P-Glycoprotein Limits Oral Availability, Brain Penetration, and Toxicity of an Anionic Drug, the Antibiotic Salinomycin

Jurjen S. Lagas,1 Rolf W. Sparidans,3 Robert A. B. van Waterschoot,1 Els Wagenaar,1 Jos H. Beijnen,2 and Alfred H. Schinkel1*

Division of Experimental Therapy, The Netherlands Cancer Institute, Amsterdam,1 Department of Pharmacy and Pharmacology, Slotervaart Hospital, Amsterdam,2 and Department of Pharmaceutical Sciences, Utrecht University, Utrecht,3 The Netherlands

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Salinomycin is a polyether organic anion that is extensively used as a coccidiostatic antibiotic in poultry and commonly fed to ruminant animals to improve feed efficiency. However, salinomycin also causes severe toxicity when accidentally fed to animals in high doses. In addition, humans are highly sensitive to salinomycin and severe toxicity has been reported. Multidrug efflux transporters like P-glycoprotein (P-gp), BCRP, and MRP2 are highly expressed in the intestine and can restrict the oral uptake and tissue penetration of xenobiotics. The purpose of this study was to investigate whether the anionic drug salinomycin is a substrate for one or more of these efflux pumps. Salinomycin was actively transported by human MDR1 P-gp expressed in polarized MDCK-II monolayers but not by the known organic anion transporters human MRP2 and murine Bcrp1. Using P-gp-deficient mice, we found a marked increase in plasma salinomycin concentrations after oral administration and decreased plasma clearance after intravenous administration. Furthermore, absence of P-gp resulted in significantly increased brain penetration. P-gp-deficient mice also displayed clearly increased susceptibility to salinomycin toxicity. Thus far, P-gp was thought to affect mainly hydrophobic, positively charged or neutral drugs in vivo. Our data show that P-gp can also be a major determinant of the pharmacokinetic behavior and toxicity of an organic anionic drug. Variation in P-gp activity might thus directly affect the effective exposure to salinomycin and possibly to other anionic drugs and toxin substrates. Individuals with reduced or absent P-gp activity could therefore be more susceptible to salinomycin toxicity.

ATP-binding cassette multidrug transporters like P-glycoprotein (P-gp, \textit{ABCB1}), BCRP (\textit{ABCG2}), and MRP2 (\textit{ABCC2}) form important defense mechanisms against exogenous toxins and other potentially harmful compounds that can be encountered in daily life. Because of their strategic localization at apical membranes of important epithelial barriers and at the canalicular membrane of hepatocytes, they can mediate active excretion of transported substrates via the liver, intestine, and kidneys. Furthermore, efflux transporters can restrict small intestinal uptake of substrates after oral ingestion, and overexpression of drug efflux transporters in tumor cells can confer resistance to cancer chemotherapy (2). The presence of these efflux transporters in blood-brain, blood-testis, and maternofetal barriers also restricts the penetration of substrates in the brain, testis, and fetus (pharmacological sanctuary sites). As many drugs and their metabolites are excellent substrates for these transporters, they can have a dramatic impact on the oral availability, tissue distribution, elimination and excretion, and toxicity risks of drugs, with potentially profound positive or negative consequences for the clinical application of these drugs.

Salinomycin (Fig. 1) is a polyether antibiotic belonging to the group of ionophores. This natural toxin is produced by a \textit{Streptomyces albus} strain (20). Salinomycin is extensively used as a coccidiostat in poultry and other livestock and is commonly fed to ruminant animals to improve feed efficiency (4, 33). However, salinomycin can also cause severe toxicity when accidentally fed to animals in relatively high doses, as described for chickens (9, 12, 19), turkeys (1, 24, 31), cats (32), pigs (14, 22, 23), alpacas (15), and horses (21, 25). Severe human poisoning with salinomycin has also been reported (30). Accidental ingestion of an estimated 1 mg/kg of salinomycin resulted in a 6-week hospital admission with prolonged rhabdomyolysis, pain, and disability. In addition, human poisoning with fatal rhabdomyolysis has been reported for the polyether ionophore antibiotic monensin, illustrating that humans are highly vulnerable to the toxicity of these types of compounds (3, 16).

