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Ivermectin induces P-glycoprotein expression and function through mRNA stabilization in murine hepatocyte cell line

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ABSTRACT

Ivermectin is widely used in human and veterinary medicine for the control of helminth infections. Ivermectin is known to interact with P-glycoprotein (P-gp/*MDR1*), being a good substrate and a potent inhibitor, however, the influence of ivermectin on the expression of the transporter has not been investigated. Expression of P-glycoprotein was investigated in cultured mouse hepatocytes acutely exposed to ivermectin. The two P-glycoprotein murine isoforms, *Mdr1a* and *Mdr1b*, mRNA levels were assessed by real-time RT-PCR. Ivermectin induced a clear time- and concentration-dependent up-regulation of *Mdr1a* and *Mdr1b* mRNA levels (as early as a 12-h exposure and up to 2.5-fold at 10 μM). Moreover, ivermectin-treated cells displayed enhanced cellular efflux of the P-glycoprotein substrate calcein that was inhibited by the P-glycoprotein blocker valspodar, providing evidence that the ivermectin-induced P-glycoprotein was functional. The mechanisms underlying these effects were investigated. Ivermectin-mediated *Mdr1* mRNA induction was independent of the two nuclear receptors CAR and PXR, which are known to be involved in drug transporters regulation. Moreover, by using reporter cell lines that detects specific ligand-activated transcription factors, we showed that ivermectin did not displayed CAR, PXR or AhR ligand activities. However, studies with actinomycin D revealed that the half-life of *Mdr1a* and *Mdr1b* mRNA were significantly prolonged by two-fold in ivermectin-treated cells suggesting a post-transcriptional mode of ivermectin regulation. This study demonstrates for the first time that ivermectin induces P-glycoprotein overexpression through post-transcriptional mRNA stabilization, thus offering insight into the mechanism of reduced therapeutic efficacy and development of ivermectin-resistant parasites.

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1. Introduction

Macrocyclic lactones (MLs), such as ivermectin, are a large family of broad spectrum antiparasitic drugs intensively used worldwide in livestock to treat diseases caused by gastrointestinal nematodes and external parasites. In humans, ivermectin is used through mass drug administration programs for the treatment of onchocerciasis, a tropical parasitic disease caused by the filarial nematode *Onchocerca volvulus*. MLs resistance has become a worsening problem in veterinary medicine and recent reports suggest that human ivermectin-resistant parasites are emerging, thus threatening the sustainable efficacy of this drug [1].

P-glycoprotein (P-gp, *MDR1*/*ABCB1*) is a plasma membrane protein belonging to the ATP-binding cassette (ABC) transporters family. This transporter is an ATP-dependent efflux pump that reduces the intracellular concentration of a broad range of hydrophobic compounds and is involved in the multidrug resistance (MDR) [2]. The activity of efflux ABC transporters can be subject to regulation by inhibition or induction and considerable evidence has accumulated to indicate that *MDR* gene expression can be rapidly and transiently induced following acute exposure to some xenobiotics [3,4]. The regulation of ABC transporters occurs by transcriptional or post-transcriptional events. At transcriptional level, the constitutive androstane receptor (CAR, NR1I3) and the pregnane X receptor (PXR, SXR, NR1I2), two nuclear receptors primarily expressed in the liver, are

Abbreviations: ABC, ATP-binding cassette; AhR, aryl-hydrocarbon receptor; CAR, constitutive androstane receptor; IVM, ivermectin; MLs, macrocyclic lactones; P-gp, P-glycoprotein; PXR, pregnane X receptor.

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now both widely recognized to play the most important role in the regulation of *MDR1* [5–7]. Other specific ligand-activated transcription factors have been implicated in regulation of drug transporters expression such as aryl-hydrocarbon receptor (AhR) [8]. Apart from transcriptional regulation, overexpression of P-gp has also been shown to be controlled partially through post-transcriptional mechanisms, such as mRNA stability alteration [9,10].

P-gp has been clearly identified as the main factor that controls the concentration of ivermectin by affecting its absorption, distribution, and elimination in the host [11,12], but also its neurotoxicity by limiting its penetration in the brain [13]. Despite the key role of P-gp in metabolism, toxicity, efficacy and drug–drug interactions of many other xenobiotics [14], the question of whether ivermectin can affect the expression levels of P-gp has not been addressed. Several arguments from the literature imply that ivermectin may modulate P-gp expression. (i) Many P-gp function modulators are often found to influence the expression of the transporter in cells [15], and ivermectin is reported as good substrate and potent inhibitor of P-gp [16,17]. (ii) Ivermectin was previously shown to induce the expression and activity of cytochrome P450 isoenzymes, including CYP1A, 2B and 3A subfamilies in vivo [18,19], suggesting that ivermectin could interact with cellular transcription factors such as AhR, CAR and PXR which are involved in chemical induction of these cytochromes [20]. Since several studies have shown that the biotransformation systems (phase I/II) and efflux proteins are regulated by the same network of transcription factors, one can speculate that ivermectin may also regulate ABC efflux transporters expression. (iii) P-gp homologs expression levels were shown to be increased in ivermectin-resistant nematodes [21–23].

In this context, this study aimed at investigating P-gp regulation in response to acute ivermectin treatment. Hepatocytes were chosen as a suitable in vitro model system for studying the potential of xenobiotics to modulate P-gp gene expression [24] and elucidating the role of specific transcription factors in the regulation of gene involved in the metabolism of xenobiotics [25,26]. The objectives were to evaluate the ability of ivermectin to modulate P-gp gene expression, to determine the functional significance of this modulation and to further establish molecular mechanisms underlying this effect. Our finding demonstrate for the first time that acute exposure to ivermectin led to over-expression of functional P-gp in mouse liver cells through increased stability of mRNA in the cell.

2. Materials and methods

2.1. Materials

1,4-Bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), pregnenolone 16α-carbonitrile (PCN), dimethylsulfoxide (DMSO), T0901317, geneticin (G418), Triton X-100, calcein-AM, actinomycin D (Act-D) and ivermectin were purchased from Sigma–Aldrich Chimie (St Quentin Fallavier, France). Luciferin was from Promega (Charbonnières-les-bains, France). Valspodar (VP) was a generous gift from Novartis (Basel, Switzerland). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, dioxin) was purchased from Promochem (Wesel, Germany). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum (FBS), glutamine, penicillin–streptomycin solution (10,000 units/ml penicillin and 10,000 μg/ml of streptomycin), trypsin–EDTA solution (0.05% trypsin, 0.53 mM EDTA) and lipofectamine 2000 were obtained from Invitrogen – Life Technologies (Cergy Pontoise, France). Tissue culture plastic flasks and culture plates were supplied by Sarstedt (Orsay, France). All other chemicals were obtained from Sigma–Aldrich, unless otherwise stated.

