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SHORT RUNNING TITLE: Interaction of aflatoxins and PCB 126 with bovine ABCG2

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ABSTRACT

The ATP-binding cassette efflux transporter ABCG2 plays a key role in the mammary excretion of drugs and toxins in humans and animals. Aflatoxins (AF) are worldwide contaminants of food and feed commodities, while PCB 126 is a dioxin-like PCB which may contaminate milk and dairy products. Both compounds are known human carcinogens. The interactions between AF and bovine ABCG2 (bABCG2) as well as the effects of PCB126 on its efflux activity have been investigated by means of the Hoechst H33342 transport assay in MDCKII cells stably expressing mammary bABCG2. Both AFB1 and its main milk metabolite AFM1 showed interaction with bABCG2 even at concentrations approaching the legal limits in feed and food commodities. Moreover, PCB126 significantly enhanced bABCG2 functional activity. Specific inhibitors of either AhR (CH233191) or ABCG2 (Ko143) were able to reverse the PCB 126-induced increase in bABCG2 transport activity, showing the specific up-regulation of the efflux protein by the AhR pathway. The incubation of PCB 126 pre-treated cells with AFM1 was able to substantially reverse such effect, with still unknown mechanism(s). Overall, results from this study point to AFB1 and AFM1 as likely bABCG2 substrates. The PCB 126-dependent increased activity of the transporter could enhance the ABCG2-mediated excretion into dairy milk of chemicals (i.e. drugs and toxins) potentially harmful to neonates and consumers.

Keywords: Bovine ABCG2, Aflatoxins, Aryl hydrocarbon receptor (AhR), PCB 126, MDCKII cells

INTRODUCTION

Aflatoxins (AF), and namely AFB1, B2, G1, and G2, are naturally occurring mycotoxins, produced by fungi of the *Aspergillus flavus* and *A. parasiticus* species, which can contaminate important food and feed commodities, including cereals and oilseeds (Kensler et al., 2011). AFB1 is considered the most important compound in terms of prevalence and toxicity for both humans and farm animals, as it causes growth stunting, immunosuppression, mutagenicity, genotoxicity, and increases the incidence of hepatocellular carcinoma (Abbès et al., 2010; Theumer et al., 2010). The effects of AFB1 are closely linked to the CYP1A/3A-mediated metabolism yielding a highly reactive intermediate, AFB1-exo-8,9-epoxide (AFBO) (Guengerich et al., 2001), which may form adducts with DNA and proteins (Mary et al., 2012). The CYP1A-related biotransformation of AFB1 can also generate AFM1, a hydroxylated metabolite that can be found in milk and thereof products when lactating animals are fed with contaminated feedstuffs (Prandini et al. 2009). Despite AFM1 is considered less toxic than the parent compound, AF as a group (AFB1, AFB2, AFG1, AFG2 and AFM1) are classified as class 1 carcinogens by the International Agency for Research on Cancer (IARC, 2012a). To protect consumer's health, very low limits have been set for AFM1 in the EU (0.050 µg/kg in dairy milk, lowered to 0.025 µg/kg in infant formulae) (EC Regulation 1881/2006). Another important AFB1 metabolite is aflatoxicol (AFL), which results from the NADPH-dependent reduction of the cyclopentanone carbonyl of AFB1. Being easily re-oxidised to AFB1, it plays an important role in prolonging the lifetime of the mycotoxin in the body acting as an AFB1 reservoir (Karabulut et al., 2014). It is worth noting that, even if AFL is considered much less toxic than AFB1, in rainbow trouts it displayed an *in vivo* carcinogenic potency similar to AFB1 (Bailey et al., 1994). Finally, AFL levels of the same order of magnitude of AFM1 have been detected in retail dairy milk (Carvajal et al.,

2003); however, no legal limits in food commodities are currently set for AFL in EU countries.

The carrier protein ABCG2, also referred to as breast cancer resistance protein (BCRP), belongs to the ATP-binding cassette (ABC) family of efflux transporters (Doyle et al., 1998). It is expressed at the apical surface of polarized cells in several tissues, showing high levels in liver, small intestine, central nervous system and the mammary gland of humans and mice (Krishnamurthy and Schuetz, 2006). In ruminants (cow, sheep and goat), ABCG2 is mostly expressed in jejunum and the mammary gland but is also present in liver and, to a lesser extent, in colon, kidney and lung (Lindner et al., 2013; Zancanella et al., 2013). The relatively broad substrate specificity and the localisation in many tissue barriers are generally associated with the role of ABCG2 in reducing the oral bioavailability and/or limiting the distribution of (toxic) chemicals into critical districts of the body. In the mammary gland, ABCG2 expression is limited to the apical membrane of alveolar epithelial cells and it is strongly upregulated during late pregnancy and lactation in humans, mice (Jonker et al., 2005) and farm animals (cows, sheep and goats) (Lindner et al., 2013). Several studies have highlighted the involvement of ABCG2 in the active transport of various drugs (e.g. fluoroquinolone antibiotics and benzimidazole anthelmintics) and toxins (e.g. the food derived carcinogenic heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, PhIP), across the blood-milk barrier, posing a potential health risk to breast-fed infants and dairy consumers (Pulido et al., 2006; Wassermann et al., 2013a; Wassermann et al., 2013b).

