et al. 1999; Rimkus and Wolf 1996).

More than two decades ago Yamagishi and colleagues reported the presence of musks in the aquatic environment and biota in Japan (Yamagishi et al. 1983; Yamagishi et al. 1981), leading to similar investigations in Europe and North America (Balk and Ford 1999a; Bester et al. 1998; Draisci et al. 1998; Gatermann et al. 2002; Käfferlein et al. 1998; Kallenborn et al. 1999; Osemwengi and Steinberg 2001; Paasivirta et al. 2002; Peck and Hornbuckle 2004; Ricking et al. 2003; Rimkus 1999; Rimkus et al. 1997; Rimkus et al. 1999; Rimkus and Wolf 1995; Tas and Balk 1997; Tas et al. 1997). These studies show that synthetic musks are

widespread in marine and freshwater environments and bio-accumulate to a high degree in fish and invertebrates. Since acute and chronic toxicity thresholds for musks in invertebrate and fish species are much higher than the environmentally measured levels (Balk and Ford 1999b; Behechti et al. 1998; Breitholtz et al. 2003; Chou and Dietrich 1999b; Wollenberger et al. 2003), the environmental risks posed by musks are assumed to be low (Balk et al. 2001). Musks show low binding affinity to estrogen receptors and their environmental impact as endocrine disruptors through this pathway is also regarded as low (Bitsch et al. 2002; Chou and Dietrich 1999a) although indirect endocrine effects such as inhibition of hormone synthesis (Kester et al. 2000; Sanderson et al. 2001) have not been examined.

However, it has been proposed that pharmaceutical and personal care products (PPCPs), a vast group of environmental contaminants including the artificial musks, might affect organisms through interference with multidrug/multixenobiotic resistance (MDR/MXR) efflux transporters (Daughton and Ternes 1999; Epel and Smital 2001; Smital et al. 2004). The activity of these transporters provides a first line of defense to prevent accumulation of xenobiotics in cells. Inhibition of this cellular defense mechanism increases the sensitivity of cells to xenobiotics by permitting normally excluded toxicants to enter the cell (Epel 1998; Kurelec 1992).

The transporter proteins responsible for MXR include p-glycoprotein (p-gp), multidrug resistance protein (MRP), and other members of the ABC (ATP-binding cassette) family of transport proteins. A characteristic feature of these efflux transporters is affinity for a diverse array of substrates. P-gp, for instance, acts on a large number of chemically unrelated substrates whose common properties are small size, moderate hydrophobicity and positively charged domains (Bain et al. 1997). Although this low specificity may provide an advantage by enabling

the system to cope with “new” chemicals (e.g. environmental pollutants), a disadvantage is that the transporter capacity is more easily saturated in the presence of many substrates and its protective role can then be lost. This subversion of the MXR defense by multiple substrates or inhibitors of efflux transporters is referred to as chemosensitization and compounds that cause this behavior are referred to as chemosensitizers (Epel 1998; Kurelec 1997; Smital and Kurelec 1998b). We hypothesized that the synthetic musks, which are small molecular weight, moderately hydrophobic compounds, might be such chemosensitizers, and preliminary studies indeed showed inhibition of efflux transporters in gill tissues of a marine mussel (Luckenbach et al. 2004).

Here we describe the inhibitory potencies of six artificial musk compounds and report the remarkable finding of continued inhibition of transport activity for 24-48 h following a short (2 h) exposure to musks. Although of low toxicity themselves, musks could therefore enhance toxicity of other compounds by blocking the MXR defense system. These results strongly affirm the hypothesis of Kurelec and collaborators, that indirect effects of environmental chemicals as efflux transporter chemosensitizers could be of major importance (Epel 1998; Kurelec 1995; Kurelec 1997; Smital and Kurelec 1998b), but, additionally, our results indicate that the effects of chemosensitizers might continue long after the exposure event.

2. Materials and Methods

2.1. Chemicals

Musk ketone (MK), musk xylene (MX), Galaxolide™ (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta- γ -[2]-benzopyran; HHCB) and Celestolide™ (4-Acetyl-1,1-dimethyl-6-*tert*-butylindane; ADBI) were gifts from IFF (Union Beach, NJ); Tonalide™ (7-Acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene; AHTN) from Bush Boake Allen

Inc. (Jacksonville, FL); and Traseolide™ (5-Acetyl-1,1,2,6-tetramethyl-3-isopropylindane; ATII) from Quest International (Mount Olive, NJ). Rhodamine B, (±)-verapamil hydrochloride and quinidine were purchased from Sigma (St. Louis, MO). Purity of musk ketone, musk xylene, verapamil and quinidine was at or above 99%. Purity data were not available for the other musk compounds used. Stocks of rhodamine B in ultrapure water and ethanol stocks of the musks, verapamil and quinidine were stored in glass flasks at 4°C in the dark.

2.2. *Animals and tissue preparation*

California mussels (*Mytilus californianus* Conrad, 1837), with valve lengths ranging from 70-95 mm, were collected from the rocky intertidal zone at Hopkins Marine Station (Pacific Grove, CA, USA) and maintained in tanks with running seawater (approximately 15°C) for at least 24 h and no longer than two weeks before experiments. Experiments were performed with gill tissue which shows high efflux transporter activity (Cornwall et al. 1995).

The mussels were opened by cutting through the adductor muscles with a sharp knife and the gills excised with fine scissors and placed in filtered seawater (FSW; 15°C). Each gill consists of two tissue lobes which were separated at their dorsal connection. To obtain tissue pieces of equal size, dermatology biopsy punches (Acuderm, Fort Lauderdale, FL) were used to excise discs (diameter 5 mm) from the tissue lobes. The tissue discs that result from this procedure consist of a double layer of lamellae (descending and ascending arm) that are connected by interlamellar tissue bridges. Approximately 55-70 discs per individual were obtained from mussels of the size used for these experiments. Mucus was removed from the gills with forceps before preparing the discs and the tissue discs were flushed and kept in FSW until use.

The gender of each animal was determined from visual inspection of gametes which are clearly distinguishable by shape (White 1937). A piece of gonad tissue was dipped into a droplet of seawater on a microscope slide, covered with a cover slip and examined under a light microscope equipped with phase contrast at 400x.