2.2. Cell culture and treatment

JWZ murine hepatic cells, also referred to as MuSH immortalized hepatocytes [27], were kindly provided by Dr. J.P. Gray (Pennsylvania, USA). These cells expressed standard levels of Retinoid X Receptor (RXR), the heterodimeric partner of the nuclear receptors CAR and PXR, while CAR and PXR transcript are barely detectable, providing a useful tool for mechanistic examination of the role of nuclear receptors in gene regulation, as previously described [27,28]. JWZ cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin, 100 μg/ml streptomycin. Cells were kept at 34 °C in 5% CO_2 and 95% humidity. Every 3–4 days, cells were trypsinized and seeded at 5×10^5 cells per 6-well cluster trays for induction studies. The cells were grown at more than 80% confluence and exposed to ivermectin or different compounds of interest at various concentrations or to the vehicle for 6, 12, 24, 48 or 72 h. All drugs were dissolved in DMSO at a final concentration of 0.1% (v/v).

2.3. Cytotoxicity assays

2.3.1. Cell viability: the MTS-tetrazolium salt assay

The cell viability in presence of ivermectin was evaluated using the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Charbonnières-les-bains, France). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay is based on the ability of viable cells to convert a soluble tetrazolium salt to a formazan product. JWZ cells were seeded at 5×10^4 cells in 96-well culture plates. 24 h after seeding, increasing concentrations of ivermectin were added; media and DMSO were used as negative and positive controls, respectively. JWZ cells were further incubated at 34 °C for 24, 48 or 72 h. Thereafter, 20 μl of the MTS/PMS mixture (MTS/PMS ratio: 20:1) was added to each well and plates were incubated at 34 °C for 4 h. At the end of incubation period, absorbance was recorded at 492 nm using a multiwell-scanning spectrophotometer.

2.3.2. Release of lactate dehydrogenase

The effect of ivermectin upon cell integrity was also determined by measurement of lactate dehydrogenase (LDH)-release as a marker of cell membrane damage using the Cytotoxicity Detection Kit (Roche Applied Science, Meylan, France). JWZ cells grown on 96-well dishes were incubated with increasing concentrations of ivermectin. After 24, 48 or 72 h of exposure 100 μl samples were withdrawn and analyzed for LDH content. In control experiments, cells were incubated with medium or 1% Triton X-100. Afterwards, results were normalized to 0% and 100% LDH release, respectively.

2.4. Functional detection of the P-gp activity (calcein assay)

P-glycoprotein transport activity was assessed following the intracellular accumulation of the P-gp fluorescent substrate calcein, as previously described [29]. Briefly, confluent JWZ cells in 24-well cluster plates were cultured with (treated cells) or without (control cells) 10 μM ivermectin for 48 h. Cells were then incubated with or without the reference P-gp inhibitor valspodar (5 μM) for 40 min. After this pre-incubation period, cells were washed with PBS buffer and 0.5 μM calcein-AM (in DMSO) was added for a 2-h incubation period at 34 °C. Medium was then removed, cells were washed 2 times with ice-cold PBS and a lysis buffer (1% Triton X-100 and SDS 0.1% in PBS) was added. The intracellular fluorescence corresponding to calcein accumulation was measured in cell lysate by using a microplate reader (TECAN, Infinite™ 200, Lyon, France), with a 485 nm excitation wavelength and 530 nm emission filter. Values were normalized to the protein

content per well determined by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Marnes-la-Coquette, France).

2.5. Total RNA isolation and RT-PCR analysis

2.5.1. Isolation of RNA and cDNA synthesis

After two washes with ice-cold PBS, the cells were harvested and total cellular RNA was isolated using Trizol Reagent (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. Total RNA was quantified using nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). RNA purity was checked by measurement of the $A_{260/280\ nm}$ ratio, which was routinely in the range of 1.8–2.0, and RNA integrity was checked upon migration in 1% agarose gel and visualization with ethidium bromide staining and UV irradiation. cDNA was synthesized from 2 μg of total RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems – Life Technologies, Courtaboeuf, France).

2.5.2. Quantification of mRNA expression by RT-PCR

Real-time quantitative PCR (RT-qPCR) was performed using an ABI Prism 7300 Sequence Detection System instrument and software (Applied Biosystems, Courtaboeuf, France). Gene-specific primers for SYBR Green assays are described in Table 1. All primers were designed using Primer Express software version 2.0 (Applied Biosystems) and synthesized by Invitrogen (Cergy Pontoise, France). All primers were entered into the NCBI Blast program to ensure specificity. Results were expressed using the comparative Ct method as described in User Bulletin 2 (Applied Biosystem). Briefly, the ΔC_t values were calculated in every sample for each gene of interest as following: $C_{t\ gene\ of\ interest} - C_{t\ reporter\ gene}$, with TATA-box binding protein (TBP) as the reporter gene. The fold change in the level of target *mRNA* between treated and untreated cells was then expressed as $2^{-\Delta C_t}$ with $\Delta C_t \pm$ S.D. where S.D. is the standard deviation of the mean of the ΔC_t value. A dissociation curve allowed us to verify the specificity of the amplification.

2.6. Transient transfection assay and DNA constructs

The pCR3-mCAR construct expressing the murine CAR was kindly provided by Dr. M. Negishi (North Carolina, USA) and the pSG5-mPXR construct expressing the murine PXR was kindly provided by Dr. J.M. Pascussi (Montpellier, France). These plasmids were described previously [28,30]. JWZ cells (1.5×10^6) were plated onto 10-cm culture dishes and grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 34 °C. 24 h after seeding, cells were transiently transfected with 8 μg/plate of plasmid construct containing the cDNA for mouse CAR (pCR3-mCAR), mouse PXR (pSG5-mPXR), or their control expression vector (pCR3 or pSG5), using lipofectamine 2000 according to the manufacturer's protocol. Cells were transferred to 6-well plates at a density of 5×10^5 cells/well 16 h after transfection. Drug treatments were performed 24 h after transfection.

2.7. Study of the CAR-, PXR- and AhR-activating capacity of ivermectin

2.7.1. Stable reporter cell lines

HG_5LN parental cells, obtained by integration of GAL4RE_5-βGlob-Luc-SVNeo in HeLa cells and containing a stably integrated GAL4-responsive gene, have already been described [31]. mCAR- and mPXR-expressing cells were obtained, as previously described [32,33], by transfecting HG_5LN cells with pSG5-GAL4(DBD)-mPXR(LBD)-puro or pSG5-GAL4(DBD)-mCAR(LBD)-puro, which enables the expression of the DNA binding domain of the DNA binding domain of the yeast activator GAL4 (Met1–Ser147) fused to the ligand binding domain of mCAR or mPXR and confers resistance to puromycin. The rodent AhR-activating capacity was assessed with the H4IIE XRE-LUC cell line, which was obtained by transfecting rat H4IIE hepatocytes cells with CYP1A1-Luc and pSG5-puro plasmids. A similar dioxin reporter cell line, HAhLP, used to assess interactions with human AhR, has previously been described [34].