It should also be noted that ABCG2 expression, and thus activity, can be transcriptionally induced via the AhR signalling pathway, as it has been demonstrated by *in vitro* studies performed in cells of human or bovine origin upon 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) incubation (Tan et al., 2010; Halwachs et al., 2013). Interestingly, the non AhR-activating compound, PCB 101, had no significant effect on caprine ABCG2 efflux activity

(Halwachs et al., 2014). Thus, the repeated exposure to AhR-ligands (e.g. PCDDs, PCDFs, and dioxin-like (DL) PCBs, collectively referred to as DL-compounds) could have the potential to enhance the ABCG2-mediated xenobiotic excretion into the milk, resulting in the increased presence of xenobiotics in dairy products. Of note, DL-compounds themselves are excreted through the mammary gland (Rychen et al., 2008), dairy products representing one of the most important source of exposure to such pollutants (Rauscher-Gabernig et al., 2013; Pizarro-Aránguiz et al., 2015). Among the different DL-congeners, several studies indicate that PCB 126 is one of the major contributors to the dioxin contamination of dairy milk (Focant et al., 2003; Turrio-Baldassarri et al., 2009; Bertocchi et al., 2015).

Although a previous *in vitro* study has demonstrated the involvement of both murine and human ABCG2 in the transport of AFB1 (van Herwaarden et al., 2006), to the best of our knowledge neither similar studies have been conducted with bovine ABCG2, nor they have included AFB1 metabolites like AFM1 and AFL. The aim of the present study was to investigate the interaction between bovine ABCG2 and the above mentioned AF, as well as the regulation of the transporter by the AhR agonist PCB 126. In order to adequately reproduce the active excretion of xenobiotics by the bovine mammary gland, primary mammary epithelial cells may best reflect the *in vivo* situation. However, the applicability of primary cells for regulation studies is limited by their constricted life span and differences in stability of cytochrome P450 (CYP) expression resulting in a heterogeneous phenotype (Hewitt et al., 2007). Moreover, primary cell cultures do not allow cost-effective and efficient analysis of ABCG2 transport activity (Hegedus et al., 2009). Therefore, as an experimental model we used the recently generated MDCKII cells stably expressing full-length bovine ABCG2 (bABCG2) from lactating mammary gland tissue in conjunction with the Hoechst H33342 accumulation assay (Wassermann et al., 2013a). The MDCKII cell line represents a well-established *in vitro* tool for the study of ABCG2-mediated substrate transport, especially

in the form of a polarized tight cell layer that allows the performance of efflux pump assays (Hegedus et al., 2009). MDCKII cells transfected with ruminant ABCG2 have already been used to identify substrates of this efflux protein (Real et al., 2001; Wasserman et al. 2103b; Gonzalez-Lobato et al., 2014). In addition, some of the Authors of the present study recently demonstrated the comparable inducibility of the AhR signalling pathway by TCDD in the caprine MDCKII-ABCG2 cell line and in primary bovine mammary epithelial cells as well (Halwachs et al., 2013; Halwachs et al., 2014). Thus, although the Hoechst accumulation assay does not distinguish between ABCG2substrates and inhibitors, our model is suitable for the study of ABCG2-drug interactions as well as of AhR-dependent regulatory mechanisms in bovine mammary epithelial cells.

MATERIALS AND METHODS

Chemicals

All chemicals, including Ko143, CH223191, media and supplements, were obtained from Sigma-Aldrich at analytical grade (Deisenhofen, Germany). PCB 126 was purchased from VWR (Darmstadt, Germany). AFB1, AFM1 and AFL were purchased from Apollo Scientific (Bredbury, United Kingdom), bisbenzimide Hoechst H33342 was purchased from AppliChem (Darmstadt, Germany). The purity of PCB126, AFB1, AFM1 and AFL was greater than 99% as indicated by HPLC analysis.

Cell culture

Madin-Darby canine kidney (MDCKII) cells stably expressing full-length bovine ABCG2 (bABCG2) as well as Mock-transfected control cells (MDCKII-Mock) lacking ruminant ABCG2 expression were generated as described (Wassermann et al., 2013a). The cells were

maintained in Dulbecco's modified Eagle's medium/Ham's F12 supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 100 U/mL penicillin and 100µg/mL streptomycin in a humidified incubator with 5% CO_2 at 37°C.

