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Cytotoxicity of anticancer candidate salinomycin and identification of its metabolites in rat cell cultures

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ABSTRACT

Salinomycin (SAL) is a polyether antibiotic, which is commonly used as a coccidiostat and has recently shown to exhibit anticancer activity. The toxic action of the drug may be connected with the extent and routes of its biotransformation. The cytotoxic potential of SAL and its combination with tiamulin and prednisolone was investigated using three cell models from rat: primary hepatocytes, hepatoma cells (FaO) and myoblasts (L6). The four biochemical endpoints were assessed: mitochondrial and lysosomal activity, total cell protein content and membrane integrity. The metabolites of SAL in the medium from cell cultures were determined using LC-MS/MS. The cytotoxicity of SAL was time-, concentration- and cells dependent. The most sensitive endpoint was the inhibition of lysosomal activity. Tiamulin increased SAL cytotoxicity, whereas the opposite results were observed for prednisolone. Primary hepatocytes were the most efficient in SAL biotransformation both in terms of its intensity and number of produced metabolites. The range of the cytotoxicity and mode of salinomycin interaction with tiamulin and prednisolone cannot be explained by the biotransformation alone.

1. Introduction

Salinomycin (SAL) has been used in veterinary for nearly fifty years for the treatment and prevention of coccidiosis in food-producing animals. Its mechanism of both pharmacological and toxicological action involves the formation of lipid-soluble complexes with monovalent cations, thereby facilitating bidirectional ion flux through lipid barriers by passive diffusion processes (Mitani et al., 1975). Since 2009 other applications of ionophores are proposed. Gupta et al. (2009) proved that salinomycin is nearly 100-fold more effective against breast cancer stem cells than the commonly used cytostatic drugs in the screening study on about 16,000 biologically active substances. The ionophore showed strong inhibition of proliferation, migration and invasion of cancer cells including colorectal, ovarian, lung, gastric and prostate cancer, and drug-resistant cell lines (Antoszczak and Huczyński, 2015). Since 2012 it has been approved for testing in clinical studies on patients with invasive head, neck, breasts and ovary carcinoma. SAL inhibited the progress of the disease over an extended period. Acute side effects were rare and the serious long-term adverse side effects were not observed (Naujokat and Steinhart, 2012).

The acute toxicity of SAL is highly dependent on animal species with turkeys and horses being the most sensitive among tested species: LD_{50} 0.6 mg/kg b.w. in comparison to LD_{50} 50 mg/kg b.w. and 21 mg/kg b.w. in rats and rabbits, respectively (Oehme and Pickrell, 1999). The narrow safety margin of SAL and susceptibility of certain species to its toxic properties may lead to poisonings of both target and non-target husbandry animals. The clinical cases of such severe, often fatal, intoxications were reported (Novilla, 2012). Signs of intoxication in animals included cardiovascular effects, necrosis of striated muscles, neuropathy and gastrointestinal disorders (Novilla, 2012; Oehme and Pickrell, 1999). Risk assessment based on analysis of such cases revealed that SAL present in feed at 2% broiler ratios concentration could already induce adverse health effects in horses (Dorne et al., 2013).

The species-dependent toxicity seems to be connected with the metabolism of that compound. Although the majority of authors suggest that SAL biotransformation is a detoxification pathway, it is not clear if the toxicity is caused only by the parent compound. The toxic clinic interactions between the SAL (or other ionophore coccidiostats) and tiamulin and the effects on drug-metabolizing enzymes have been reported (Stripkovits et al., 1992; Plumlee et al., 1995). Tiamulin inhibits CYP3A enzymes involved in SAL metabolism. On the other hand, prednisolone induces those enzymes and may increase SAL's metabolism.

A number of studies have been performed using cell cultures in cytotoxicity studies of SAL and its interaction with drugs (Cybulski et al., 2015; Sommer et al., 2016; Zou et al., 2017). Hepatocytes as main

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cells involved in the drug metabolism a proper model to study the metabolites of the ionophore. The hepatoma cells maintain some physiological and metabolic profile of healthy liver cells and are widely used to assess drugs cytotoxicity simultaneously with their biotransformation (Wilk-Zasadna et al., 2015). The myoblasts line (L-6) isolated from primary cultures of rat thigh (Yaffe, 1968) represent right, sensitive tool for myotoxicity testing of the ionophore which might be relevant to *in vivo* scenario.

The aim this study was to describe the cytotoxicity and biotransformation of SAL and its interaction with tiamulin or prednisolone using three rat cell models of different metabolic activities: primary hepatocytes, hepatoma (FaO) cells and myoblasts (L6). The battery of tests estimating different endpoints (Weyermenn et al., 2005) were used to evaluate basal cytotoxicity. Subsequently, the direction and intensity of the interaction with tiamulin and prednisolone were measured using combination index (CI). The drug and its metabolites in the culture media were determined by LC-MS/MS analysis.