2.7.2. Cell culture conditions

HG_5LN-mCAR, HG_5LN-mPXR and H4IIE XRE-LUC cell lines were cultured in DMEM F12, supplemented with 5% of fetal calf serum (FCS) and 1% antibiotic (penicillin/streptomycin) in a 5% CO_2 and 95% air-humidified atmosphere at 37 °C. HG_5LN cell medium was supplemented with 1 mg/ml geneticin (G418) and HG_5LN-mPXR and -mCAR cell medium with 1 mg/ml geneticin and 0.5 μg/ml puromycin. To test ivermectin for its mCAR-, mPXR- and rAhR-activating capacity, cells were grown in DMEM F12 supplemented with 5% dextran-coated, charcoal-treated fetal calf serum (DCC-FCS).

2.7.3. Living cell luciferase assay

Reporter cells were seeded at a density of 2.5×10^4 cells per well in 96-well white opaque tissue culture plates (Greiner, France) and grown in 150 μl DCC-FCS. Ivermectin or the reference effector (agonist or antagonist) was added 24 h after seeding and the cells were then incubated for 24 h with the compounds. Various concentrations were tested through serial dilution using an automated workstation (biomek® 2000, Beckman Coulter). To ensure 100% cell viability, ivermectin concentration range used was set from 10^{-8} M to 3.3×10^{-6} M. Each concentration was tested in quadruplicate in at least two experiments which included both negative (solvent) and positive (reference agonist or antagonist) controls. At the end of incubation, medium was removed and replaced with 0.3 mM luciferin-containing culture medium. Intact living cell luminescence was measured for 2 s using a Microbeta Wallac luminometer. Results were expressed as a fold-induction of the basal luciferase activity measured in untreated cells, which was set to 1. Reference agonist ligand was used in HG_5LN-mPXR and H4IIE XRE-LUC cells (PCN 10 μM and TCDD (dioxin) 10 nM, respectively). Due to the high constitutive activity of CAR in HG_5LN-mCAR cells, a reference antagonist ligand was used as control (T0901317 10 μM). All effectors were dissolved in DMSO at 10 mM and then diluted in culture medium.

Table 1
Primer sequences used in quantification of the gene expression by qRT-PCR.

Targeted gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Mdr1a (*Abcb1a*)	CATGACAGATAGCTTTGCAAGTGTAG	GGCAAACATGGCTCTTTTATCG
Mdr1b (*Abcb1b*)	AAGCCAGTATTCTGCCAAGCAT	CTCCAGACTGCTGTTGCTGATG
Cyp3a11	TCACACACACAGTTGTAGGCAGAA	GTTTACGAGTCCCATATCGGTAGAG
Cyp2b10	TTTCTGCCCTTCTCAACAGGAA	ATGGACGTGAAGAAAAGGAACAAC
TBP	ACTTCGTGCAAGAAATGCTGAA	GCAGTTGTCCGTGGCTCTCT

2.8. Determination of Mdr1 mRNAs stability

The half-lives of *Mdr1a* and *Mdr1b* mRNA were determined by an Act-D chase assay, as previously described [35]. JWZ cells were pre-treated with ivermectin (10 μM) or DMSO for 24 h. Thereafter, the transcription inhibitor actinomycin D was added to inhibit further RNA synthesis. Act-D was used at a final concentration of 5 μg/ml for 1 h, then, to avoid Act-D toxicity, medium was replaced and Act-D, in the presence or absence of ivermectin 10 μM, was added at a concentration of 1 μg/ml for the following 24 h. Cells were harvested, at various times (0, 3, 6, 10 and 24 h) after the addition of 1 μg/ml Act-D, total RNA was isolated and levels of *Mdr1a* and *Mdr1b* mRNAs were quantified by RT-qPCR as described above. TBP mRNA levels were also monitored as control. The data were represented as percentage decrease in mRNA levels *versus* time using a semilogarithmic scale. The mRNA half-life values were determined with the use of regression curves.

2.9. Statistical analysis

All experiments were conducted at least in triplicate and results are expressed as mean ± standard deviation (S.D.). Statistical analysis was performed using one-way analysis of variance (ANOVA) with a Tukey post-test to compare the effect of ivermectin over control cells, while individual comparisons between pairs of data were performed using the Mann–Whitney test (GraphPad Instat, San Diego, CA, USA). Statistical significance was accepted as $p < 0.05$.

3. Results

3.1. Effects of ivermectin on cell viability

The aim of these studies was to determine subtoxic concentrations of ivermectin which could be used in the subsequent studies on hepatocytes JWZ cells. The cytotoxicity of ivermectin towards JWZ cells was examined using the MTS assay and LDH release. The dose-dependent viability of JWZ cells treated with ivermectin for 24, 48 or 72 h is presented in Fig. 1. These results showed that ivermectin had cytotoxic effects in a concentration- and time-dependent manner. A 24-h treatment with ivermectin up to 25 μM induced no significant mitochondrial toxicity or cell-growth inhibition towards JWZ cells, as revealed by the MTS assay with more than 90% cell viability; however LDH release around 35% was observed. When JWZ cells were exposed to ivermectin for 72 h at concentration below 12.5 μM, no cytotoxic effects were observed. The effective LD_{50} values (concentration that causes 50% lethality) at 24, 48 and 72 h were determined graphically and were approximately 75, 30 and 20 μM, respectively. Subsequent studies were performed following an ivermectin incubation period up to 72 h and with concentrations up to 10 μM, a time and concentration exposure of ivermectin that gave more than 80% cell viability and moderate LDH release (less than 20%).

3.2. Time-dependent effects of ivermectin on P-glycoprotein mRNA expression

We first analyzed the effects of ivermectin treatment as a function of time on the expression of the 2 genes encoding the murine P-glycoprotein, *Mdr1a* and *Mdr1b* (*Abcb1a* and *Abcb1b*) in JWZ mouse cultured hepatocytes. JWZ cells were exposed to 10 μM ivermectin for 6, 12, 24, 48, or 72 h and mRNA levels were determined by RT-qPCR. Fig. 2 shows a time-dependent up-regulation in *Mdr1a* and *Mdr1b* mRNA when cells were exposed to 10 μM ivermectin. A significant increase in mRNA level was