2.3. *Competitive substrate/transporter inhibition assay and long-term inhibition*

The fluorescent dye rhodamine B was used as an indicator of efflux transporter activity. Inhibition of transporter activity by a test compound is indicated by increased fluorescence due to higher accumulation of rhodamine B in the cell (Neyfakh 1988). Test solutions were prepared in FSW with 1 μ M rhodamine B. Stocks of the test compounds dissolved in ethanol were added to FSW to the desired concentrations and all solutions adjusted to 1% ethanol along with an ethanol control. Separate experiments indicated that this ethanol concentration had no effect of on transport activity (data not shown). Incubations were performed in glass petri-dishes (diameter 5 cm) in a volume of 10 mL.

Gill tissue discs were incubated in test solutions with gentle rocking for 90 min at 15°C in the dark (n = 2-5 tissue discs per dish). After exposure, the tissue discs were briefly washed twice in FSW to remove external rhodamine B, frozen on dry ice or in liquid nitrogen, and stored at -20°C in the dark. For analysis, rhodamine B was extracted by sonicating the tissue in 200 μ L of n-butanol until fully homogenized. N-butanol was used for extractions, since, of the several extraction media tested (water, PBS, some organic solvents), fluorescence signals from rhodamine B were strongest with this solvent. The sonicates were then centrifuged at 13,000 g for 5 min and the supernatant transferred to black 96-well microplates (Packard Instrument Co, Meriden, CT). The amount of dye in the supernatant was determined with a fluorescence microplate reader (Packard FluoroCount; emission: 540 nm, excitation: 580 nm). Rhodamine B

extracts were kept dark and on ice at all times. At each reading, background fluorescence was determined with pure n-butanol and subtracted from each value. Background fluorescence values of gill tissue with no rhodamine added were similar to that of the n-butanol extraction medium.

To examine the duration of inhibitory effects after exposure to test compounds, 6-9 gill tissue discs were incubated with 1 μM of test compounds for 2 h. Transporter activity was then assayed in one third of the discs immediately after exposure and in the others after 24 h and 48 h of washing in fresh FSW (50 mL). FSW was changed after 24 h. During exposure and washing, dishes were gently rocked at 15°C in the dark. The transporter activity assay was performed with 1 μM rhodamine B as described above.

2.4. *Data analysis*

Transport activities in treated gill discs were normalized relative to non-treated controls that were run for each individual. The ratios, which indicate relative increases in fluorescence, were used as a measure of inhibition by each compound at the specified concentrations. The potencies of compounds for inhibiting MXR transporter activity are calculated as IC₁₀ and IC₅₀ values, which indicate concentrations causing 10% and 50% of the maximal inhibition observed for a compound. IC₁₀ and IC₅₀ values were determined by probit analysis using a macro program for Microsoft Excel written by Josef Greve, Fraunhofer-Institut für Umweltchemie und Ökotoxikologie, Schmallenberg, Germany.

All experiments were performed at least three times using different individuals for each experiment and average values and standard deviations were determined. Statistical analyses were performed using Student's t-Test. For correlation analyses, Pearson correlation coefficients

were determined. Statistical analyses were conducted using JMP software (SAS Institute Inc., Cary, NC).

3. Results

3.1. Method evaluation

Biopsy punch and extraction procedure. The biopsy punch assay allowed processing large numbers of samples in a standardized way to examine effects of test compounds on efflux transporter activity in gill tissue of mussels. After exposure to rhodamine B and test compounds the dye was extracted from the biopsy tissue discs in a defined volume of solvent and the amount of dye then measured fluorometrically. This approach gave results comparable to the epifluorescence microscopy procedure of measuring accumulation of fluorescent dye in living mussel gills (Cornwall et al. 1995; Eufemia and Epel 1998; Galgani et al. 1996), but was less susceptible to subjective errors connected with the microscopic assay, such as the selection of a tissue area to be measured.

Sample homogeneity. The use of biopsy punches allowed us to obtain samples of equally sized tissue discs from gills from one individual. Standard deviations of dry weight, protein content and the amount of MXR transporter p-glycoprotein (detected with antibody C219 using western blot techniques) varied by less than 10% of the average values in discs from the same animal (data not shown). We furthermore found that transporter activity, indicated by the amount of rhodamine B accumulated by the tissue, was consistent among tissue discs from the same individual (e.g. S.D. from 18 discs from one individual incubated in FSW with 1 μ M rhodamine B were \pm 6.5%).

Individual variation in transporter activity. There were considerable differences in basal efflux transporter activity between individual animals. In control tissues, low basal efflux

transporter activity is indicated by high accumulation of rhodamine B, whereas high basal efflux activity is indicated by low levels of rhodamine fluorescence (since more dye is effluxed from the cells). We found that these basal rhodamine fluorescence levels varied up to 3.3-fold between individuals. A significant correlation was found between transporter activity and mussel size (quantified by valve length) ($P = 0.003$, $n = 27$), but there was no relationship to gender ($P = 0.32$, $n = 27$).

These differences in basal activity also correlated with increased uptake of rhodamine (and correspondingly rhodamine fluorescence) when transporter activity was maximally repressed by an inhibitory compound. Using a ratio of maximal fluorescence/basal fluorescence [$R_{F(\max)/F(\text{basal})}$] to compare basal and repressed transporter activity, individuals with low basal transporter activity exhibited a low $R_{F(\max)/F(\text{basal})}$, whereas animals with high basal activity had a high ratio. For example, in a low-activity individual with 37.3 fluorescence units in its control, the $R_{F(\max)/F(\text{basal})}$ was 1.5; whereas in a high-activity individual with 11.3 fluorescence units in the control, the $R_{F(\max)/F(\text{basal})}$ was 3.8. A strong positive correlation ($P = 6.2 \times 10^{-8}$; $r^2 = 0.7$; $n = 27$) was found for this relationship (Figure 1).