2. Materials and methods

2.1. Chemicals and reagents

Analytical standards of salinomycin monosodium salt hydrate (SAL, CAS: 55721–31-8), tiamulin (T, CAS 55297-96-6), prednisolone (P, CAS 50-24-8) and monensin sodium (MON, CAS: 22373-78-0) were purchased from Sigma-Aldrich (Germany). Triton X-100, dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), bovine calf serum (BCS), neutral red dye (NR), coomassie brilliant blue R-250 dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsin-EDTA, antibiotic solution (10,000 U/ml of penicillin, 10 mg/ml of streptomycin), collagenase IV, insulin, hydrocortisone were purchased from Sigma – Aldrich (Germany). Acetonitrile, methanol, ammonium formate, formic acid, all HPLC or LC-MS grade were purchased from J.J.Baker (Germany). All other chemicals were purchased from commercial suppliers and were of the highest available purity.

2.2. Isolation and culture of rat hepatocytes

The procedure of isolation was carried out according to the bioethical principles and in compliance with the permission of Local Ethical Commission (University of Life Sciences in Lublin, Poland). Hepatocytes were obtained from male Wistar rats weighing 250–350 g and fed *ad libitum*. The cells were isolated from rat livers using the two-step collagenase perfusion technique described by Seglen (1976). Hepatocytes were cultured using William's medium E (Gibco, USA) supplemented with 10% FBS, 1 μM insulin and hydrocortisone, 200 μM glutamine and antibiotics in a humidified incubator at 37 °C, in an atmosphere of 5% CO_2. The cells were seeded on 96-well plates coated with fibronectin (Corning BioCoat) at a density of 5×10^5 cells/well in 100 μl of medium and were incubated until attached. After that (ca 4 h) the medium was replaced with fresh medium containing the studied drugs.

2.3. Cell line cultures

The rat hepatoma (FaO) cell line was purchased from the European Collection of Cell Cultures (ECACC 89042701, UK). These cells were cultured in F12 nutrient mixture (Kaighn's modification) (Gibco, USA). The rat myoblasts (L6) cell line was purchased from the American Type Culture Collection (ATCC). These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ATCC). The media were supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin and streptomycin. The cells were maintained in 75 cm^2 cell culture flasks (Nunc, USA) in a humidified incubator at 37 °C, in an atmosphere of 5% CO_2. The medium was refreshed every two or three days and the cells were trypsinized with 0.25% trypsin–0.02% EDTA after reaching 70–80% confluence. Single cell suspensions were prepared and adjusted to a density of 2×10^5 cell/ml (FaO) and 1×10^5 cell/ml (L6). The cell suspension was transferred to 96-well plates (100 μl/well) and incubated for 24 h before the exposure to the studied drugs.

2.4. Exposure to drugs

The concentration ranges of drugs were chosen according to their solubility and their plasma level. Each drug was dissolved in DMSO. The final concentration of DMSO was 0.1% or 0.2% in the medium. The same final concentration of the solvent and solution 1% Triton-× 100 were used as negative and positive control, respectively. The medium used for test solutions and in control preparation did not contain serum and antibiotics. All drug solutions in the medium were freshly prepared and protected from light. SAL was tested in eight concentrations from 0.39 to 50 μg/ml. Tiamulin was added in two (1 and 10 μg/ml) or just one concentration (1 μg/ml) to cell lines or isolated rat hepatocytes, respectively. Prednisolone was tested in one concentration – 1 μg/ml independent of the type of cells. Each concentration of SAL or the combination of the drug with tiamulin or prednisolone were tested in six replicates during three (cell lines) or four (isolated rat hepatocytes) independent experiments. The cytotoxicity was assessed after 24 and 48 h of cells lines exposure or after 12 and 24 h of isolated rat hepatocytes exposure. The medium was not changed during the incubation time.

2.5. Cytotoxicity assessment

2.5.1. MTT assay

The metabolic activity of living cells was assessed by the measurement of the activity of dehydrogenases (Mosmann, 1983). After incubation of the cells with drugs, 10 μl of the MTT solution (5 mg/ml in PBS) was added to each well of 96-well plates and incubated. After 3 h the MTT solution was removed and the intracellular formazan crystals were dissolved in 100 μl DMSO. The plate was shaken for 15 min at room temperature and transferred to a microplate reader (Multiscan RC Labsystems, Thermo, USA) to measure the absorbance at 570 nm, using blank as a reference. Cytotoxicity was expressed as a percentage of the negative control (0.1% or 0.2% DMSO).