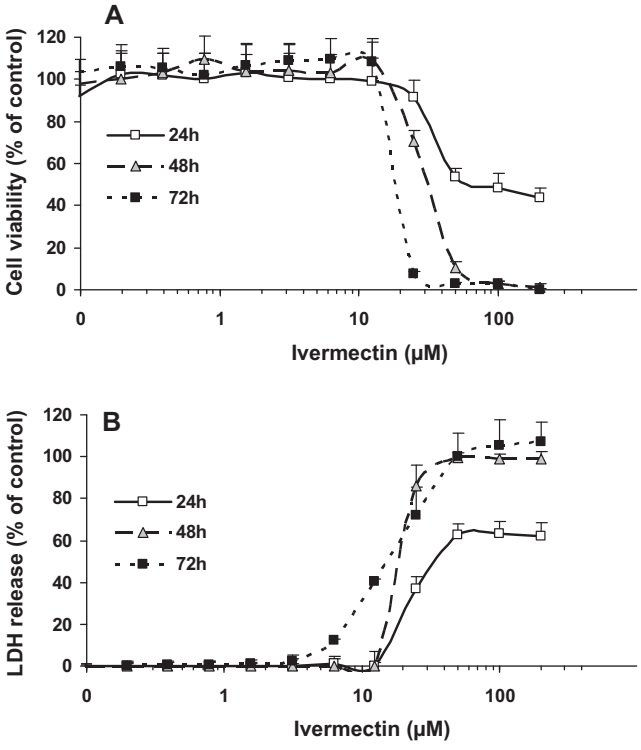

Fig. 1. Cytotoxic effects of ivermectin on JWZ cells as a function of time and concentration. Cytotoxicity was measured by the MTS assay (A) and LDH release (B) following 24, 48 or 72 h incubation at 34 °C, 5% CO_2 and 95% relative humidity. For the MTS assay, medium and DMSO were used as positive (100% cell viability) and negative (0% cell viability) controls, respectively. For LDH assay, medium and Triton 1% were used as negative (0% LDH release) and positive (100% LDH release) controls, respectively, and results were normalized to 0% and 100% LDH-release. All measurements were expressed as mean ± S.D ($n = 8$).

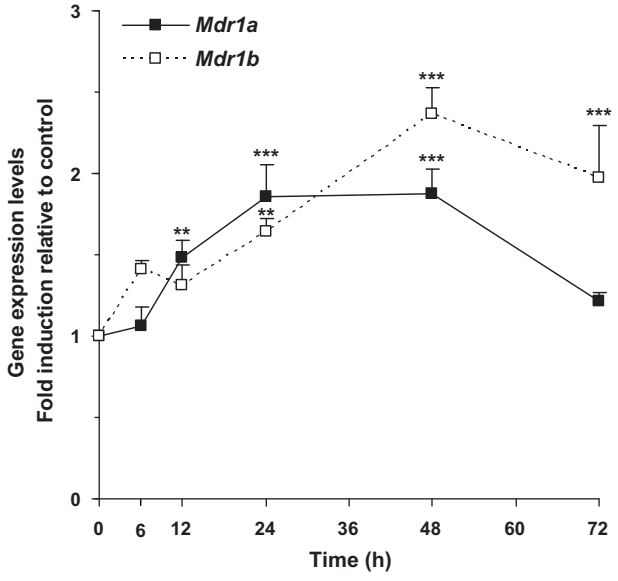

Fig. 2. Time-dependent effects of ivermectin on *Mdr1a* and *Mdr1b* mRNA levels. Mouse JWZ hepatocytes were treated with 10 μM ivermectin (IVM) or with the vehicle alone (DMSO) for 6, 12, 24, 48 or 72 h. *Mdr1a* and *Mdr1b* mRNA expression was then determined by real-time qPCR using gene-specific primers as described in Section 2. The mRNA expression levels were expressed as -fold increase over the corresponding control (untreated cells) given the arbitrary value of 1. Data are reported as the mean ± S.D. of three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$ *versus* untreated control.

observed as early as 12 h after treatment for *Mdr1a* (1.5-fold, $p < 0.01$) and 24 h for *Mdr1b* (1.6-fold, $p < 0.01$). The two P-glycoprotein mRNAs were maximally up-regulated after 48 h (1.9- and 2.4-fold over control for *Mdr1a* and *Mdr1b*, respectively). The up-regulation of *Mdr1* expression appeared to be transient with a lower induction after a longer exposure to ivermectin (1.2- and 2.0-fold of control levels with a 72 h exposure for *Mdr1a* and *Mdr1b*, respectively). TBP mRNA, which was used as a loading control, remained stable over the time period of the study (data not shown).

3.3. Concentration-dependent effects of ivermectin on P-glycoprotein mRNA expression

The dose-response relationship for ivermectin effects towards P-glycoprotein expression was then characterized. JWZ hepatocytes were either untreated or exposed to various concentration of ivermectin ranging from 0.5 to 10 μM for 48 h. Results are shown in Fig. 3. Even at low concentration such as 0.5 μM, ivermectin was able to alter the expression of *Mdr1a* with a slight but significant 1.3-fold increase ($p < 0.05$). When higher concentrations were used, *Mdr1a* and *Mdr1b* mRNA levels were significantly increased by ivermectin treatment in a clear concentration-dependence manner up to 2.1- and 2.5-fold for *Mdr1a* and *Mdr1b*, respectively.

3.4. P-glycoprotein functional activity in JWZ hepatocytes under influence of ivermectin

We then determined whether the induction of *Mdr1* gene expression by ivermectin resulted in a modulation of P-glycoprotein transport function. To measure P-gp transport activity in JWZ cells, we used a fluorometric assay previously described [29]. This assay utilizes lipophilic nonfluorescent calcein-AM which penetrates into the cell, where it is immediately cleaved by esterases to highly fluorescent calcein. Because calcein-AM is a high-affinity substrate for P-gp, intracellular calcein fluorescence inversely correlates with P-gp activity and, therefore, is a measure of P-gp-mediated transport. Thus, an increase in P-gp activity results in reduced intracellular calcein fluorescence and *vice versa*.

As expected, the presence of valspodar, the P-glycoprotein-selective blocker, in untreated cells resulted in a significant increase in calcein accumulation of more that 1.6-fold compared with control (Fig. 4). This result indicates that P-gp actively extruded calcein from JWZ cells and that valspodar clearly inhibit P-gp transport function in these cells. When JWZ cells were cultured with 10 μM ivermectin for 48 h before measuring the intracellular calcein accumulation, a treatment which was shown to increase *Mdr1* gene expression, intracellular calcein fluorescence was reduced by more than 30% compared with untreated cells, indicating that the P-gp-mediated efflux of calcein was increased. These results demonstrate that the induction of *Mdr1* mRNA expression observed with 10 μM ivermectin following a 48-h exposure is associated with an increase in P-gp transport function. It is important to note that this effect was abolished with valspodar (Fig. 4), indicating that the reduction in intracellular calcein fluorescence was specifically mediated by P-gp.

3.5. Role of transcription factors in the ivermectin-induced modulation of P-glycoprotein mRNA expression

Because CAR, PXR and AhR have been recognized as transcription factors playing an important role in the xenobiotic-induced regulation of the expression of efflux ABC transporters such as P-gp [6,36,37], we investigated their role in the ivermectin-induced up-regulation of *Mdr1* mRNA expression through two different approaches.