WST-1 cytotoxicity assay

Cytotoxicity of all tested substances was investigated using WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay (Roche Applied Science, Mannheim, Germany), as previously described (Halwachs et al., 2014). In brief, MDCKII cells were seeded at a density of 1.2 x 10^4 cells in 96-well culture plates and were incubated with increasing concentrations of AF or PCB 126 for 4 h or 24 hours, respectively. Triton X-100 (0.1%) was used as a positive control. WST-1 reagent [5% (v/v) in supplemented medium] was added and cell viability was measured as the increase in total absorption at 450 nm over 1 h using a microplate reader (Tecan Genios, Crailsheim, Germany).

MDCKII-bABCG2 and MDCKII-Mock cells treatment

Stock solutions of PCB 126, AFB1, AFM1 and AFL were prepared in DMSO. Final working concentrations for all compounds and the solvent control were prepared in culture medium and DMSO concentration did not exceed 0.5% (v/v). All the experiments were performed in six replicates on MDCKII-bABCG2 and MDCKII-Mock (used as reference) cells, seeded into 96-well culture plates (4 x 10^4) and grown to subconfluency over 48 hours. To investigate the interaction of AF with mammary bABCG2, cells were incubated for 4 hours with increasing concentrations of AFB1, AFM1 or AFL (0.15 nM, 1.5 nM, 15 nM, 150 nM) or the solvent alone . The lowest tested concentration was selected based on the legal limit for AFM1 in milk (EC Regulation 1881/2006) (see the concluding remarks for further explanation). To test the effect of PCB 126 on bABCG2 transport activity, cells were incubated for 24 hours with different concentrations of PCB 126~~, starting from that approximating the legal limit for the~~

~~sum of DL-PCB and dioxins in milk~~ (0.01 nM, 0.1 nM, 1 nM, 10 nM) ~~(EC Regulation 1259/2011)~~. In addition, the involvement of AhR in regulating bABCG2 transport activity was studied by the pre-incubation of cells for 12 hours with the specific AhR inhibitor CH 223191 (10μM), followed by the incubation of cells for 24 hours with PCB 126 (10 nM). The specificity of ABCG2-mediated cellular H33342 excretion was verified incubating cells with a specific ABCG2 inhibitor (Ko143, 5μM) (Allen et al., 2002) for 4 h after PCB126 treatment (24 hours). Finally, to assess the modulation of bABCG2-AFM1 interaction by PCB 126, cells were pre-incubated for 24 hours with PCB 126 (10 nM); then cell culture medium was replaced and supplemented with AFM1 (0.15 nM) for 4 hours.

Hoechst H33342 accumulation assay

The functional ABCG2 activity was investigated with the Hoechst H33342 accumulation assay. After entering the cell by passive diffusion, the dye is actively secreted through efflux transporters, including ABCG2. ABCG2-interacting molecules can be generally identified through their inhibition of cellular H33342 secretion (Hegedus et al., 2009). After MDCKII-bABCG2 and MDCKII-Mock cells were treated with AF or AFM1 following PCB216 pre-incubation at the previously described conditions, H33342 stain (10μM) was added and its accumulation was measured after 20 minutes, at 37 °C and 5% CO_2. To evaluate the modulation of bABCG2 activity by PCB 126, H33342 (5μM) accumulation was determined at different time points (15, 30, 45, 60 min). The measurement of H33342 (5μM) accumulation in the presence of the specific inhibitors (i.e. CH 223191 or Ko143) was performed after 30 minutes.

Subsequently, cells were washed twice with ice-cold PBS and lysed with 0.1% SDS in PBS. The intracellular fluorescence was measured by means of a fluorescence microplate reader (360/465 nm). Total cell protein concentration was determined with the bicinchoninic

(BCA) protein assay (Pierce, Rockford, IL, USA) and intracellular Hoechst H33342 accumulation was calculated as fluorescence units (RFU) per mg protein. The functional efflux activity of bABCG2 was expressed as relative H33342 accumulation, defined as the ratio of MDCKII-bABCG2 cells compared to that determined in MDCKII-Mock reference cells (Wassermann et al., 2013a).

Statistical analysis

To enable statistical analysis, all the experiments were performed independently three times. Data analysis was performed with Microsoft Excel software (Office 2010 suite). Outliers in the respective test series were removed by means of the Grubb's test. Since all data were normally distributed according to the D'Agostino & Pearson normality omnibus test, differences between mean values were determined by one-way ANOVA followed by a Tukey's Multiple Comparison test using GraphPad Prism software (4.03 version, La Jolla, CA). Statistical significance was assumed at P values of ≤ 0.05.