Our interest in salinomycin was sparked by the widespread poisoning of cats with salinomycin that occurred in 1996 in The Netherlands and in Switzerland (32). Two brands of cat food from one manufacturer were contaminated with salinomycin, which resulted in an outbreak of acute polyneuropathy. In The Netherlands, an estimated 100,000 cats were exposed to the contaminated food and the reported number of cases of acute paralysis, many with a fatal outcome, was 823. The incidence among exposed cats was thus about 1%. We wondered if salinomycin could be a substrate of one or more of the apical efflux transporters, as these can restrict the oral uptake and reduce the bioavailability of harmful substrate compounds, thus protecting the body. This was shown for the anticancer agents paclitaxel in \textit{Mdr1a/1b}−/− mice and topotecan in \textit{Bcrp1}−/− mice and for the carcinogen PhIP in MRP2-deficient rats (7, 10, 29).

Salinomycin is an organic anionic drug with a pKa of around

* Corresponding author. Mailing address: Division of Experimental Therapy, The Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam 1066 CX, The Netherlands. Phone: 31 20 512 2046. Fax: 31 20 512 2050. E-mail: a.schinkel@nki.nl.

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4.4 (Fig. 1), implying that at physiological pH, >99% of the drug is present in its anionic form. With a molecular mass of 751 Da, it is also relatively large. We therefore expected that salinomycin might be a substrate for MRP2 and/or Bcrp1, which are known to transport a broad spectrum of organic anions, in contrast to MDR1 P-gp, which primarily transports hydrophobic neutral or positively charged compounds (2). Nevertheless, we started out by testing whether salinomycin is transported by any of these three apical efflux transporters.

## MATERIALS AND METHODS

### Animals
Mice were housed and handled according to institutional guidelines complying with Dutch legislation. The animals used in this study were male wild-type (WT) and Mdr1a/+/ mice of a >99% FVB genetic background between 9 and 15 weeks of age. Animals were kept in a temperature-controlled environment with a 12-h light, 12-h dark cycle and received a standard diet (AM-II; Hope Farms, Woerden, The Netherlands) and acidified water ad libitum.

### Chemicals
Salinomycin, SV sodium salt pentahydrate was obtained from Sigma-Aldrich (Steinheim, Germany), heparin (3,000 U/ml) from Leo Pharma BV (Breda, The Netherlands), methoxyflurane (Metofane) from Medical Developments Australia Pty. Ltd. (Springvale, Victoria, Australia) and bovine serum albumin, fraction V, from Roche (Mannheim, Germany). The organic solvents methanol, acetone (both high-performance liquid chromatography grade), and diethyl ether originated from Merck (Darmstadt, Germany). GlaxoSmithKline (Uxbridge, United Kingdom) kindly provided elacridar (GF120918).

### Transport assays
Polarized canine kidney MDCK-II cell lines were used in transport assays. Human MDR1- and MRP2-transduced and murine Bcrp1-transduced MDCK-II subclones and their growth conditions were described previously (8, 11). Transepithelial transport assays using Transwell plates were carried out as described previously, with minor modifications (26). Experiments were performed using heparinized capillary tubes (Oxford Labware, St. Louis, MO). Blood samples were centrifuged at 2,100 × g for 5 min at 4°C, and the plasma fraction was collected and stored at −20°C until LC-MS/MS analysis. For tissue distribution studies, salinomycin was injected i.v. as a single bolus of 1 mg/kg body weight (5 ml/kg) into a tail vein (n = 4). After 180 min, blood, liver, brain, and small intestinal contents (SIC) were isolated, plasma was collected, and the samples were stored at −20°C until LC-MS/MS analysis.