3.5.1. Induction of Mdr1a and Mdr1b mRNA by ivermectin in CAR- and PXR-overexpressing cells

Nuclear receptors-overexpressing hepatocytes have proved to be useful models in elucidating the role of transcription factors in the induction of ABC efflux transporters or cytochromes by xenobiotics [25,26]. We therefore firstly evaluated the effects of ivermectin in JWZ hepatocytes overexpressing the functional mouse CAR (Fig. 5A) or PXR (Fig. 5B). As previously described,

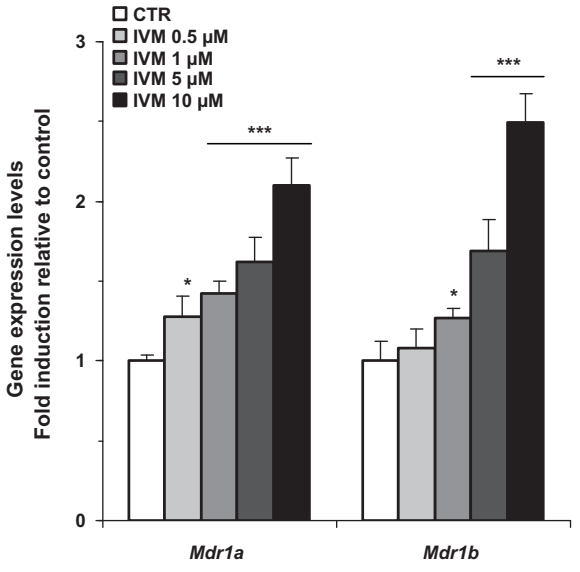

Fig. 3. Concentration-dependent effects of ivermectin on *Mdr1a* and *Mdr1b* mRNA levels. Mouse JWZ hepatocytes were either untreated (CTR) or exposed to increasing concentrations of ivermectin (IVM), ranging from 0.5 to 10 μM, for 48 h. Changes in *Mdr1a* and *Mdr1b* mRNA levels, normalized with respect to TBP mRNA levels, were determined by real-time qPCR using gene-specific primers as described in Section 2. The mRNA expression levels were expressed as -fold induction relative to control (DMSO) and are reported as the mean ± S.D. of six independent experiments. $^{*}p < 0.05$; $^{***}p < 0.001$ *versus* control.

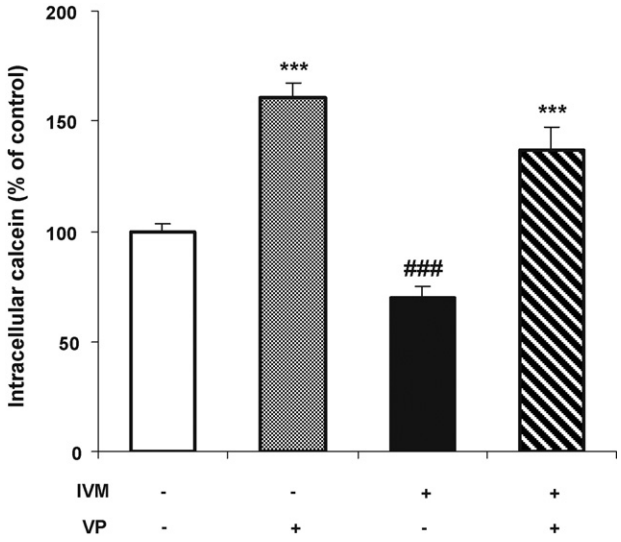

Fig. 4. P-glycoprotein transport activity in JWZ cells exposed to ivermectin. Intracellular calcein fluorescence was assessed in untreated cells or following a 48-h treatment with ivermectin (IVM, 10 μM) with or without a 40-min pre-exposure to the P-gp-specific inhibitor valspodar (VP, 5 μM). Data are expressed as percentage of control; each data represents mean ± S.D. ($n = 4$). $^{***}p < 0.001$, significantly greater than control; $^{###}p < 0.001$, significantly lower than control.