Physiological indices and long-term exposure studies. Experiments in which dissected gill tissue was kept in FSW for 48 h necessitated an assessment of physiology over this time period. Oxygen consumption rates measured in isolated gill tissue kept in FSW at 15°C were stable over a period of at least 3 days. Ciliary movement in gills was similarly stable for 3 days (indeed, ciliary movement was unaffected after 7 days incubation). There were also no evident signs of morphological deterioration in the tissue. Efflux transporter activity tended to slightly increase over time, as indicated by the lower amounts of rhodamine B in control tissues. After 24 h, fluorescence values were lower by an average of 8%, after 48 h 13% lower than initial

values. A control was therefore run at each time point and activity was normalized relative to the control tissue.

3.2. *Inhibitory effects by test compounds, dose-effect relationships*

Musks and the p-gp inhibitor reference compounds quinidine and verapamil caused a dose-dependent increase in accumulation of rhodamine B in mussel gill tissue, indicating an inhibition of efflux transporter activity. IC₁₀ and IC₅₀ values were determined from the dose-effect curves (range of tested concentrations: 0.01-100 μM for musk compounds and quinidine, 0.001-10 μM for verapamil; using 6-11 concentrations per series, including controls). In most cases, curve fits (r^2) of the probit regressions were between 0.7 and 0.9. Musk concentrations above 5-10 μM did not increase rhodamine B fluorescence, indicating maximum inhibition of the transporter activity at these levels (Figure 2, A-F).

The IC₁₀ values, used as indicators for low effect concentrations, showed clear effects in the 0.1-0.4 μM concentration range for musks and quinidine and at 0.01 μM for verapamil (Figure 2, Table 2). The range of IC₅₀ values, which were used as a measure for the inhibitory potencies of the test compounds, was 0.7-2.6 μM for the musks and quinidine and 0.08 μM for verapamil (Figure 2, Table 2).

The nitromusks were more effective inhibitors than the polycyclic musks. When combining the values for the two musk groups, the IC₅₀ values for the nitromusks (IC₅₀_{nitromusks} = 0.82 ± 0.53 μM, n = 11) were significantly lower than for the polycyclic musks [IC₅₀_{polycyclics} = 2.34 ± 0.82 μM, n = 12; P = 0.0001 (Student's t-Test)].

3.3. *Combinatory effects*

Synthetic musks are usually used in combinations in fragrances and environmental samples often contain several different musk compounds. We therefore tested whether combinations of musks act in a synergistic, antagonistic, or additive fashion in inhibiting transporter activity. Treatments containing (1) a nitromusk mixture (musk xylene and musk ketone), (2) a polycyclic musk mixture (Galaxolide and Celestolide) and (3) a nitromusk/polycyclic musk mixture (musk xylene and Galaxolide) were tested. For these experiments, each compound was applied at 0.5 μM to achieve a final total concentration of 1 μM musks. An additional experiment was run with a mixture of two nitromusks (musk xylene and musk ketone) and two polycyclics (Galaxolide and Celestolide) with each compound at 0.25 μM . All compounds were also tested separately at 1 μM .

In all cases, inhibition of transporter activity by mixtures of different musk compounds was intermediate to treatments with single compounds (Figure 3). This indicates that the inhibitory effects of the musks in the mixtures were additive.

3.4. *Long-term inhibitory effects*

After exposing mussel gill discs to test compounds for 2 h, rhodamine uptake was measured directly after the exposure (0 h) and after 24 and 48 h of washing the tissue in FSW. Immediately after exposure (0 h), a sample of the discs was incubated in rhodamine B for 90 minutes and rhodamine uptake was found to be 38–84% higher in musk treatments compared to respective controls due to transporter inhibition (Figure 4, A-F). After 24 h of washing, another sample of discs was similarly incubated in rhodamine and levels in the tissues previously exposed to the musks were still 30-74% higher than the controls, indicating continuing inhibition of transporter activity. The differences between treatments vs. controls were

significant for musk xylene, Galaxolide and Traseolide ($P < 0.05$, paired t-Test), and there were clear trends of increased rhodamine levels also for musk ketone, Celestolide and Tonalide, indicating an inhibitor related effect after 24 h (Figure 4). A similar measurement of transport activity at 48 h showed that activity had now reached the control levels for Traseolide and Tonalide, indicating complete recovery from the inhibitory action of the musks. However, there was still a trend of reduced activity (i.e., higher fluorescence) for the tissues previously incubated in musk xylene, musk ketone, Galaxolide and Celestolide. The higher fluorescence was consistently seen with these four compounds, indicating an inhibitory effect of these musks even after this time period (Figure 4).

Verapamil, a p-gp reference inhibitor, exhibited strong long-term inhibition, indicated by rhodamine tissue levels that were 88 and 35 % higher than the controls at 24 and 48 h, respectively. In contrast, a different p-gp reference inhibitor, quinidine, showed no long-term effects, with almost complete reversal of inhibition seen after 24 h of washing (Figure 4).

4. Discussion

This study shows that nitromusk and polycyclic musk compounds inhibit the activity of efflux transporters in the marine mussel *Mytilus californianus* and that these inhibitory effects last for 24-48 h after termination of exposure to the musks. As part of this work, we also describe an improved efflux transporter assay that allows processing large numbers of tissue samples from one individual and quantifying the potency of chemicals to inhibit MXR transporter activity.

4.1. MXR efflux inhibition by musks

Both nitromusks and polycyclic musks inhibited efflux transporter activity, with the nitromusks being the more effective inhibitors. The tested musks inhibited transporter activity at a concentration range similar to that for quinidine but were an order of magnitude less effective than verapamil (Figure 2, Table 2). Furthermore, the inhibitory effects of combinations of musks were additive (Figure 3).

Individual mussels differed in basal transporter activity and in sensitivity to treatment with test agents. Differences in size and age may partly explain the variation in activity levels, but the variability is likely to reflect the heterogeneity of the natural population. Thus, stress response levels may be determined by genetic variation and site specific conditions, such as time of water immersion during tides, wave exposure, temperature, and so forth (Roberts et al. 1997).