### Pharmacokinetic calculations and statistical analysis
Pharmacokinetic parameters were calculated by noncompartmental methods using the software package WinNonlin Professional version 5.0. The areas under the plasma concentration-time curves (AUCs) were calculated by using the trapezoidal rule. For oral experiments at a dose of 10 mg/kg, the AUC could not be reliably extrapolated to infinity as clearance up to 6 h was very slow, so only the AUC from 0 to 6 h (AUC0-6) was calculated. For oral and i.v. experiments at a dose of 1 mg/kg, both the AUC0-6 and the AUC extrapolated from zero time to infinity (AUC0-∞) were calculated. The peak drug concentration in plasma (Cmax) and the time of maximum drug concentration in plasma (Tmax) were estimated from the original data. Plasma clearance (CL) after i.v. administration was calculated by the formula CL = dose/AUC0-6. The Wilcoxon rank sum test was used for statistical analysis. Differences were considered statistically significant at P < 0.05. Data are presented as means ± standard deviations (SD).

## RESULTS

### In vitro transport of salinomycin
To determine whether the organic anion salinomycin was transported by one or more of the apical ATP-binding cassette multidrug transporters in vitro, we used the polarized canine kidney cell line MDCK-II and its subclones transduced with human MDR1 and MRP2 and murine Bcrp1. The parental and transduced cell lines were grown to confluent polarized monolayers on porous membrane filters, and vectorial transport of 5 μM salinomycin across the monolayers was determined. In the MDCK-II parental cell line, the translocation of salinomycin in the basolateral direction was slightly higher than in the apical direction (Fig. 2A). In MDR1-transduced MDCK-II cells, apically directed translocation was significantly increased and basolaterally directed translocation was markedly decreased (Fig. 2B). In the presence of the P-gp inhibitor elacridar at 5 μM, this transport was completely inhibited, resulting in a translocation pattern similar to that of the MDCK-II parent cell line (Fig. 2C and D). In the murine Bcrp1- and human MRP2-transduced MDCK-II cell lines, vectorial translocation of salinomycin was not different compared to that in parental MDCK-II cells (Fig. 2E and F). These results demonstrate efficient transport of salinomycin by human MDR1 but not by murine Bcrp1 and human MRP2.

### Plasma pharmacokinetics of salinomycin in WT and Mdr1a/ 1b−/− mice
To test whether the in vitro-observed MDR1 P-gp-
mediated transport of salinomycin is also relevant in vivo, we studied salinomycin plasma pharmacokinetics in WT and Mdr1a\textsuperscript{−}\textsuperscript{−}/Mdr1b\textsuperscript{−}\textsuperscript{−}/H11002/H11002 mice. For oral administration, we performed an experiment at 10 mg/kg body weight (20% of the published oral 50% lethal dose in mice). At 15 to 30 min after administration, four out of five Mdr1a\textsuperscript{−}\textsuperscript{−}/Mdr1b\textsuperscript{−}\textsuperscript{−}/H11002/H11002 mice displayed symptoms of salinomycin poisoning whereas these signs were absent in WT mice. The affected Mdr1a\textsuperscript{−}\textsuperscript{−}/Mdr1b\textsuperscript{−}\textsuperscript{−}/H11002/H11002 mice had paralyzed fore and hind limbs, and their respiratory frequency was decreased. Therefore, we had to terminate this experiment for the Mdr1a\textsuperscript{−}\textsuperscript{−}/Mdr1b\textsuperscript{−}\textsuperscript{−}/H11002/H11002 mice. For WT mice, the AUC\textsubscript{0–6} was 2,130 ± 178.0 h \cdot \mu g/liter, with a \textit{C}\textsubscript{max} of 517.4 ± 152.4 \mu g/liter at approximately 0.5 h (Table 1). We repeated the oral study with 1 mg/kg salinomycin (Fig. 3). No signs of toxicity were observed at this dose. The AUC\textsubscript{0–6} for Mdr1a\textsuperscript{−}\textsuperscript{−}/Mdr1b\textsuperscript{−}\textsuperscript{−}/H11002/H11002 mice was increased 3.1-fold compared to that for WT mice (250.8 ± 27.3 versus 80.2 ± 12.3 h \cdot \mu g/liter; \textit{P} < 0.01; Table 1 and Fig. 3), and the \textit{C}\textsubscript{max} for both WT and Mdr1a\textsuperscript{−}\textsuperscript{−}/Mdr1b\textsuperscript{−}\textsuperscript{−}/H11002/H11002 mice was reached approximately 2 h after administration. Comparison of the AUC\textsubscript{0–6} for WT mice at 1 or 10 mg/kg shows that a 10-fold increase in the dose resulted in a 34-fold greater AUC (Table 1). This, together with the shift in the estimated \textit{T}\textsubscript{max}, suggests that the