RESULTS

Effect of AFB1 and its metabolites on Hoechst H33342 accumulation in MDCKII-bABCG2 cells

The effects of AFB1 and its metabolites, AFM1 and AFL, on the efflux activity of mammary bABCG2 were examined by the Hoechst H33342 accumulation assay following cell incubation with concentrations ranging from 0.15 to 150 nM of each compound for 4 h. Before initiation of transport experiments, the cytotoxicity of the different test substances in MDCKII cultures was determined by the WST-1 assay. For each compound, at each tested concentration, cell viability remained unaffected compared to both untreated and DMSO

control cells (Fig. 1A). Based on the results of previous studies reporting the maximum time of fluorescent dye uptake (Wassermann et al., 2013a), the effects of AFB1, AFM1 or AFL on H33342 accumulation in MDCKI-bABCG2 cells were measured 20 minutes after dye supplementation. As depicted in Fig. 2, AFB1 and AFM1 interact with mammary bABCG2, causing a statistically significant inhibition of H33342 excretion in MDCKI-bABCG2 cells compared to control cells (Fig. 2). The enhancement of H33342 intracellular permanence was already evident at the lowest tested concentrations (0.15 nM)~~, which for AFM1 approximates those found in milk under field conditions~~. However, no concentration-dependent effect could be observed with either compound, with the exception of AFB1 at 150 nM, which induced a more than two-fold increase of intracellular dye levels. Conversely, a limited (up to 35%) but not statistically significant increase in H33342 accumulation was detected in AFL-treated cells.

Effect of PCB 126 on Hoechst H33342 accumulation in MDCKII-bABCG2 cells

To investigate the effects of the AhR signalling pathway activation by PCB 126 on the functional activity of bABCG2, MDCKII-bABCG2 cells were incubated with PCB 126 at concentrations ranging from 0.01 to 10nM for 24 h, a time-point that resulted in the highest induction rate of CYP1A1 transcription in a bovine mammary epithelial cell line (BME-UV1) (Girolami et al., 2015). PCB 126 at the used concentrations did not impair cell viability, as assessed by the WST-1 assay (Fig. 1B). Based on the results from a previous study performed with TCDD using a similar *in vitro* model expressing caprine ABCG2 (Halwachs et al., 2014), fluorescence dye concentration was measured in MDCKII-bABCG2 cells at 5, 15, 30, 45 and 60 minutes. PCB 126 caused a statistically significant reduction in H33342 levels at all tested concentrations starting from 30 minutes after fluorescence dye supplementation; such an effect was apparently concentration-unrelated (Fig. 3). The involvement of the AhR

signalling cascade in the regulation of mammary bABCG2 transport activity was then investigated by means of a specific AhR inhibitor (CH223191). As depicted in Figure 4, in PCB 126-incubated cells and supplemented with CH223191, H33342 levels were comparable to those of untreated controls, as a consequence of the impairment of PCB 126-mediated AhR signalling pathway activation. Moreover, the specificity of bABCG2-mediated H33342 transport was determined using a specific ABCG2 inhibitor (Ko143). In MDCKII-bABCG2 cells incubated with PCB 126, cellular H33342 excretion was significantly inhibited by Ko143 and resulted in steady-state substrate levels similar to that observed in Ko143-treated control cells. (Fig. 4).

Effect of AFM1 on Hoechst H33342 accumulation in MDCKII-bABCG2 cells after pre-incubation with PCB 126

To further investigate PCB 126-mediated induction of bABCG2, we tested the effect of AFM1, the main milk AFB1 metabolite, on bABCG2 secretory activity in PCB 126 pre-treated MDCKII-bABCG2 cells. To this end, cells were pre-incubated with 10 nM PCB 126 for 24 hours and then treated with AFM1 (0.15 or 150 nM) for 4 hours. bABCG2-mediated H33342 efflux was measured after 20 minutes from dye supplementation. Results are depicted in Fig. 5. Pre-treatment with the AhR-ligand PCB 126 elicited a significant decrease in the intracellular H33342 levels compared to the vehicle control due to an enhanced cellular excretion of the Hoechst dye by bABCG2. In line with the results of the experiments described above (Fig. 2), both AFM1 concentrations caused a significant increase in intracellular H33342 indicating a competitive inhibition of bABCG2-mediated H33342 efflux by AFM1. Interestingly, AFM1 counteracted the stimulatory effect of PCB 126 on bABCG2 efflux activity, resulting in H33342 levels higher than those recorded in cells exposed to PCB 126 alone and similar to those of the control cells.

DISCUSSION

Recent research performed with the same MDCKII-bABCG2 cell culture model used in the present study identified several potential bABCG2 substrates, including antibiotic (i.e. enrofloxacin) and non-antibiotics (i.e. albendazole) veterinary drugs (Wassermann et al., 2013a). Our results show for the first time the interaction of mammary bABCG2 with AFB1 and its metabolite, AFM1, suggesting that both could be substrates of the efflux pump. The lack of a substantial concentration dependent-effect with both compounds might be explained by the limited concentration range used. ~~However, the aim of the present study was primarily to elucidate if AFB1 and its metabolites could be substrates of bABCG2 in the mammary gland under conditions reflecting the *in vivo* exposure of dairy cows. Hence, the selected concentrations were in the range of the maximum content of AFB1 and AFM1 in feed and food, respectively~~. A dye excretion trend similar to that recorded for AFB1 or AFM1 was observed upon the incubation with AFL, yet results did not reach the statistical significance. This is difficult to explain. In general, ABCG2 mediates the transport of hydrophobic molecules (Krishnamurthy and Schuetz, 2006), which may certainly be the case for AFB1 but presumably not for the more hydrophilic AFM1 and AFL, which in our study showed a remarkably different degree of interaction with the efflux protein. However, numerous studies showed that ABCG2 represents a cellular efflux pump with a very broad substrate spectrum and even among structurally-related compounds, some may be substrates while other not (see Szafraniec et al., 2014 for a review).