Despite this variation among test animals, differences between the different musk species in blocking rhodamine efflux through MXR transporters were clearly visible. These differences in inhibitory capacity most likely result from differences in affinity to substrate binding sites of the transporter, correlated with differences in K_{ow} . Bain and LeBlanc (1996) found that the inhibitory efficiency of a chemical against human p-gp activity was highest for moderately hydrophobic compounds with a log K_{ow} (octanol-water coefficient) in the range of 3.6-4.5. This is consistent with our data showing that the nitromusks, with log K_{ow} values of 4.3 and 4.9, were more efficient inhibitors than the more hydrophobic polycyclic musk compounds, with log K_{ow} values 5.6-6.3 (Table 1). In fact, IC₅₀ values for the musk compounds were strongly correlated with respective log K_{ow} values ($P=0.0006$, $r^2=0.9$, $n=6$; Figure 5), consistent with the lower inhibitory potency of compounds with a higher hydrophobicity. Verapamil, which was the most effective p-gp inhibitor in our study, also fits the regression line calculated for the musks.

Quinidine, however, was less effective as an inhibitor and did not fit this regression line; consistent with its K_{ow} , which is below the K_{ow} range for high affinity p-gp substrates (Figure 5).

The sensitivity of rhodamine efflux to verapamil, quinidine, and other known p-gp inhibitors/substrates indicates that the MXR phenomenon in *Mytilus* is associated with a p-gp like efflux transporter (Cornwall et al. 1995; Galgani et al. 1996; Minier et al. 1993; Smital et al. 2003). Other transporters, such as an MRP (Lüdeking and Köhler 2002), may be present, but the substrate and inhibitor profile suggests a dominant activity of a p-gp type transporter which is most likely the target of the musk compounds.

4.2. *Long-term inhibitory effects of musks*

Inhibitory effects of musk compounds and verapamil were still present 24-48h after removal of the inhibitors (Figure 4). To our knowledge, this is the first demonstration of long-term inhibitory effects of MXR modulators and was unexpected since efflux transporters are involved in removal of xenobiotics from cells and therefore quicker recovery of the transporter activity would have been anticipated.

The inhibition indicates that the musks and verapamil remain accessible to the efflux transporters for 24-48 h after removal of the compounds from the medium. This could be related to the hydrophobicity of the compounds and/or their affinity for the transporters. Hydrophobic compounds will accumulate in the cell membrane during exposure and could affect efflux transporters indirectly through membrane effects (Ferté 2000) or the membranes could directly serve as reservoirs for slow release of the chemicals which could then bind to the active sites of the transporter proteins. Alternatively, the chemicals might have a high affinity for specific sites of the transporters such as substrate binding sites or other functional sites (e.g. ATP-binding site) from where they are only slowly released, with resultant long-term inhibition.

4.3. Potential environmental relevance of synthetic musks as chemosensitizers

It was first suggested by Kurelec (1997) that environmental pollutants may act as chemosensitizers by compromising the MXR system, allowing other toxicants, which would normally be excluded by the MXR transporters, to enter the cell. Kurelec pointed out that chemosensitizers could include chemicals of low toxicity, which would present an unanticipated environmental or human health risk. A broad range of anthropogenic chemicals such as pesticides, pharmaceuticals and some polyaromatic hydrocarbons have been found to inhibit MXR transporters by blocking efflux of fluorescent or radio-labeled substrates in human cells or cells of aquatic organisms (Bain and LeBlanc 1996; Britvic and Kurelec 1999; Cornwall et al. 1995; Eufemia and Epel 1998; Kurelec 1992; Smital and Kurelec 1998b; Toomey and Epel 1993). When water samples from differently contaminated field sites were tested for their inhibitory potential for MXR transporters, greater inhibition by samples containing high levels of anthropogenic pollutants was detected (Smital and Kurelec 1997). This suggests that levels of anthropogenic pollutants currently found in the environment have the potential to interfere with normal animal defense mechanisms. Recent extensions of this work have shown that these MXR chemosensitizers increased the potency of toxic or mutagenic transporter substrates (Britvic and Kurelec 1999; Smital and Kurelec 1998a).

Our data indicate that synthetic musks are also chemosensitizers and could therefore have indirect effects by allowing normally excluded toxicants to permeate cells. Earlier studies have pointed to other indirect toxic effects of nitromusks. For example, musk ketone and musk xylene both induce P450 dependent oxygenases (Lehman-McKeeman et al. 1995; Mersch-Sundermann et al. 1996; Mersch-Sundermann et al. 2001; Suter-Eichenberger et al. 1999) and therefore could cause increased transformation of other environmental chemicals which are

mutagenic in their transformed form such as benzo(a)pyrene, 2-aminoanthracene and aflatoxin B1 (Mersch-Sundermann et al. 1996; Mersch-Sundermann et al. 2001).

Do the effects of synthetic musks as MXR inhibitors apply to real world situations? The concentration range in which musks are effective inhibitors [10^{-6} – 10^{-7} M (ppb) range] is several orders of magnitude higher than concentrations reported for water samples from the environment [10^{-9} – 10^{-12} M (ppt) range] (Rimkus 1999). However, musks are concentrated in sediments (Winkler et al. 1998) which could make them available to bottom dwellers. More importantly, their hydrophobicity results in bio-accumulation and levels in tissues of aquatic organisms are 10^1 – 10^4 fold higher, and in the lipid fraction may be $>10^5$ fold above environmental levels (Gatermann et al. 2002; Rimkus 1999). Association with lipid may be especially relevant to MXR transporters, which reside in the cell membrane and hence are more directly exposed to fat-soluble compounds such as the musks. These considerations indicate that even if ambient concentrations are low, long-term exposure will lead to tissue burdens which could inhibit MXR function indirectly via membrane effects or directly via high-affinity binding to transporter sites.

The unexpected long-term effects of the musks are troubling. One consequence of such long-term inhibition of transporters is that short-term events could have continuing consequences. Thus, the effects of short-term incidents such as storm-water runoffs or chemical spills could continue after such events if there is accumulation of chemosensitizers in the tissue, or prolonged inhibition as a result of the acute exposure.

Musks are typically present in the environment with other contaminants (Fromme et al. 2001; Kallenborn et al. 1999) which could well include other transporter modulators. As shown, the effects of combinations of p-gp inhibitors are additive and therefore musks could be part of a

suite of pollutants which contribute to chemosensitizing effects in nature. In fact, as first suggested by Daughton and Ternes (1999), this could be an unanticipated consequence of chemicals such as the PPCPs. Additive effects of low concentrations of many compounds could affect the MXR transporters and this would be magnified by the sheer numbers of different components of the PPCPs.