FIG. 2. Transepithelial transport of salinomycin (5 \mu M) in MDCK-II cells either nontransduced (A and C) or transduced with human \textit{MDRI} (B and D), human \textit{MRP2} (E), or murine \textit{Bcrp1} (F) cDNA in the absence (A, B, and F) or presence (C, D, and E) of elacridar (5 \mu M). At \textit{t} = 0 h, salinomycin was applied in one compartment (apical or basolateral) and the percentage of salinomycin transported to the opposite compartment at \textit{t} = 2 and 4 h was measured by LC-MS/MS and plotted (\textit{n} = 3). Translocation from the basolateral to the apical compartment (●) and translocation from the apical to the basolateral compartment (▲) are shown. Data represent means \pm SD.
plasma pharmacokinetics of salinomycin is nonlinear in mice, which might be the result of saturation of absorption-limiting, drug-metabolizing, and/or drug-excreting systems at high salinomycin concentrations.

After i.v. administration of 1 mg/kg salinomycin, there were no signs of toxicity, despite the high initial plasma concentrations. *Mdr1a* 

\[
\text{WT} \quad \text{Mdr1a}^\text{b}^-^-^\text{KO} \quad \text{KO/WT ratio}
\]

Table 1. Plasma pharmacokinetics after oral or i.v. administration of salinomycin

<table>
<thead>
<tr>
<th>Strain or parameter</th>
<th>10-mg/kg oral dose</th>
<th>1-mg/kg oral dose</th>
<th>1-mg/kg i.v. dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC(_{0-}\infty) (h · µg/liter)</td>
<td>C(_{\text{max}}) (µg/liter)</td>
<td>T(_{\text{max}}) (h)</td>
</tr>
<tr>
<td>WT</td>
<td>2,130 ± 178.0(^g)</td>
<td>517.4 ± 152.4</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Mdr1a</em>(^b^-^-^\text{KO})</td>
<td>ND(^b)</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>KO/WT ratio</td>
<td>3.2</td>
<td>3.1</td>
<td>3.1</td>
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\(^{a}\) Data are means ± SD; \(n = 5\) for both oral and i.v. administrations.

\(^{b}\) ND, not determined in view of toxicity.

\(^{c}\) \(P < 0.01\) compared to WT mice.

\(^{d}\) \(P < 0.05\) compared to WT mice.

\(^{e}\) KO, knockout.

Brain penetration, liver accumulation, and intestinal excretion of salinomycin. To investigate whether P-gp deficiency affects the tissue distribution and disposition of salinomycin, we determined salinomycin concentrations in the brain, liver, and SIC at 180 min after i.v. administration of 1 mg/kg. The brain salinomycin concentration in *Mdr1a*\(^b^-^-^\text{KO}\) mice was increased about 6-fold, whereas the plasma salinomycin concentration in *Mdr1a*\(^b^-^-^\text{KO}\) mice at this time point was 3.7-fold higher than that in WT mice (Table 2). To correct for the higher drug concentration in the plasma of *Mdr1a*\(^b^-^-^\text{KO}\) mice, we calculated the brain/plasma ratio. In *Mdr1a*\(^b^-^-^\text{KO}\) mice, this ratio was significantly greater than in WT mice (0.20 ± 0.03 versus 0.13 ± 0.01; \(P < 0.05\); Table 2), suggesting that P-gp limits the penetration of the brain by salinomycin, albeit to a modest extent.