To the best of our knowledge, the only available information about ABCG2-mediated transport of AF regards the corresponding murine and human proteins (i.e. Bcrp1, and BCRP, respectively). Using a transwell transport assay based on polarized MDCKII cells transfected with Bcrp1 or BCRP, van Herwaarden and collaborators (2006) demonstrated that AFB1 is a substrate of both transporters, while data about AFM1 are lacking. In the same study, the

comparison of milk and plasma AFB1 concentrations between wild-type and Bcrp1$^{-/-}$ mice after the intravenous administration of the mycotoxin proved the involvement of Bcrp1 in the *in vivo* mammary excretion of AFB1. Nevertheless, despite being a highly conserved transporter (Wu et al. 2008), an increasing number of studies describe differences in expression, function and specificity of ABCG2 in different species, including ruminants (Schnepf and Zolk, 2013; Wassermann et al., 2013a; Lewis et al., 2015). Thus, the use of an *in vitro* model expressing the mammary bovine isoform allowed to test the species- and tissue-specific affinity and the likelihood of active secretion of very low levels ~~(approximating the maximum permitted content)~~ of AFM1 into dairy milk.

It has been previously demonstrated that AhR ligands such as TCDD and prochloraz, beside inducing several phase I and phase II xenobiotic metabolizing enzymes (XME) (Rudzok et al., 2009; Abel and Haarmann-Stemmann, 2010), increase bovine and caprine ABCG2 expression and activity, resulting in a reduction of H33342 intracellular accumulation (Halwachs et al., 2013; Halwachs et al. 2014). However, no specific data have been reported so far about the induction of bABCG2 by PCB 126, which represents the most important DL-compound in the contamination of dairy milk, being characterized by the highest carry-over and the slowest elimination rate in exposed cows (Huwe and Smith, 2005). From the food safety viewpoint, it should be also noted that PCB 126 has been recently classified as a Group 1 human carcinogen (IARC, 2012b), along with TCDD. Our results show that pre-treatment of MDCKII-bABCG2 cells with PCB 126 for 24 hours resulted in an increased bABCG2-mediated excretion of Hoechst H33342. The Hoechst dye has been described also as a substrate for the ABCB1 (P-gp) efflux transporter (Sarver et al., 2002), and it has been reported that TCDD increases P-gp expression and activity in rat brain capillaries (Wang et al., 2011). However, the specific ABCG2 inhibitor Ko143 significantly depressed ABCG2 efflux activity in PCB126-treated MDCKII-bABCG2 cells and resulted in substrate levels

similar to Ko143-incubated control cells, as in the MDCK-ABCG2 model expressing the caprine isoform (Halwachs et al., 2014). Thus, the observed effect of PCB 126 on H33342 excretion can be clearly attributed to the induction of mammary bABCG2 efflux activity. Similarly, the AhR inhibitor CH 223191 blocked the inducing effect of PCB 126 on bABCG2 transport, supporting the AhR dependence of the PCB-induced regulatory mechanism. Such an effect has been reported in human intestinal Caco-2 cells, where the AhR inhibitor alpha-naphtoflavone reversed the stimulatory effect of AhR inducer beta-naphtoflavone on ABCG2 efflux activity (Buesen et al., 2003; Ebert et al., 2005). Moreover, phenobarbital, another general XME inducer, failed to enhance the expression of ABCG2 and other efflux proteins in liver and in several extrahepatic organs from *in vivo* treated cattle (Zancanella et al., 2013).

Interestingly, in the present study the incubation of MDCKII-bABCG2 cells with AFM1 was able to counteract the stimulatory effect of PCB 126 on bABCG2 efflux activity. Although the mechanism(s) of such an effect remain(s) to be established, it can be hypothesised that AFM1 may compete with the Hoechst dye in the PCB 126 pre-incubated MDCKII-bABCG2 cells, thereby decreasing the ABCG2-mediated H33342 excretion to levels similar to untreated control cells. This would further support the potential role of AFM1 as a substrate of mammary bABCG2.