2.5.2. NRU assay

The assay, based on staining of living cells by neutral red, was performed according to the protocol described by Borenfreund and Puerner (1985). After the incubation, the medium containing the drug was removed and the cells were washed with PBS. Then 100 μl/well of NR solution (50 μg/ml) was added for 3 h. After this time the cells were washed with PBS. The dye from viable cells was released by extraction with a mixture of acetic acid, ethanol and water. After 10 min of shaking, the absorbance of the dissolved NR was measured at 540 nm using blank as a reference. Cytotoxicity was expressed as a percentage of the negative control (0.1% or 0.2% DMSO).

2.5.3. LDH leakage assay

The integrity of the plasma membrane was assessed through the test of lactate dehydrogenase (LDH) release (Korzeniewski and Calleawert, 1983), which was monitored using the commercially available Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Poland). The medium (100 μl/well) without cells was transferred into the corresponding wells of an optically clear 96-well flat bottom microplate and 100 μl reaction mixture was added to each well. Then the plates were incubated for 30 min at room temperature in darkness. After that time, 50 μl/well 1 M HCl was added to stop the reaction. The absorbance was measured at 492 nm in a microplate reader using blank as a reference.

2.5.4. TPC assay

The assay was based on staining total cellular protein (Bradford,

1976). After the incubation, the medium containing drug was removed and 100 µl of coomassie brilliant blue R-250 dye was added to each well. The plate was shaken for 10 min. Then the stain was removed and the cells were rinsed twice with 100 µl of washing solution (glacial acetic acid/ethanol/water). After that, 100 µl of the desorbing solution (1 M potassium acetate) was added and plates were shaken again for 10 min. The absorbance was measured at 595 nm in a microplate reader using blank as a reference. Cytotoxicity was expressed as a percentage of the negative control (0.1% or 0.2% DMSO).

2.6. Analysis of drugs interactions

The nature of the interaction between salinomycin and tiamulin or prednisolone was analyzed using combination index (CI) introduced by Chou and Talalay (1984) for quantification of synergism or antagonism of two drugs. The L6 and FaO cells were incubated for 24 and 48 h with SAL in concentrations from 0.39–50 µg/ml and with tiamulin (1 or 10 µg/ml) or prednisolone (1 µg/ml). The isolated rat hepatocytes were incubated for 12 and 24 h with SAL in concentrations from 0.39–50 µg/ml and with tiamulin (1 µg/ml) or prednisolone (1 µg/ml). The tested concentrations of both tiamulin and prednisolone were previously proved to be non-cytotoxic for all tested cell models.

The CI (combination index) was calculated, which is mathematically compiled algorithm for the pharmacological interaction of two drugs and denominates its nature. CI = (C)1/(x)1 + (C)2/(x)2, where (C)1 or (C)2 represents the concentrations of drug 1 or 2 used in combination to achieve x% cell viability. Values of CI ≤ 0.9, between 0.9 and 1.1, and ≥1.1 indicate synergism, additive effect, and antagonism, respectively.

2.7. Determination of SAL metabolites

2.7.1. Sample preparation

The medium from microplate wells was collected for the identification of SAL metabolites. An aliquot of 200 µl of each sample was measured to an eppendorf tube. Next, 5 µl of MON solution 10 µg/ml (used as internal standard), 100 µl of 40% sodium acetate solution and 500 µl of acetonitrile was added. The samples were vortex-mixed for 30 s and centrifuged (14,000 rpm, 5 min). The upper organic phase was transferred to a clean glass tube and evaporated to dryness under the stream of nitrogen at the temperature 45 °C. The dry residue was reconstituted in 50% acetonitrile and analyzed with LC-MS/MS.

2.7.2. LC-MS/MS determination

The analysis was performed on 1200 (Agilent, USA) coupled with 5500 QTrap (Sciex, Canada). The system was operated by Analyst 1.6.2 software. SAL metabolites were separated on Poroshell 120 EC-C18, 2.1 mm × 100 mm, 2.7 µm column (Agilent, USA) coupled with precolumn. Gradient elution was applied with acetonitrile: methanol mixture (60:40, v:v) used as phase A and 0.01 ammonium formate pH 4.0 (phase B). The gradient was as follows: 0–1 min 20% A, 1.5–14 min 95%, from 14.5 min re-equilibration with 20% A. The column temperature was kept at 45 °C, the flow of the mobile phase was 250 µl/min, and the injected volume was 10 µl.

The detection was performed in electrospray positive ionization mode (ESI+). The parameters of an ion source and collision cell were optimized for salinomycin and were as follows: the source temperature 475 °C, capillary voltage +5.5 kV, curtain gas 20 psi, both nebuliser gas and turbo heater gas 20 psi, declustering potential 65 eV, entrance potential 10 eV, collision energy 65 eV, collision exit potential 20 eV. The collision gas was set as High.