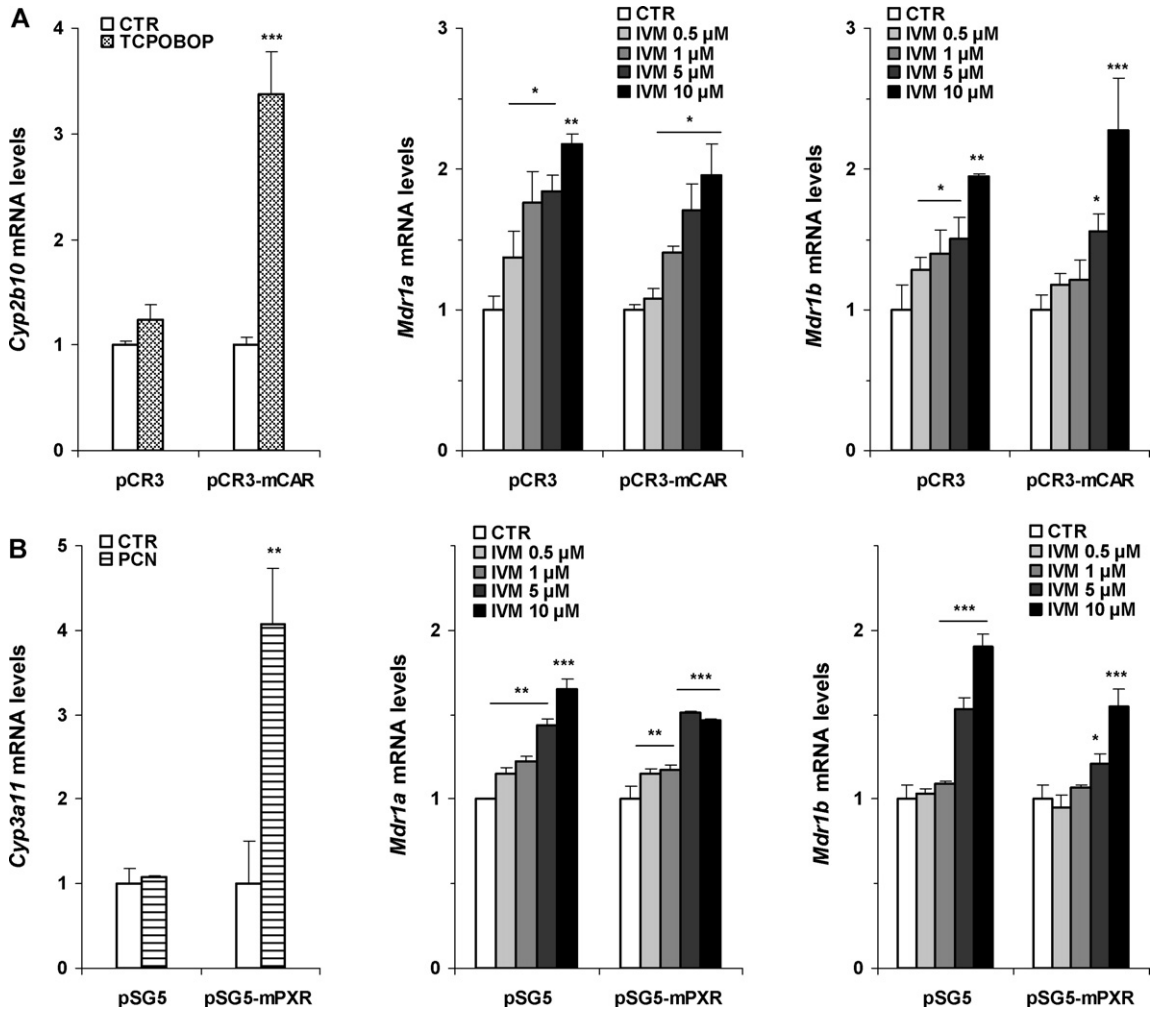

Fig. 5. Influence of the nuclear receptors CAR (A) and PXR (B) on the ivermectin-induced modulation of *Mdr1a* and *Mdr1b* gene expression. JWZ cells were transiently transfected with mouse CAR (pCR3-mCAR), mouse PXR (pSG5-mPXR), or their respective control expression vector (pCR3 and pSG5). 24 h after transfection, cells were treated with the vehicle alone (0.1% DMSO; CTR), TCPOBOP (250 nM), PCN (1 μM) or increasing concentrations of ivermectin (IVM) for 48 h. Changes in *Mdr1a*, *Mdr1b*, *Cyp2b10* and *Cyp3a11* mRNA levels, normalized with respect to TBP mRNA levels, were determined by real-time qPCR using gene-specific primers as described in Section 2. The mRNA expression levels were expressed as -fold induction relative to control and are reported as the mean ± S.D. ($n = 6$). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ *versus* control.

Cyp2b10 and *Cyp3a11*, the prototypical target genes of CAR [38] and PXR [39], were used as a positive indicator for CAR or PXR activation, respectively; while TCPOBOP, a synthetic direct agonist of mouse CAR, and PCN, a synthetic agonist of mouse PXR, were used as positive controls.

In untransfected JWZ cells, mouse CAR and PXR constitutive expression levels were extremely low under basal conditions, which correlated with the impaired induction of *Cyp2b10* by TCPOBOP or *Cyp3a11* by PCN, respectively (Fig. 5). In JWZ cells transiently transfected with pmCAR or pmPXR, TCPOBOP or PCN were able to increase either *Cyp2b10* (3.4-fold, $p < 0.001$, Fig. 5A) or *Cyp3a11* (4.1-fold, $p < 0.001$, Fig. 5B) expression, demonstrating that these nuclear receptors could be further activated in our model by an agonist. Fig. 5 shows that a 48-h exposure of ivermectin modified the expression level of *Mdr1a* and *Mdr1b* in the exact same manner in CAR- or PXR-overexpressing cells compared with JWZ cells transfected with the control plasmid: the overexpression of these nuclear receptors did not further up-regulate *Mdr1* in ivermectin-treated cells and no significant differences in fold-induction levels were observed compared with control JWZ cells. These results provide support for the hypothesis that the ivermectin-induced regulations were independent of the activation of CAR or PXR nuclear receptors.

3.5.2. Activation of PXR, CAR and AhR by ivermectin

In order to provide more data on the capacity of ivermectin to activate specific transcription factors, we used bioluminescent reporter cell lines which have already proved to be a valuable model to detect nuclear receptor ligands [31,40]. These cells expressed the GAL4-DNA binding domain fused to different nuclear receptor ligand binding domains and allow the detection of compounds that activate some specific transcription factors.

Ivermectin was tested for its ability to activate rodent transcription factors (murine CAR, murine PXR and rat AhR) by using three different reporter cell lines (HG_5LN-mCAR, HG_5LN-mPXR and H4IIE XRE-LUC) in which PXR, CAR and AhR agonists or antagonists induced or repressed luciferase gene expression. Activation was measured in these cells in the presence of increased concentrations of ivermectin from 10^{-8} M to 3.3×10^{-6} M. The vehicle alone (DMSO) was used as negative control (luciferase activity baseline set to 1) and known reference effectors were used as positive control, as already described [34]. Results are presented in Table 2. As expected, the basal luciferase activity of the mPXR and rAhR cell lines after treatment with their reference agonist was increased (2.3- and 10.1-fold, respectively) and the luciferase activity of the mCAR cell line was significantly decreased for more than fourfold after treatment with the antagonist ligand T0901317

Table 2
Modulation of luciferase expression by ivermectin in stable transfected cells.

Cell lines	Transcription factor	Luciferase activity (fold induction to CTR)							
		CTR (DMSO)	IVM concentration						Reference effector
			10^{-8} M	3.3×10^{-8} M	10^{-7} M	3.3×10^{-7} M	10^{-6} M	3.3×10^{-6} M	
HG_5LN-mCAR	mCAR	1.00 ± 0.05	1.03 ± 0.07	nd	1.05 ± 0.05	nd	1.04 ± 0.05	nd	T0901317 10 μM (reference antagoniste) 0.24 ± 0.02***
HG_5LN-mPXR	mPXR	1.00 ± 0.03	1.02 ± 0.03	nd	1.04 ± 0.03	nd	1.03 ± 0.03	nd	PCN 10 μM (reference agoniste) 2.33 ± 0.05***
H42E XRE	rAhR	1.00 ± 0.15	1.09 ± 0.07	1.03 ± 0.01	1.12 ± 0.20	1.15 ± 0.11	1.05 ± 0.05	1.07 ± 0.12	TCDD (dioxin) 10 nM (reference agoniste) 10.83 ± 0.86***

The luciferase activity of HG_5LN-mCAR, HG_5LN-mPXR and H42E XRE cells was measured in untreated, ivermectin-treated and reference ligand-treated cells. Concentration range of ivermectin was from 10^{-8} M to 3.3×10^{-6} M. Reference effectors used were T0901317 (10 μM), PCN (10 μM) and TCDD (dioxin, 10 nM) for mCAR, mPXR and rAhR, respectively. The incubation period with compounds was 24 h. Results are expressed as a fold-induction of the basal luciferase activity measured in untreated cells (CTR), which was set to 1, and are presented as mean ± S.D. (quadruplet). ***$p < 0.001$ *versus* control. nd: not determined; m: murine; and r: rat.

(Table 2). Ivermectin, at all concentrations tested, did not modulated luciferase gene expression in HG_5LN-mCAR, HG_5LN-mPXR or H4IIE XRE-LUC cell lines (Table 2): the activation in ivermectin-treated cells was not statistically different from untreated cells. These results showed that ivermectin was not a CAR, PXR or AhR ligand.