In the liver and SIC, no differences in concentration between WT and *Mdr1a*\(^b^-^-^\text{KO}\) mice were found at 180 min after administration (Table 2), in spite of the clear differences in the concentration in plasma. The concentration of salinomycin in the liver was 7.4 ± 1.0 µg/g in WT mice and 6.5 ± 1.1 µg/g in *Mdr1a*\(^b^-^-^\text{KO}\) mice, representing 33.9% ± 4.2% and 29.5% ± 3.8% of the administered dose, respectively. These high concentrations in the liver at 180 min suggest that initially high concentrations of salinomycin in plasma upon i.v. administration result in a substantial accumulation of salinomycin in the liver and that subsequent biliary excretion and/or sinusoidal clearance is relatively slow.

![FIG. 3](http://aac.asm.org/) Plasma concentration-time curves of salinomycin in male FVB WT (△) and *Mdr1a*\(^b^-^-^\text{KO}\) (■) mice after oral (A) or i.v. (B) administration of salinomycin at a dose of 1 mg/kg. Data represent mean concentrations ± SD (\(n = 5\) for oral or i.v. administration). The insert in panel B shows a semilog plot of the data.
DISCUSSION

In this study, we demonstrate that the organic anion salinomycin, belonging to the group of ionophore polyether antibiotics, is an efficiently transported substrate of human and murine P-gp, but not of MRP2 or Bcrp1, even though the latter two are known to transport many negatively charged compounds. From our studies with Mdr1a/1b−/− mice, it is clear that P-gp deficiency results in pronounced effects on the pharmacokinetics and toxicity of salinomycin in mice. Mdr1a/1b−/− mice displayed substantially increased oral bioavailability and toxicity of salinomycin, decreased salinomycin clearance from plasma, and significantly increased penetration of the brain by salinomycin.

Thus far, P-gp was thought to affect mainly hydrophobic, positively charged or neutral drugs in vivo. Although P-gp was shown to be a low-affinity transporter of the negatively charged hydrophobic anticancer agent methotrexate in vitro (6), to date there are, to the best of our knowledge, no reports that P-gp affects the oral availability, plasma clearance, or toxicity of negatively charged compounds in vivo. Our data show that P-gp can be a major determinant of the pharmacokinetics and toxicity of an organic anionic drug. Variation in P-gp activity might thus directly affect the effective exposure to salinomycin and possibly other anionic drugs and toxin substrates, further expanding the already broad spectrum of compounds critically influenced by P-gp in vivo.

During the preparation of the manuscript, Chen et al. reported that the acidic (anionic) form of the statin drug lovastatin showed a significantly higher brain/plasma ratio in Mdr1a/1b−/− than in WT mice (5). Although the neutral lactone form of lovastatin showed a markedly higher brain/plasma ratio, which was increased at least eightfold in P-gp-deficient mice, this result may reflect reduced brain penetration by acidic lovastatin due to P-gp activity, in line with our observation of brain penetration by salinomycin. Plasma clearance of lovastatin (lactone or acidic form) was not noticeably altered in P-gp−/− mice. Knowledge of anionic and other compounds that are affected by P-gp in vivo is useful for veterinary medicine, since there are domestic animals known to be deficient in P-gp. For example, some dog breeds (e.g., collies) and their crosses were found to have a deletion of the Mdr1a gene, resulting in loss of protein activity and greatly increased sensitivity to neurotoxicity induced by the P-gp substrate ivermectin, a drug used to treat worm infections (18). A spontaneous mutation in a subpopulation of outbred CF-1 mice also resulted in Mdr1a deficiency and ivermectin hypersensitivity (17). Interestingly, an isolated herd of Murray Grey cattle in Australia showed unusually high concentrations of the ivermectin analogue abamectin in their central nervous systems and corresponding toxicity (27). This strongly suggests a P-gp deficiency in some inbred populations of this species as well. It is therefore tempting to speculate that a possible low-frequency P-gp deficiency in domestic cats might explain the low-frequency hypersensitivity of cats exposed to salinomycin-contaminated food (32). Unfortunately, the currently available necropsy material of a limited number of cats that succumbed to salinomycin poisoning does not allow unambiguous assessment of their Mdr1-type P-gp status. If P-gp deficiency does indeed occur in a small subfraction of Dutch, Swiss, and possibly other domestic cat populations, this might have implications for their treatment with veterinary drugs.

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