Analytes were detected as sodium adducts, $[M + Na]^+$. Salinomycin (m/z 774.1 to m/z 431.2) and monensin (m/z 693.4 to m/z 461.4) were monitored in multiple reaction monitoring (MRM) mode. Based on theoretical biotransformation of salinomycin, 63 multiple reaction monitoring (MRM) transitions were designed and included in the method. Additionally, information dependent analysis (IDA) experiments were performed in full scan mode (enhanced product ion spectra of four most abundant MRM transitions). The method is described in detail elsewhere (Olejnik et al., 2018).

2.7.3. Data analysis

The signals for further analysis were selected with the LightSight 2.3 software. Extracts from control samples (media incubated with corresponding concentrations of salinomycin) were used as reference chromatograms. Based on this analysis, seven MRM transitions were chosen as containing potential SAL metabolites. These MRM transitions (with 16 chromatographic peaks) were then quantified with Multiquant 2.1. software.

2.8. Statistical analysis

The results of the tests were expressed as mean ± SD (standard deviation). The experiments were performed in three or four independent repetitions. Statistical analysis was performed using Prism5 (Version 5.0; GraphPad; San Diego, CA, USA). One way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* test was applied. The values indicated cytotoxicity concentration (EC_{50}) at two time points were calculated according to the Hill's equation (sigmoidal model of concentration-response curve) and expressed as a mean ± SEM (n = 3 or n = 4). Statistical evaluation was performed using ANOVA followed by Tukey's post-hoc test. Values of $P \leq 0.05$ were considered statistically significant.

3. Results

3.1. Cytotoxicity

The viability of primary rat hepatocytes, rat hepatoma (FaO) cells and myoblasts after exposure to drugs was time-, concentration- and assay- dependent (Fig. 1). The viability of cells co-treated with tiamulin was decreased compared to SAL alone. The opposite effect was observed when the cells were exposed to SAL with prednisolone. In primary rat hepatocytes, SAL affected mostly lysosomal activity and membrane integrity. SAL did not cause detachment of study cells, based on total protein content assay. In hepatoma (FaO) cells, the significant effect was noted already at 0.39 µg/ml of SAL alone or in both drug combinations in all studied endpoints. The myoblasts metabolism and lysosomal activity were significantly affected in the concentration of 0.39 µg/ml of SAL alone or with tiamulin or prednisolone (Fig. 1, Supplementary material).

The obtained EC_{50} results are shown in Tables 1 and 2. In primary rat hepatocytes, the lowest EC_{50} values were noted in NRU and LDH assay. The EC_{50} values for SAL co-action with tiamulin were lower than for SAL alone in MTT, NRU and LDH assays in these cells. In case of rat hepatocytes co-treated with prednisolone, the EC_{50} values were not significantly changed when compared to the results for SAL alone (Table 1).

The EC_{50} results obtained in primary rat hepatocytes for 24 h exposition were lower in MTT and LDH assay but higher in NRU and TPC assay when compared to the values in rat hepatoma (FaO) cells (Tables 1 and 2). The EC_{50} values (< 0.39 µg/ml, the lowest studied concentration) for SAL alone and its co-action with tiamulin or prednisolone were found in MTT (48 h) and NRU (24, 48 h) assay in FaO cells. The EC_{50} values for SAL co-action with 10 µg/ml of tiamulin was significant lower in MTT (24 h) and LDH (24 and 48 h) assay when compared to the results for SAL alone or co-action with 1 µg/ml of tiamulin. In case of co-action of SAL with prednisolone, the EC_{50} values significantly increased in TPC (24 and 48 h) and LDH (48 h) assay when compared to the values for SAL alone (Table 2).

The EC_{50} values for primary rat hepatocytes and hepatoma (FaO) cells were lower to the results obtained for myoblasts at 24 h time of