Overall, although caution is needed in extrapolating data from *in vitro* studies to field conditions, our findings point to bovine ABCG2 as a transport protein for both AFB1 and AFM1, even at concentrations in the range of the maximum content of AFB1 (5 µg/Kg) in feed (EC Regulation 574/2011) and AFM1 (0,05 µg/Kg) in dairy milk (EC Regulation 165/2010). Taking into account the high expression of ABCG2 in bovine mammary gland during lactation (Jonker et al., 2005; Lindner et al., 2013), such results provide new insight about the mechanism of AFM1 mammary excretion in dairy cows. Moreover, it is suggested that ABCG2 may be also important in the absorption/bioavailability of AFB1 in the bovine

species, due to the relatively high enteric expression of ABCG2 in cattle (Lindner at al., 2013; Zancanella et al., 2013). The protective role of Bcrp1 in limiting AFB1 systemic exposure has been already demonstrated in mice, where wild-type animals showed lower plasma levels after the oral administration of AFB1 compared to Bcrp1$^{-/-}$ mice (van Herwaarden et al., 2006). As the Hoechst transport assay does not distinguish between ABCG2 substrates and inhibitors (Hegedus et al., 2009), our results should be further validated by efflux studies using polarized cell models overexpressing the bovine transport protein (Real et al., 2011; Wasserman et al., 2013b).

Taking into account the importance of ABCG2 in the active transport of a wide array of xenobiotics in milk (Robey et al., 2009), it is anticipated that the upregulation of the bovine isoform driven by PCB 126, even at concentrations approaching the legal limits in feed (EC Regulation 277/2012) and food (EC Regulation 1259/2011), may cause a significant enhancement of the mammary excretion of several toxic substances (e.g. AF metabolites, primarily AFM1) and veterinary drugs in DL-compound exposed animals. This scenario would assume a remarkable relevance both from a clinical point of view, increasing the exposure risk for suckling calves, and in the field of food safety, potentially affecting milk and tissue residue levels of xenobiotics.

ACKNOWLEDGMENTS

This project was supported by the University of Torino: Ricerca Finanziata dall'Università (ex 60%) 2014 - Ruolo della proteina di efflusso ABCG2 bovina nell'escrezione mammaria dell'Aflatossina B1 e modulazione indotta dai composti diossino-simili. Part of the results of the study have been the subject of an oral presentation at the 13th EAVPT Congress in Nantes

and published as a short abstract in the Proceedings (*Journal of Veterinary Pharmacology and Therapeutics*, 38, Suppl. 1, 50.).

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FIGURE LEGENDS

Fig. 1. Cell viability of MDCKII cells determined by the WST-1 assay, after incubation with increasing concentrations of AF for 4 hours (A) or PCB126 for 24 hours (B). Triton X-100 was used as a positive control. Results are expressed as percentage of untreated control cells (mean ± SEM, n =18).

Fig. 2. Effect of AFB1, AFM1 and AFL on Hoechst H33342 accumulation in MDCKII-bABCG2 cells. Relative efflux activity of bABCG2 was defined as the ratio in H33342 accumulation of MDCKII-bABCG2 cells compared to that determined in MDCKII-Mock cells, and expressed as RFU/mg protein. The results represent mean ± SEM (n =18; *P < 0.05 or less compared to DMSO control cells; $^{\#}$P< 0.05 or less compared to other tested concentrations).

Fig. 3. Effect of PCB 126 on bABCG2 transport activity by measuring Hoechst H33342 accumulation over 60 minutes in MDCKII-bABCG2. Relative efflux activity of bABCG2 was defined as the ratio in H33342 accumulation of MDCKII-bABCG2 cells compared to that determined in MDCKII-Mock cells, and expressed as RFU/mg protein. The results represent mean ± SEM (n = 18, *P < 0.05 or less).

Fig. 4. Effect of specific ABCG2 (Ko143) and AhR inhibitors (CH223191) on PCB 126-induced bABCG2 efflux activity. Relative efflux activity of bABCG2 was measured as RFU/mg protein, and defined as the ratio in H33342 accumulation of MDCKII-bABCG2 cells compared to that determined in MDCKII-Mock cells. The results represent fold change

compared to DMSO control cells (mean ± SEM, n = 18, *P < 0.05 or less compared to DMSO control cells; #P< 0.05 or less compared to PCB 126-treated cells).

Fig. 5. Accumulation of Hoechst H33342 in MDCKII-bABCG2 cells pre-incubated for 24h with PCB 126 and then incubated in the presence or absence of AFM1. Relative efflux activity of bABCG2 was defined as the ratio in H33342 accumulation of MDCKII-bABCG2 cells compared to that determined in MDCKII-Mock cells, and expressed as RFU/mg protein. The results represent mean ± SEM (n = 18, *P < 0.05 or less compared to DMSO control cells).

Figure 1.