4.4. Musks and other environmental chemosensitizers – a human health risk?

This work on the musks, although focused on aquatic organisms, also points to unsuspected effects of these chemicals on human health. MXR efflux pumps are widely distributed in mammalian tissues where they are a crucial part of the cellular defense against cytotoxins (Cordon-Cardo et al. 1990). The effects of environmental contaminants as chemosensitizers have not been studied in humans; however, inhibitors of efflux transporters used in cancer therapy can lead to increased permeability of healthy tissues to transporter substrates (Luker et al. 1997), indicating that chemosensitizers could pose a health threat.

Since synthetic musks are present in human tissue samples, the question arises, whether they could also be relevant as chemosensitizers in humans. Concentrations of musk xylene found in body fat and breast milk are in the range of 0.2-0.3 $\mu\text{mol/kg}$, and in blood plasma in the nM range (Käfferlein et al. 1998). These values represent body burdens that are one to two orders of magnitude below the effective concentrations seen in the present study on mussel tissue, however, they could contribute to additive effects with other compounds.

This study especially points to the need to screen musks and other environmental chemicals that accumulate in humans to determine if they are also chemosensitizers of MXR-related transporters. Especially critical will be to ascertain whether they cause long-term effects similar to those seen in our study. Effects on efflux systems could result in unanticipated

accumulation of toxicants in humans and confound safety predictions of seemingly innocuous chemicals.

5. References

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Table 1: Names, CAS numbers, formulas, structures, molecular weights and log octanol water partition coefficients (K_{ow}) for artificial musks and MXR model substrates and inhibitors.

Chemical and trade names	CAS RN	Formula	Structure	Molecular weight	Log K_{ow}
Musk xylene 1- <i>tert</i> -Butyl-3,5-dimethyl-2,4,6-trinitrobenzene	81-15-2	$C_{12}H_{15}N_3O_6$		297.3	4.9 ^a
Musk ketone 1- <i>tert</i> -Butyl-3,5-dimethyl-2,6-dinitro-4-acetylbenzene	81-14-1	$C_{14}H_{18}N_2O_5$		294.3	4.3 ^a
Galaxolide; HHCB 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta- γ -[2]-benzopyran	1222-05-5	$C_{18}H_{26}O$		258.4	5.9 ^a
Celestolide [®] ; Crysolide [®] ; ADBI 4-Acetyl-1,1-dimethyl-6- <i>tert</i> -butylindane	13171-00-1	$C_{17}H_{24}O$		244.4	5.9 ^a
Tonalide [®] ; Tetralide [®] ; Fixolide [®] ; AHTN 7-Acetyl-1,1,3,4,4,6-hexamethyl-tetrahydronaphthalene	21145-77-7	$C_{18}H_{26}O$		244.4	5.7 ^a
Traseolide [®] ; ATII 5-Acetyl-1,1,2,6-tetramethyl-3-isopropylindane	68140-48-7	$C_{18}H_{26}O$		258.4	6.3 ^a
Quinidine	56-54-2	$C_{20}H_{24}N_2O_2$		324.4	2.8 ^b
Verapamil	52-53-9	$C_{27}H_{38}N_2O_4$		454.6	4.5 ^b
Rhodamine B	81-88-9	$C_{28}H_{31}ClN_2O_3$		479.0	2.0 ^c

a: Balk et al. (2001), b: Wang et al. (2003), c: Liu (2004)

Table 2. IC10 and IC50 values (concentrations causing 10 and 50% of the maximum inhibition) of nitromusks, polycyclic musks and reference inhibitors verapamil and quinidine for efflux transporters in mussel gills. The values were achieved using probit regression (see Figure 2). Means \pm S.D. are given. n: number of experiments.

		IC10	IC50	n
Nitro-musks	Musk xylene	0.14 \pm 0.12	0.97 \pm 0.63	4
	Musk ketone	0.09 \pm 0.12	0.74 \pm 0.49	7
Polycyclic musks	Galaxolide (HHCB)	0.37 \pm 0.16	2.43 \pm 0.45	3
	Celestolide (ADBI)	0.39 \pm 0.34	2.32 \pm 1.18	3
	Tonalide (AHTN)	0.35 \pm 0.31	2.05 \pm 0.31	3
	Traseolide (ATII)	0.32 \pm 0.04	2.56 \pm 0.07	3
	Verapamil	0.01 \pm 0.02	0.08 \pm 0.07	6
	Quinidine	0.24 \pm 0.20	1.59 \pm 0.85	3

Figure Legends

Figure 1. Correlation between basal transporter activity [$F(\text{basal})$] and relative increase in fluorescence in mussel gill tissue with transporter activity inhibited maximally vs. basal transporter activity [$R_{F(\text{max})/F(\text{basal})}$].

Figure 2. Dose-effect curves for in vivo inhibition of MXR transporters in mussel gill tissue by nitromusks (musk xylene and musk ketone), by polycyclic musks (Galaxolide, Celestolide, Tonalide and Traseolide) and by reference inhibitors (verapamil and quinidine). Gill tissue discs from the marine mussel *Mytilus californianus* were exposed to different concentrations of test compounds and rhodamine B (1 μM). Inhibition of the transporters was quantified by retention of RB in the tissue. Probit regression was used to obtain the curves. Each curve represents data from an individual animal. (A) Musk xylene, (B) Musk ketone, (C) Galaxolide (HHCB), (D) Celestolide (ADBI), (E) Tonalide (AHTN), (F) Traseolide (ATII), (G) Verapamil, (H) Quinidine.

Figure 3. Inhibitory effects by musks as single compounds and combinations. Retention of rhodamine B was measured in gill tissue discs exposed to 1 μM RB and a 1 μM total of test compounds. The bars represent percent increase of fluorescence vs. respective controls. The values are means (\pm S.D.). $n = 7$ for the following experiments: solvent, 1 μM MX, 1 μM MK, 1 μM HHCB, 1 μM ADBI; $n = 3$ for MX + HHCB, MX + MK, HHCB + ADBI, MX + MK + HHCB + ADBI. MX: musk xylene, MK: musk ketone, HHCB: Galaxolide, ADBI: Celestolide.