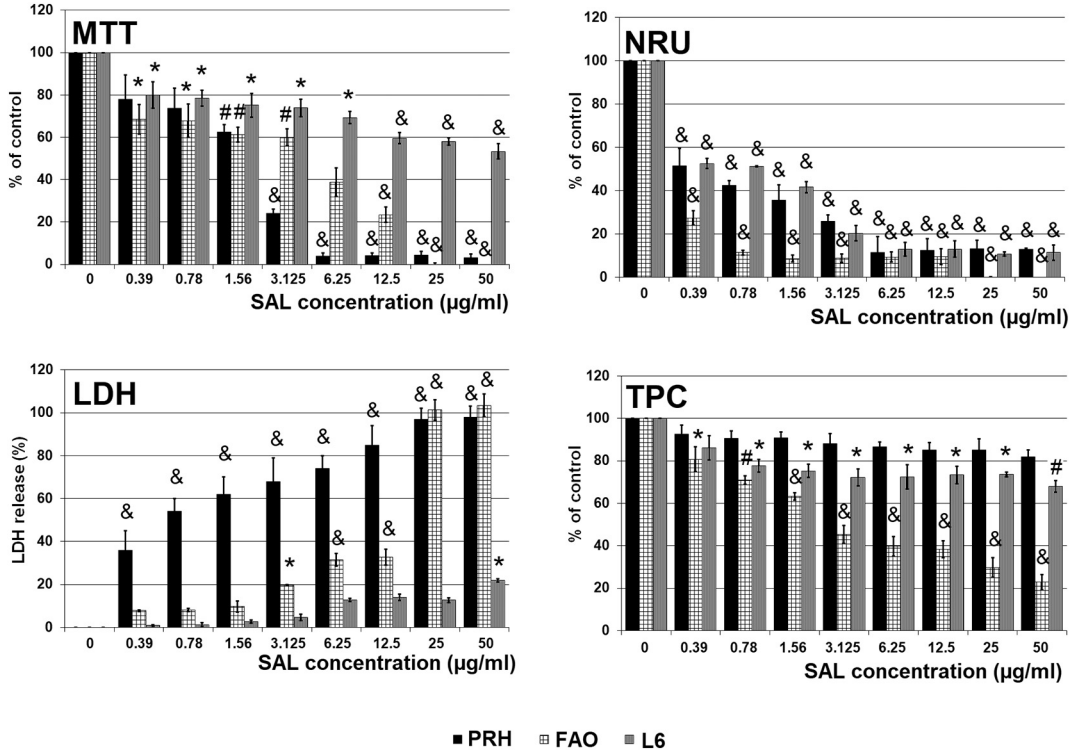

Fig. 1. Effects of 24 h incubation of primary rat hepatocytes (PRH), hepatoma cells (FaO) and myoblasts (L6) with salinomycin (SAL) on metabolism activity (MTT), lysosomal activity (NRU), membrane integrity (LDH)and total protein contents (TPC). Results were calculated as % of solvent control (mean ± SD) (n = 3, cell lines or n = 4, hepatocytes; independent biological replicates). Statistical significance was evaluated by ANOVA and Dunnet's post-test (*p ≤ 0.05; #p ≤ 0.01; & ≤ 0.001).

Table 1
The effective concentrations (EC_{50}, µg/ml) determined in primary rat hepatocytes by MTT, NRU, LDH, TPC assays after 12 and 24 h exposure to salinomycin (SAL) and its combination index (CI) with tiamulin, 1 µg/ml (SAL + T) or prednisolone, 1 µg/ml (SAL + P); data are presented as mean ± SEM (n = 4, independent biological replicates).

Primary rat hepatocytes						
Assay	Time (h)	SAL	SAL + T	CI	SAL + P	CI
MTT	12	4.7 ± 0.8[a]	2.7 ± 0.7[b]	0.71	3.6 ± 1.5[ab]	1.06
	24	2.5 ± 0.2[a]	2.2 ± 0.5[a]	1.03	2.3 ± 0.8[a]	1.11
NRU	12	1.8 ± 1.1[a]	0.6 ± 0.4[a]	0.56	< 0.39	–
	24	0.5 ± 0.2	< 0.39	–	< 0.39	–
LDH	12	2.0 ± 0.5[a]	< 0.39	–	2.2 ± 0.3[a]	1.35
	24	0.7 ± 0.3[a]	< 0.39	–	0.5 ± 0.1[a]	0.86
TPC	12	> 50	> 50	–	> 50	–
	24	> 50	> 50	–	> 50	–

The different small letters (a-b) within lines indicate significant differences (P ≤ 0.05).
CI value higher than 1 indicates antagonism, CI not different from 1 indicates addition, and CI < 1 indicates synergism.

exposition (Tables 1 and 2). The lowest EC_{50} values for SAL and co-action with tiamulin and prednisolone in NRU assay were noted in myoblasts. The significant decrease of these values for SAL co-action with 10 µg/ml tiamulin was reported when compared to SAL alone or its co-action with 1 µg/ml tiamulin. In case of myoblasts treated with SAL prednisolone mixture, the EC_{50} values also not statistically changed when compared to SAL alone except for TPC (48 h) assay (Table 2).

3.2. Interaction of salinomycin with tiamulin or prednisolone

The combination index analysis adopted to determine the type of interaction between SAL and tiamulin or prednisolone on primary rat hepatocytes, hepatoma (FaO) cells and rat myoblasts are shown in Tables 1 and 2. The interaction between SAL and tiamulin in hepatocytes showed a considerable synergistic (CI = 0.6–1.0) mode in MTT (12 and 24 h) and NRU (12 h) assay. In contrast, the interaction between the ionophore and prednisolone in hepatocytes showed antagonistic (CI = 1.1–1.4) in MTT (12 and 24 h) and LDH (12 h) or additive effects (CI = 0.9, LDH, 24 h) assay (Table 1).