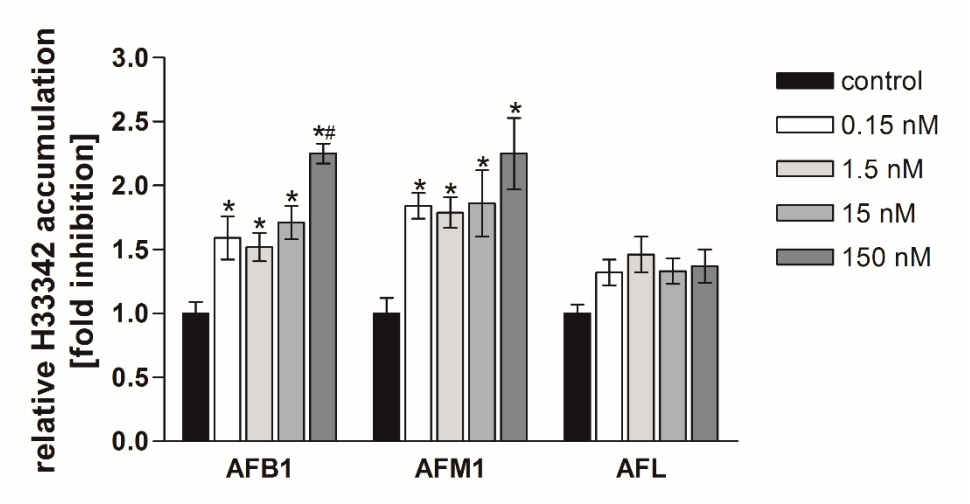


Figure 2.

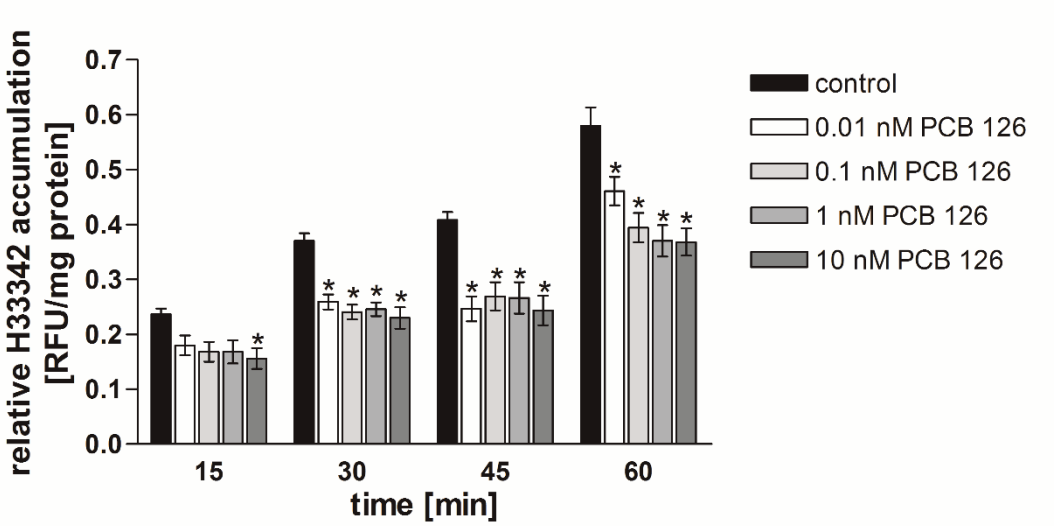


Figure 3.

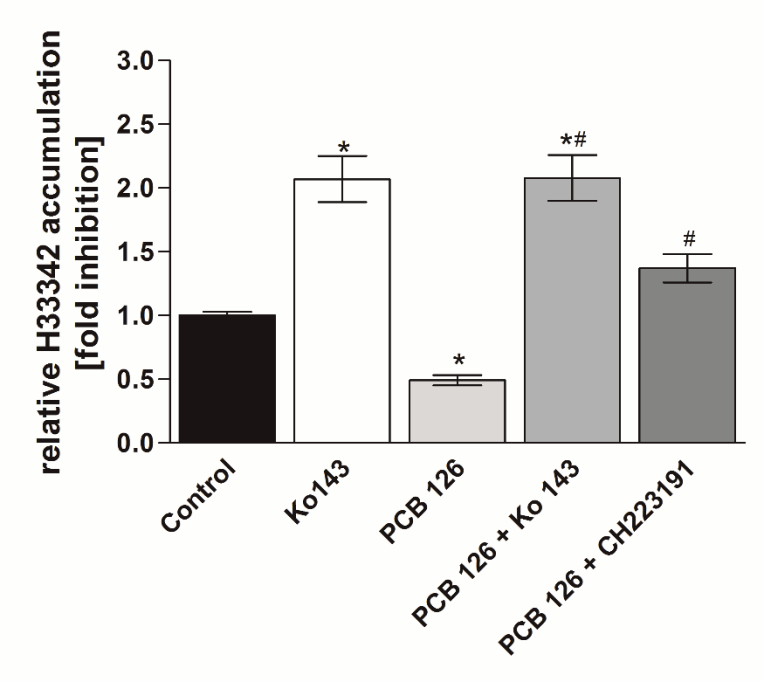


Figure 4.