Figure 4. Long-term inhibition of efflux transporters by nitromusks (musk xylene and musk ketone), polycyclic musks (Galaxolide, Celestolide, Tonalide and Traseolide) and reference inhibitors (verapamil and quinidine). The data represent mean fluorescence vs. respective controls ($\% \pm$ S.D.). $n = 3$ (11 for verapamil). A paired t-Test was used to determine significant differences between treatments and the respective controls at each time point; * indicates $P < 0.05$. (A) Musk xylene, (B) Musk ketone, (C) Galaxolide (HHCB), (D) Celestolide (ADBI), (E) Tonalide (AHTN), (F) Traseolide (ATII), (G) Verapamil, (H) Quinidine.

Figure 5. Correlation of MXR inhibitory efficiency (indicated by IC_{50} values) and $\log K_{ow}$ of test compounds. For the calculation of the regression curve (with 95% confidence intervals) only musk compounds were included.

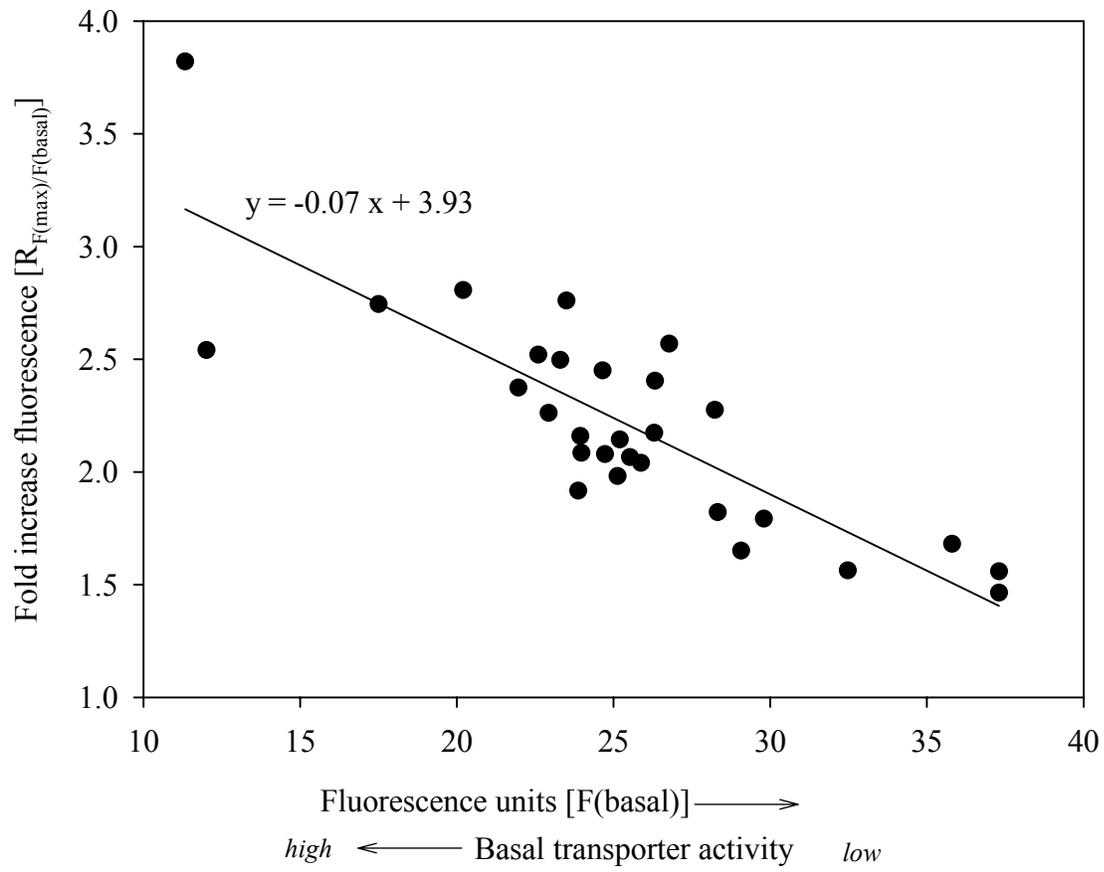


Figure 1.