The mode of interaction between SAL and tiamulin at concentration of 1 µg/ml in hepatoma (FaO) cells displayed additive (CI = 0.9–1.0) effects in MTT (24 h), TPC and LDH (24, 48 h) assays. Strong synergistic mode of interaction (CI = 0.3–0.8) was observed between SAL and tiamulin at higher concentration, 10 µg/ml. In case of SAL co-action with prednisolone, the CI (1.1–2.7) values showed an antagonistic mode of interaction in hepatoma cells (Table 2).

The mode of interaction between the ionophore and tiamulin in rat myoblasts displayed strong synergistic effects (CI = 0.1–0.6). The additive and poor antagonistic effects (CI = 1.0–1.1) of SAL co-action with prednisolone were observed in TPC and LDH after 48 h myoblasts exposition (Table 2).

3.3. Metabolites of salinomycin

Many signals of SAL–related compounds were present in the samples. The majority of them were, however, detected also in control samples. Finally, 16 potential metabolites were identified in primary hepatocytes model. They were mainly hydroxy-derivatives with one (4 compounds), two (7) or three (3) atoms of oxygen attached; two metabolites were dehydrogenation products. Among identified metabolites, only three were present in the culture media from FaO cells and none was found to be produced by myoblasts (L6). The results are presented in Table 3, in Fig. 2 and in Supplementary material.

Despite this high number of detected metabolites, the efficiency of SAL biotransformation was low. In FaO cells the percentage of the

Table 2
The effective concentrations (EC_{50}, μg/ml) determined in L6 and FaO cells by MTT, NRU, LDH, TPC assays after 24 and 48 h exposure to salinomycin (SAL) and its combination index (CI) with tiamulin, 1 μg/ml (SAL + T1) and 10 μg/ml (SAL + T10) or prednisolone, 1 μg/ml (SAL + P1); data are presented as mean ± SEM (n = 3, independent biological replicates).

Cell line	Assay	Time (h)	SAL	SAL + T (1)	CI	SAL + T (10)	CI	SAL + P (1)	CI
FaO	MTT	24	4.7 ± 0.8[a]	4.2 ± 0.5[a]	1.08	0.6 ± 0.2[b]	0.43	4.0 ± 0.9[a]	1.19
		48	< 0.39	< 0.39	–	< 0.39	–	< 0.39	–
	NRU	24	< 0.39	< 0.39	–	< 0.39	–	< 0.39	–
		48	< 0.39	< 0.39	–	< 0.39	–	< 0.39	–
	LDH	24	15.7 ± 0.8[a]	15.7 ± 1.7[a]	1.12	5.5 ± 0.1[b]	0.74	16.7 ± 0.2[a]	1.19
		48	4.6 ± 0.1[a]	4.7 ± 0.2[a]	0.98	3.5 ± 0.2[b]	0.54	6.6 ± 0.3[c]	2.67
	TPC	24	2.7 ± 0.6[a]	2.5 ± 0.4[a]	0.93	2.2 ± 0.5[a]	0.95	9.8 ± 0.9[b]	1.59
		48	0.9 ± 0.3[a]	1.1 ± 0.6[ab]	0.99	< 0.39	–	1.8 ± 0.4[b]	1.25
L6	MTT	24	> 50	28.2 ± 4.1[a]	0.37	0.7 ± 0.2[b]	0.13	> 50	–
		48	> 50	5.3 ± 0.5	0.15	< 0.39	–	> 50	–
	NRU	24	0.9 ± 0.2	< 0.39	–	< 0.39	–	< 0.39	–
		48	< 0.39	< 0.39	–	< 0.39	–	< 0.39	–
	LDH	24	> 50	> 50	–	4.9 ± 1.3	0.30	> 50	–
		48	49.2 ± 0.7[a]	23.5 ± 1.5[a]	0.56	1.7 ± 0.2[b]	0.19	48.6 ± 0.6[a]	1.02
	TPC	24	> 50	44.4 ± 2.4[a]	0.63	4.2 ± 1.1[b]	0.25	> 50	–
		48	29.1 ± 1.9[a]	0.7 ± 0.2[b]	0.15	< 0.39	–	29.9 ± 2.0[a]	1.02

The different small letters (a-c) within lines indicate significant differences ($P \leq 0.05$).
CI value higher than 1 indicates antagonism, CI not different from 1 indicates addition, and CI < 1 indicates synergism.

metabolized drug was 4% and in primary hepatocytes – 28%. The cells co-exposed to tiamulin were slightly less metabolically potent – they reduced SAL concentration by 3% and 15%, respectively. The addition of prednisolone was significant only for FaO cells, where it increased metabolism rate to 12%.