3.6. Effect of ivermectin on Mdr1a and Mdr1b mRNA stability

The level of mRNA expression is the result of the balance between the transcription and the elimination rate, through processing and degradation. The absence of influence of CAR and PXR on ivermectin-induced up-regulation of P-glycoprotein encoding genes prompted us to study the effect of ivermectin on *Mdr1a* and *Mdr1b* mRNA stability. Therefore, the half-lives of *Mdr1* mRNA in control and ivermectin-treated cells were examined, using an Act-D chase experiment. JWZ cells were stimulated with ivermectin for 24 h to induce *Mdr1* mRNA expression, after which the transcription inhibitor Act-D was added in the presence of ivermectin for another 24 h. Steady-state levels of *Mdr1a* and *Mdr1b* mRNA were determined at various time points after adding Act-D. There was a significantly slower rate of decline for ivermectin-treated cells *versus* control (Fig. 6). The half-life of *Mdr1a* and *Mdr1b* mRNA under control conditions were 8.6 h (Fig. 6A) and 10.4 h (Fig. 6B), respectively. By contrast, in ivermectin-induced cells, *Mdr1a* and *Mdr1b* mRNA half-lives were 17.3 h (Fig. 6A) and 17.2 h (Fig. 6B), respectively. This twofold increase of the half-life of the P-gp mRNA demonstrates that ivermectin treatment leads to the stabilization of the P-gp mRNA. It has to be noted that the degradation profiles for TBP mRNA in untreated and ivermectin-treated cells were similar (data not shown). Taken together, these results clearly indicated that up-regulation of *Mdr1* mRNA following exposure to ivermectin occurs, at least partially, at post-transcriptional level through a specific increase in *Mdr1* mRNA stability.

4. Discussion

Despite the well documented interactions of ivermectin, a commonly used anthelmintic, with P-gp, there is no data on a possible modulation of the expression of this transporter by ivermectin. We have used murine cultured hepatocytes to study the effects of short-term exposure to ivermectin on P-gp expression and function.

In this study, ivermectin was found to up-regulate the two mouse P-gp isoforms *Mdr1a* and *Mdr1b* gene expression in both a time- and dose-dependent manner, demonstrating the specificity of the ivermectin-induced effect. *Mdr1* mRNA levels were rapidly

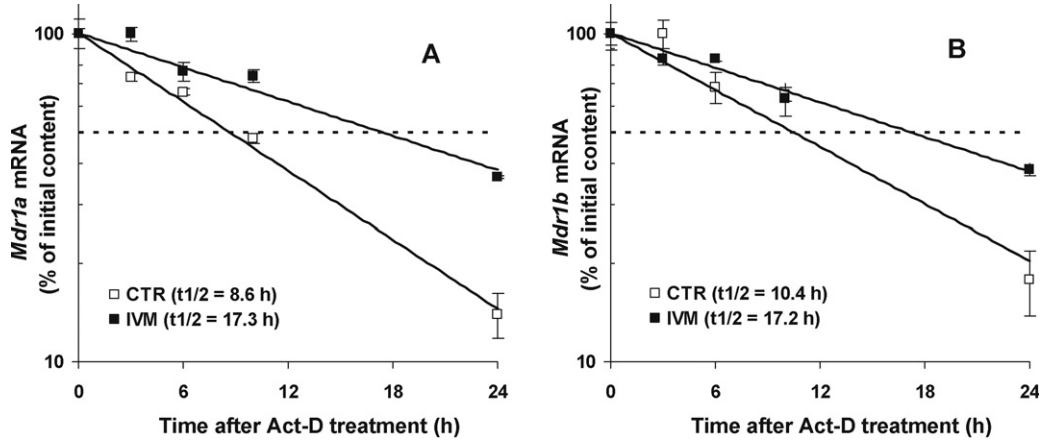

Fig. 6. Effect of ivermectin on P-glycoprotein mRNA decay pattern after actinomycin D addition. JWZ cells were treated with DMSO (0.1%, CTR, white square) or ivermectin (10 μM, IVM, black square) for 24 h, after which the transcription inhibitor actinomycin D (Act-D 5 μg/ml for 1 h and 1 μg/ml for the following 24 h) was added to inhibit nascent RNA synthesis. Cells were harvested at various time-points (3, 6, 10 or 24 h post Act-D treatment), total RNA was isolated and *Mdr1a* (A) and *Mdr1b* (B) mRNA levels were determined by qRT-PCR, as described in Section 2. The data were plotted as the percentage of mRNA remaining using a semilogarithmic scale and were reported as the mean ± S.D. The mRNA level at zero time point (before the Act-D treatment) was considered as 100%. The half-lives of mRNA ($t_{1/2}$) were determined with the use of regression curves.

induced, as early as 12 h after ivermectin addition for *Mdr1a*, with concentration as low as 0.5 μM, and the maximal effect for both *Mdr1a* and *Mdr1b* was observed 48 h after incubation. Furthermore, the induction of *Mdr1* was found to be transient: the rapid increase in *Mdr1* mRNA level reached a plateau at 24 h or 48 h and then decreased after longer exposure times, such as 72 h. This kind of transient induction of *MDR1* has already been described in hepatocytes after exposure to hepatocyte growth factor [41], or with two PXR activators, rifampicin and hyperforin [42]. The time- and concentration-dependent effect suggests that increased levels of P-gp in ivermectin-treated cells likely correspond to an acute induction in response to the anthelmintic drug. This is, to our knowledge, the first report showing that ivermectin is able to up-regulate the expression of this crucial transporter. Knowing that ivermectin is a good substrate of P-gp, our results are in agreement with the fact that, among other factors, the ability of drugs to interact with ABC transporters can lead to overexpression of these proteins [15]. The expression of the *MDR1* gene can be rapidly and transiently induced after a short-term exposure to a variety of xenobiotics in many cell lines, such as rodent cultured hepatocytes [3,43], human cancer cell lines [44,45], but also after acute in vivo exposure of a tumor to cytotoxic chemotherapy [4]. Our results dealt with the expression of murine P-gp and it would be hazardous to extrapolate this conclusion to other species such as humans. Additional data are needed in order to explore the ability of ivermectin to regulate the P-gp gene expression in human hepatocytes. Nevertheless, it has been demonstrated that *mdr1a* and *mdr1b* gene expression in rodent and *MDR1* in humans are, at least partially, controlled by the same negative regulatory element [46]. In addition, P-gp mRNA levels were increased in a dose- and time-dependent manner upon transient exposure to a variety of cytotoxic drugs including doxorubicin [44,45] and daunorubicin [45,47] in human cells but also in rodent cells [3,48], demonstrating that similar regulatory effect of xenobiotic on P-gp expression in human and rodent cells can be observed.

The possible mechanisms involved in ivermectin up-regulation of *Mdr1a* and *Mdr1b* gene expression were investigated. The rapidity of the response upon ivermectin treatment excludes gene amplification, gene rearrangement or mutations and supports transcriptional activation or post-transcriptional regulation as the underlying mechanism.