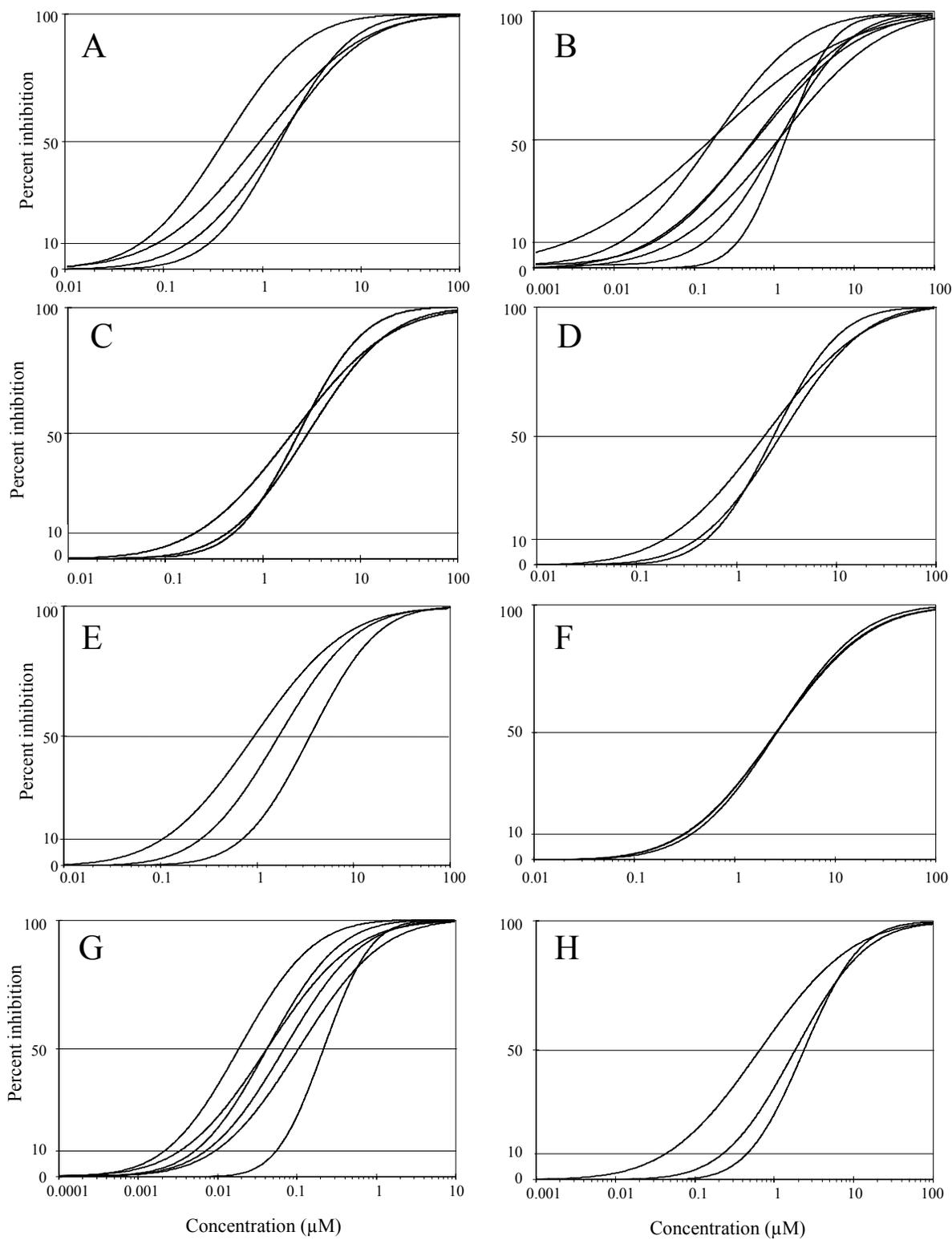


Figure 2.

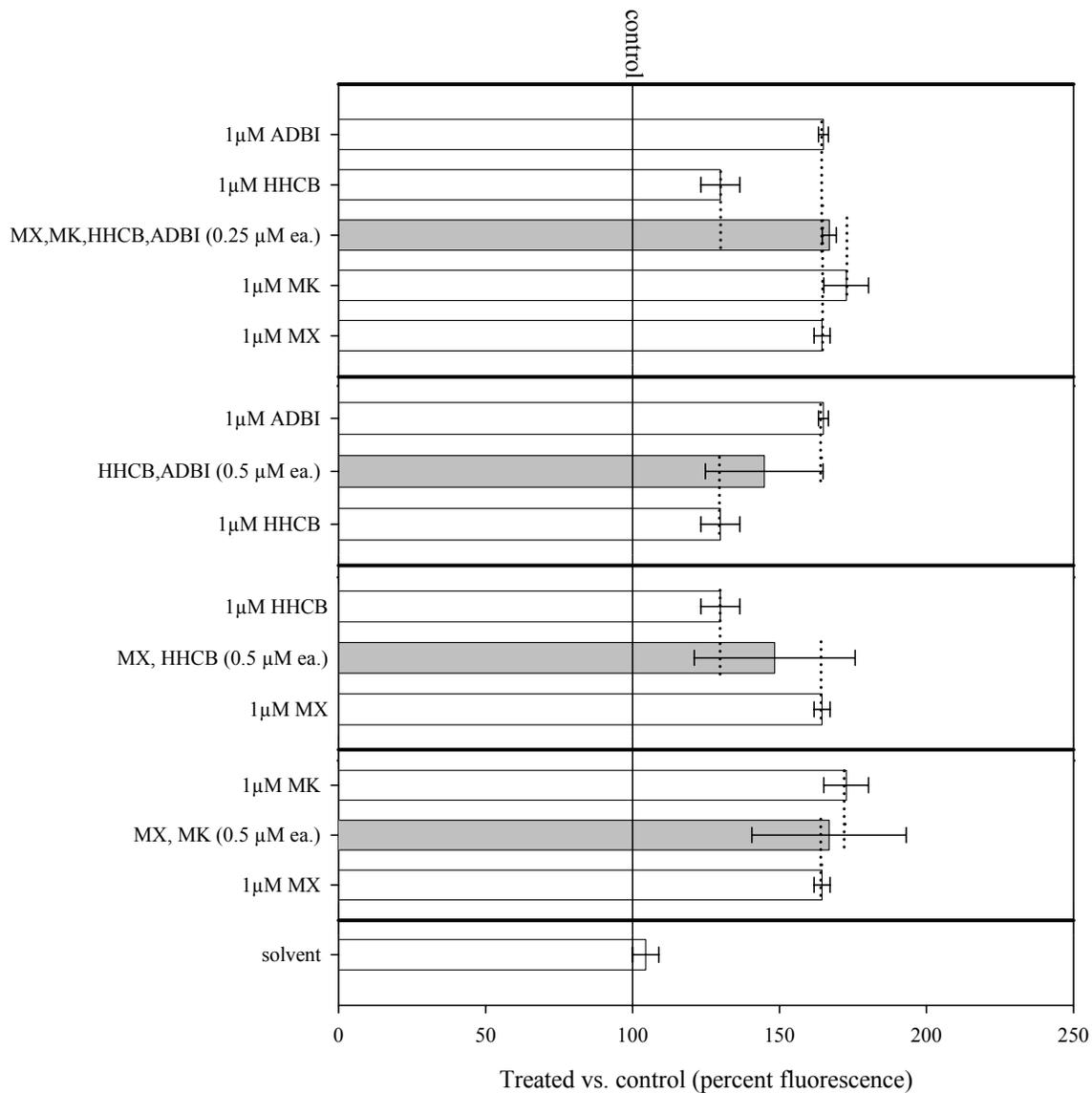


Figure 3.