The quantification of identified metabolites was not possible because no reference standards are available; therefore the signals were compared to SAL peak area. Among detected compounds, only one was present in higher concentration (above 10% SAL), and only two additional ones gave signals exceeding 1% of SAL.

4. Discussion

In vitro tests performed in cell cultures derived from liver can establish biotransformation rate and pathways and assess its impact on toxic properties. For that purpose, three different model cell cultures from rat were selected to study the differences between primary hepatocytes, hepatoma (FaO) cells and myoblasts (L6) in response to SAL alone or in combination with tiamulin or prednisolone.

SAL impairs the flow of ions (K^+/Na^+) through cellular membranes, which is responsible for both pharmacological and toxicological action. It leads to Ca^{2+} overload within cells followed by decreased cellular energy and inhibits lysosomal protein degradation by affecting pH inside the organelle (Grinde, 1983; Boehmerle and Endres, 2011; Sommer et al., 2016; Zou et al., 2017). The determined median effective concentrations of the ionophore (EC_{50} < 0.39 μg/ml) alone or in co-action with tiamulin or prednisolone confirm strong inhibition of lysosomal activity. The effects of the drug on primary rat hepatocytes also led to the disintegration of the cell membrane. Our findings are in accordance with other research, in which ionophore inhibited lysosomal activity (in different cell cultures) leading to the cell death (Yue et al., 2013; Sommer et al., 2016; Xipell et al., 2016; Zou et al., 2017).

It is well known that simultaneous administration of tiamulin and ionophore antibiotics may lead to a toxic interaction (also to death) in animals (Islam et al., 2008; Weisman et al., 1980). However, there are no studies on the interaction of SAL with tiamulin or prednisolone performed at cell cultures. Our study confirmed that the ionophore was more cytotoxic in combination with tiamulin. The synergistic

Table 3
The metabolites of salinomycin identified using mass spectrometry in the cell medium from rat myoblasts (L6), rat hepatoma (FaO) and rat primary hepatocytes (PRH).

Metabolite ID	Relative RT (compared to SAL)	Transition	Biotransformation pathway	L6	FaO	PRH
M1	0.5599	822.1/463.2	Tri-hydroxylation	–	–	+
M2	0.6247	806.1/447.2	Di-hydroxylation	–	–	+ +
M3	0.6530	806.1/447.2	Di-hydroxylation	–	–	+
M4	0.6649	822.1/463.2	Tri-hydroxylation	–	–	+
M5	0.6776	790.1/431.2	Hydroxylation	–	+	+ +
M6	0.6873	806.1/463.2	Di-hydroxylation	–	–	+ +
M7	0.7246	822.1/463.2	Tri-hydroxylation	–	–	+
M8	0.7729	806.1/447.2	Di-hydroxylation	–	–	+ +
M9	0.7870	790.1/431.2	Hydroxylation	–	+ +	+ + +
M10	0.8235	806.1/447.2	Di-hydroxylation	–	–	+ +
M11	0.8362	806.1/463.2	Di-hydroxylation	–	–	+ + +
M12	0.8451	790.1/431.2	Hydroxylation	–	+	+ +
M13	0.8928	790.1/447.2	Hydroxylation	–	–	+ + + +
M14	0.9397	772.1/429.2	Dehydrogenation	–	–	+ +
M15	0.9609	806.1/463.2	Di-hydroxylation	–	–	+
M16	0.9691	772.1/429.2	Dehydrogenation	–	–	+

+ metabolite was detected, and the peak area was at least two times higher than in corresponding control (no cells, the same concentration of salinomycin).
+ + peak area of metabolite constituted at least 0.1% of peak area of salinomycin.
+ + + peak area of metabolite constituted at least 1% of peak area of salinomycin.
+ + + + peak area of metabolite constituted at least 10% of peak area of salinomycin.