We firstly examined the role of some transcription factors since transcriptional activation is the most common mechanism described for up-regulation of *MDR1* gene expression, and several regulatory elements have been identified and characterized in the *MDR1* promoter region. Since the nuclear receptors CAR and PXR are cellular sensors capable of responding to chemical exposure [49–51] and have been implicated in *MDR1* regulation [37,52], we investigated their potential role in the ivermectin-induced modulation of P-gp. We therefore used mouse hepatocytes transiently overexpressing the functional mouse CAR or PXR. Similar patterns and extent of the dose-dependent modulations of P-gp induced by ivermectin were observed in CAR- or PXR-overexpressing cells and in control cells, which express CAR and PXR at a very low level. This strongly suggests that ivermectin may have CAR- and PXR-independent effects on gene expression. Furthermore, using bioluminescent cell lines expressing transcription factors, we have demonstrated that ivermectin was not a CAR, PXR or AhR ligand. In accordance with our results, it was previously shown that toxic insult increased *MDR1* expression through CAR-independent mechanisms [53]. However, the role of other transcription factors, including nuclear receptors, in the ivermectin-induced regulations cannot be discarded since number of them such as PPARγ, as well as Nrf2, are described to be involved in efflux ABC transporters transcriptional regulation [8,37].

Besides transcriptional activation, post-transcriptional regulation at the level of mRNA stability was shown to be an important mechanism for P-gp overexpression in vitro [54] and in vivo [10,55]. In our study, *Mdr1* mRNA decay was examined. mRNA half-life in untreated cells was found to be around 9 h which is in the range of values reported in the literature [35,56]. Interestingly, stability of *Mdr1a* and *Mdr1b* mRNA was markedly increased by ivermectin treatment, demonstrating that this drug reduced the degradation rate of *Mdr1* mRNA thus contributing, at least in part, to the increase of P-gp gene expression. Interestingly, variety of drugs – such as cycloheximide [57], doxorubicin [58], vinblastine [58], colchicine [58], daunorubicin [47] or etoposide [47], were previously shown to induce *MDR1* gene expression in mammalian cells upon acute treatment, as short as a 24-h exposure, through enhanced mRNA stability rather than increased transcription. In further agreement with our results, atorvastatin, a P-gp substrate [59] and inhibitor [60], as ivermectin is, was able to induce P-gp mRNA expression [61]. More interestingly, regulation of *MDR1* mRNA expression in human hepatocytes by atorvastatin was recently associated with a modulation in mRNA degradation [9]. The detailed mechanism that causes stabilization of P-gp mRNA in all of the above studies is currently unknown. The stability of a particular mRNA is partially controlled by specific interactions between its structural elements and trans-acting factors: mRNA binding proteins and some microRNAs. Recently, miRNAs, which contribute to post-transcriptional processing through 3′-UTR-interference, have been involved in the regulation of *ABCB1* mRNA degradation [62,63]. Since several compounds have been shown to influence P-gp mRNA stability, it is reasonable to think that ivermectin may interact with a putative trans-acting stabilizing factor which is activated upon drug exposure, resulting in increased mRNA stability. However, the mRNA stabilizing mechanism which is responsible for the differential stability of P-gp mRNA in ivermectin-treated cells *versus* untreated cells remains to be elucidated.

In our conditions, when JWZ hepatocytes were exposed to 10 μM ivermectin, *Mdr1* mRNA level was increased up to 2.5-fold, due to a twofold increase in *Mdr1* mRNA half-life, compared with untreated cells. It is now recognized that a two to fourfold fluctuation in mRNA half-life can have significant effects on mRNA and protein abundance [64]. Therefore, to investigate whether the induction of the P-gp mRNA level by ivermectin was reflected at the protein and transport activity level, an assay previously used to screen for P-gp–drug interactions [29] was performed. When cells were treated with 10 μM ivermectin for 48 h, conditions which caused a 2.5-fold increase in *Mdr1* gene expression, the P-gp transport function was increased by more than 30%. Consistently with our observation, an important correlation between P-gp modulation at the level of mRNA and the transport activity [3,65,66], but also between P-gp protein levels and efflux activity following treatment with various drugs [67] has been reported. Interestingly, many compounds defined as P-gp substrates acted to induce P-gp gene expression and activity, but rarely displayed an inhibitory activity on the transporter [61]. Our results demonstrate that ivermectin, a competitive substrate displaying both substrate and inhibitory activity, was also able to up-regulated mRNA expression and consequently transport activity of the P-gp.

P-gp is expressed in multiple key organs in drug disposition such as small intestine, blood–brain barrier, kidney and liver and it is well established that induction of the P-gp activity can cause reduced drugs biodisposition and decreased therapeutic efficacy [68]. It was recently shown that even moderate increases in the expression of *Mdr1* genes are sufficient to cause doxorubicin resistance in vivo [69], suggesting a possible impact on the pharmaco- and toxicokinetic of many drugs and endogenous P-gp substrates. The potential involvement of ivermectin in

P-gp-mediated drug–drug interactions when administered with other drugs that are P-gp substrates should therefore be taken into account in human and veterinary therapy. Furthermore, since ivermectin disposition in host is mainly controlled by P-gp efflux at the intestinal [11] and hepatic level [12,13,70], up-regulation of its expression may contribute to the autoinduction of ivermectin elimination, resulting in a decrease of the body disposition and the intracellular levels of this anthelmintic agent in the host organism. This transport regulation may be considered as a first step in lowering the drug disposition and therefore drug efficacy. Acquired resistance to ivermectin is becoming a major clinical concern in the prevention/treatment of parasites and even though the factors responsible for the development of resistance to antiparasitic compounds are not perfectly understood, one of them is the exposure of parasites to subtherapeutic drug concentrations [71]. Therefore, this study offers insight into the potential for resistance development through modulation of ivermectin pharmacokinetic in the mammalian host. Furthermore, P-gp homologs are thought to play an important role in the resistance of nematode parasites against MLs [21], and data from the literature suggests that induction of P-gp expression in response to ivermectin treatment may also exist in nematodes. Indeed, P-gp homologs were shown to be overexpressed in ivermectin-resistant worms such as *Haemonchus contortus* [22] and *Caenorhabditis elegans* [23], and after exposure of the sea lice *Lepeophtheirus salmonis* to the ML emamectin [72]. Up-regulation of these proteins would serve to eliminate MLs from the parasite, limiting the accumulation of a toxic concentration at the target receptors, therefore offering protection of the parasite. Interestingly, multidrug resistance phenotype has been associated to an overexpression of some P-gp homologs through post-transcriptional regulation, including increase in mRNA stability in *Bacillus subtilis* [73], but also in the protozoan parasites *Entamoeba histolytica* [74]. Altogether, these data demonstrate that exposure to MLs, ivermectin in particular, potently modulates P-gps in both mammals and parasites and that partial similarities in the mechanisms of ABC efflux transporters regulation may exist between mammals and parasites. The elucidation of the molecular mechanisms of overexpression of ABC efflux transporters in nematodes could provide useful markers for monitoring MLs resistance and possible targets for intervention in drug resistant parasites.

In summary, this report demonstrates for the first time that acute ivermectin treatment induces P-gp gene expression and activity in mouse hepatocytes through post-transcriptional regulation affecting P-gp mRNA degradation. These results have significant clinical implications regarding alteration of drug disposition and drug–drug interactions in host and offer insight into the potential for emergence of multidrug resistance during anthelmintic treatment in parasites.

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