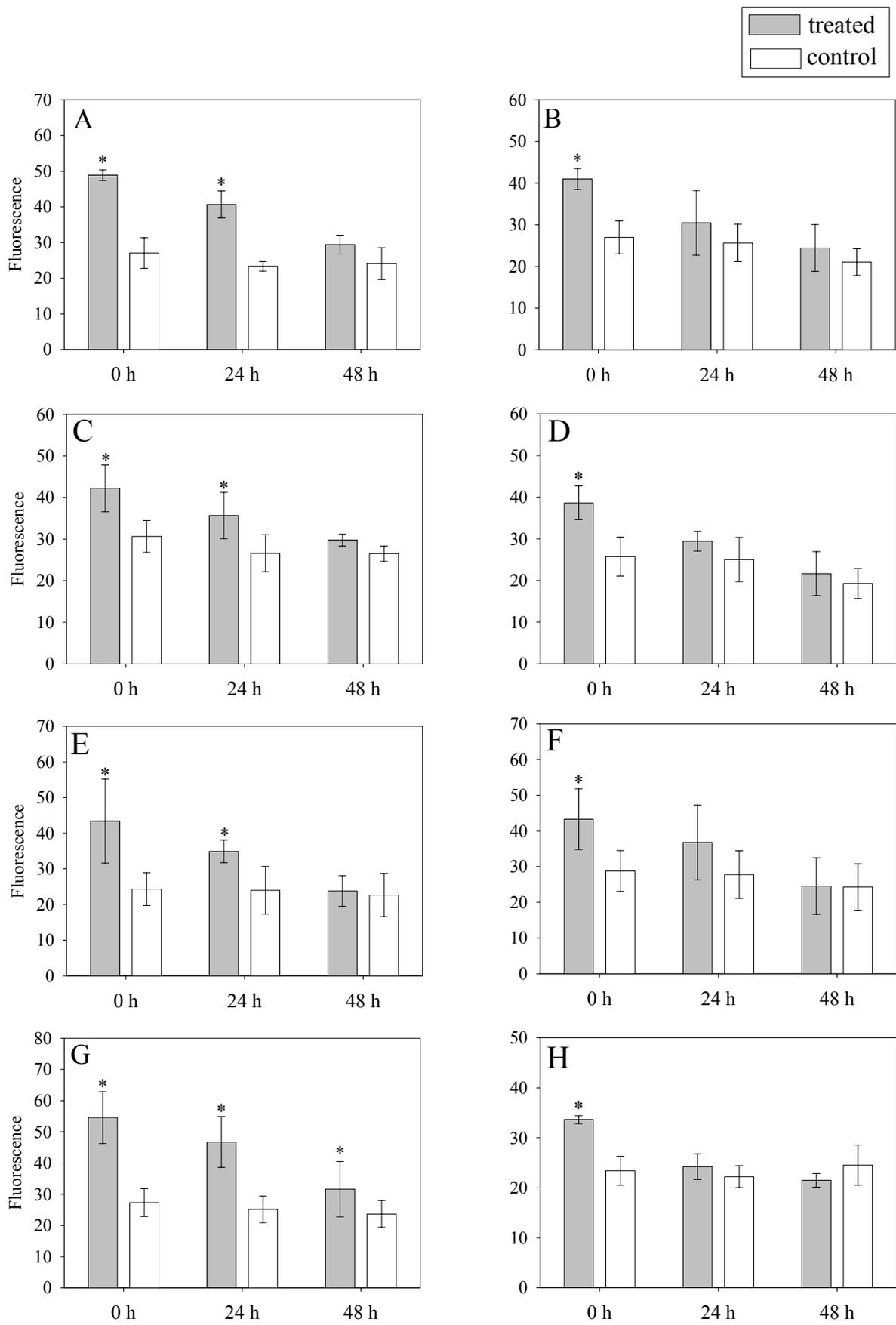


Figure 4.

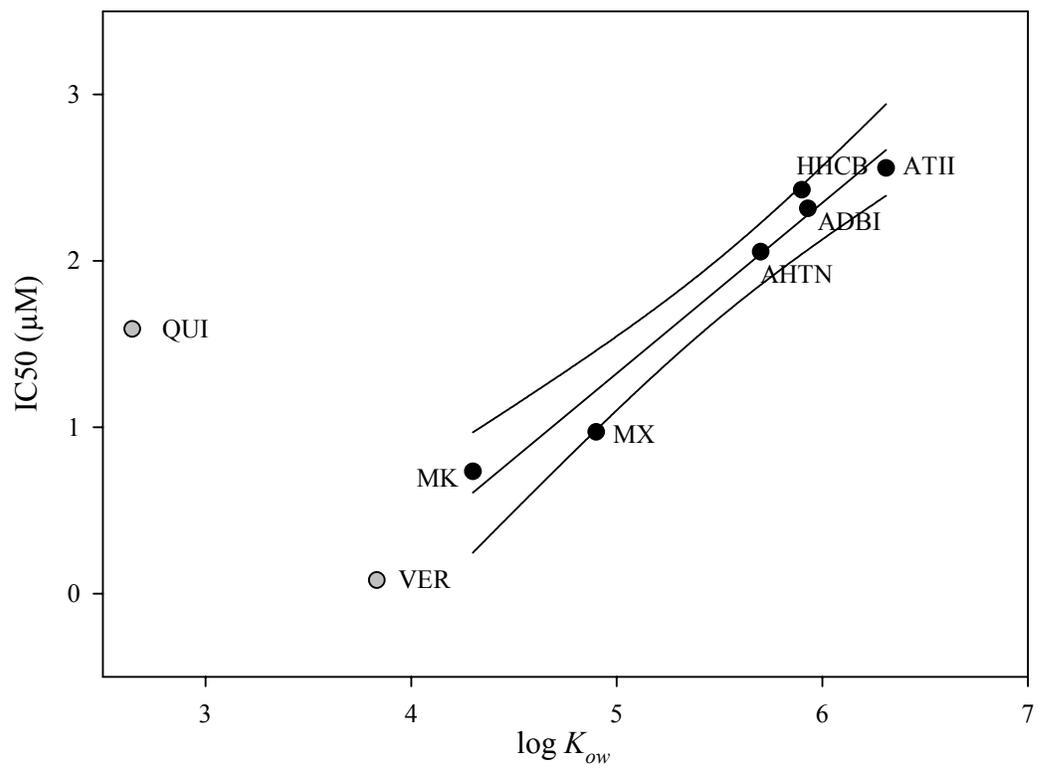


Figure 5.