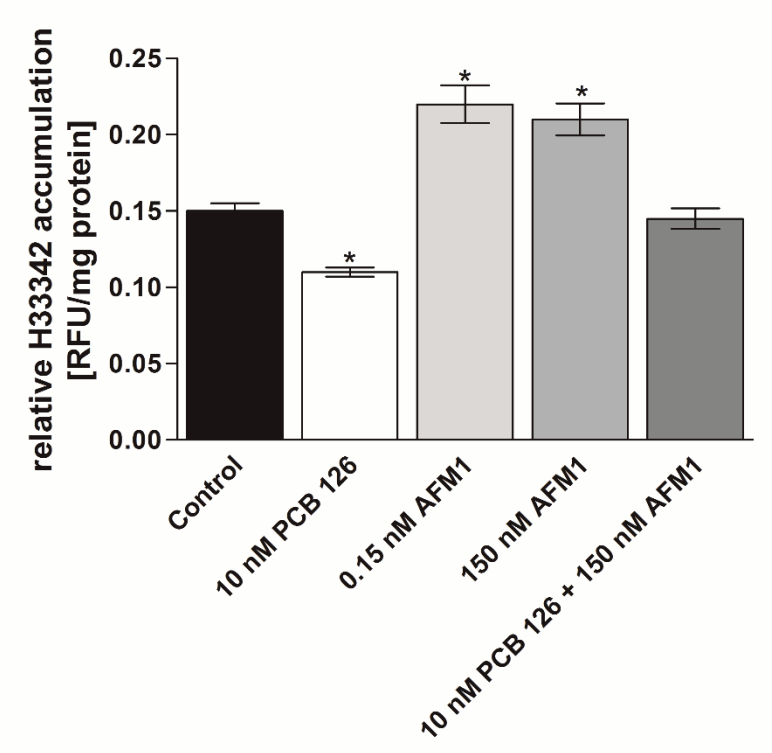
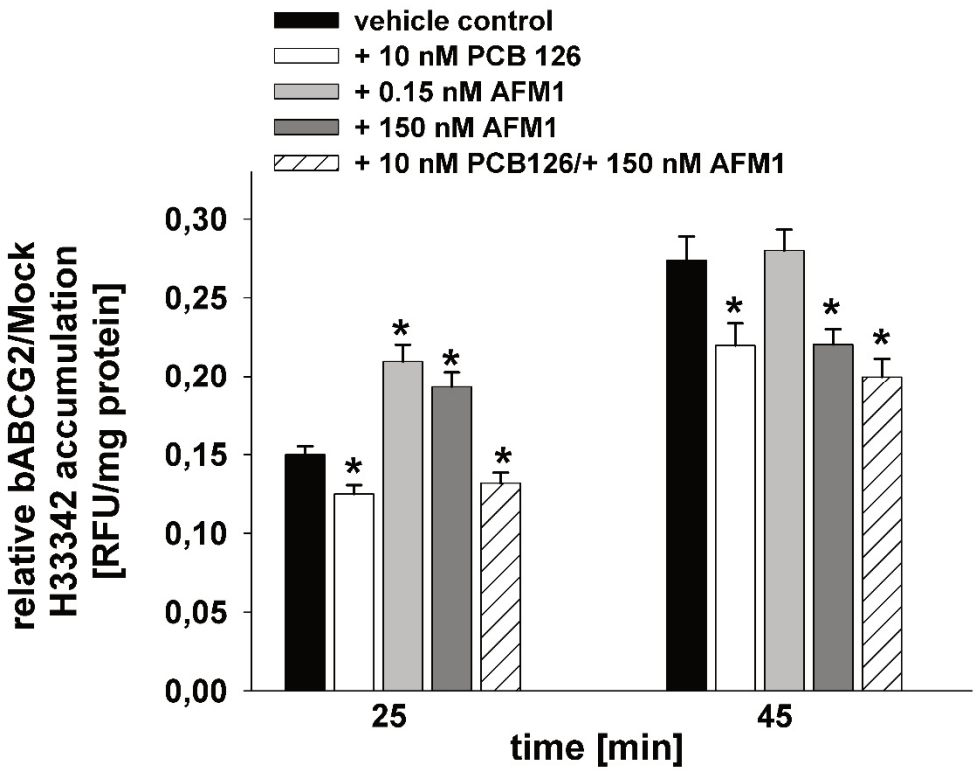


Figure 5.


vehicle control
+ 10 nM PCB 126
+ 0.15 nM AFM1
+ 150 nM AFM1
+ 10 nM PCB126/+ 150 nM AFM1
relative bABCG2/Mock H33342 accumulation [RFU/mg protein]
0,30
0,25
0,20
0,15
0,10
0,05
0,00
*
25
45
time [min]