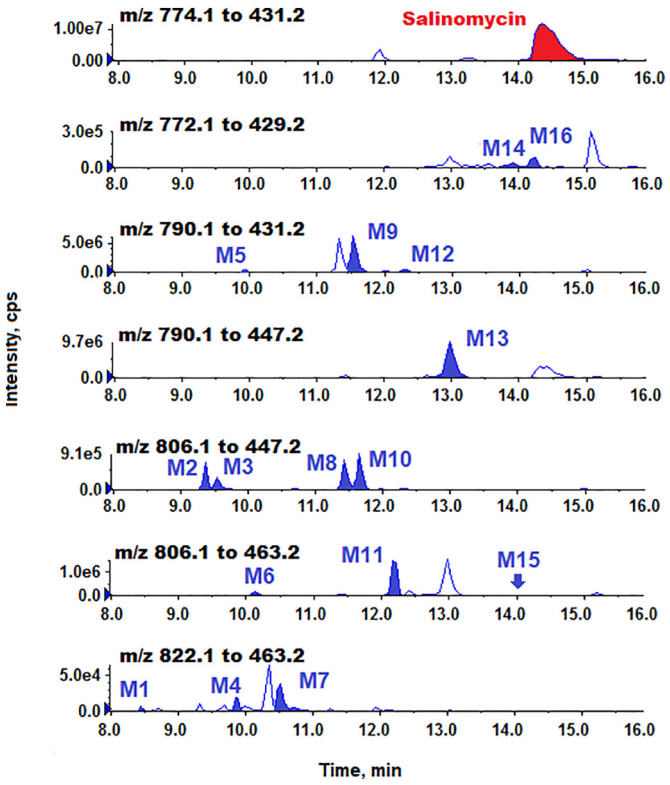

Fig. 2. Selected reaction monitoring chromatogram of extract from culture medium of primary rat hepatocytes exposed to salinomycin, 50 μg/ml for 24 h. The chromatograms represent signals of salinomycin (m/z 774.1 to 431.2), dehydro-salinomycin (m/z 772.1 to 429.2), hydroxy-salinomycin (m/z 790.1 to 431.2 and m/z 790.1 to 447.2), dihydroxy-salinomycin (m/z 806.1 to 447.2 and m/z to 463.2) and trihydroxy-salinomycin (m/z 822.1 to 463.2). The metabolites identified LightSight software are marked as M1 to M16.

interaction between tiamulin and SAL was recorded by a combination index (CI below 1). The interaction of the ionophore with tiamulin involves their influence on the cytochrome P-450, which plays an important role in the oxidative biotransformation of SAL (Nebbia et al., 1999).

On the other side, the decreased cytotoxicity of the ionophore was observed in cell cultures co-treated with prednisolone. The antagonistic interaction between prednisolone and SAL was recorded in our study (CI above 1). Many studies suggest prednisolone potential to induce CYP3A4 izoenzymes (Matoulkova et al., 2014). Another glucocorticoid (dexamethasone) has been proven to show the same action in a study on monensin metabolism (Szucs et al., 2004).

Numerous studies followed describing the effects of SAL as a potent killer of cancer stem cells. Notably, the compound can efficiently target tumor stem cells in several types of cancer with a relatively low toxicity to normal cells. Very low concentrations, from 0.1 μM (0.08 μg/ml) to 10–30 μM (7.51–22.5 μg/ml) of SAL, were already cytotoxic to different cell lines (Gupta et al., 2009; Lieke et al., 2012; Scherzed et al., 2013; Jangamreddy et al., 2013). Our cytotoxic concentrations on the ionophore were in the range of cited concentrations (< 0.52 to 21 μM) for hepatoma (FaO) cells. However, the values this concentration for myoblasts (L6) were higher than 50 μg/ml (67 μM).

This study clearly shows that cytotoxicity of SAL was dependent on used cell models. The L6 cells were less susceptible to the cytotoxic effect of SAL than rat hepatocytes and hepatoma (FaO) cells cultures. We showed that EC_{50} values were significantly lower for hepatocytes and hepatoma cells than for normal myoblasts cultures. Likewise, human (HepG2) and chicken (LMH) hepatoma lines were more sensitive to SAL cytotoxicity than rat myoblasts (L6) (Cybulski et al., 2015).

The determination of the rate and pathways of SAL biotransformation was performed concurrently with the cytotoxicity to assess the impact of the metabolites on the strength of toxic effects. In all models, the extent of biotransformation was low (maximum 28%) and it is unlikely that the metabolism of SAL was responsible for the observed differences among the tested cells. In fact, primary hepatocytes – the most metabolically efficient model, were at the same time the most susceptible to the ionophore.

After the co-exposure of cells with tiamulin or prednisolone, the level of SAL metabolism slightly changed. As expected (Nebbia et al., 1999; Szucs et al., 2000), tiamulin reduced the rate of biotransformation, especially in primary hepatocytes. Still, this effect seems too subtle to fully explain the observed interaction in cytotoxicity study.

The detected 16 potential metabolites of SAL were mainly hydroxylation products, which well confirms the data from animal studies (Rychen et al., 2017). To our best knowledge, the only previous data on the *in vitro* biotransformation of SAL come from our study on HepG2 cell cultures (Olejnik et al., 2018). The three metabolites identified in FaO cells (M5, M9 and M12) were also produced by human hepatoma cells. The primary hepatocytes, however, were much more metabolically active, which resulted in the formation of di- and tri-hydroxy derivatives of SAL, found previously in chicken *in vivo*. The data for rats are scarce but also in this species SAL is extensively biotransformed and the metabolites seem to be similar to those observed in chicken (Anadón et al., 2004).

5. Conclusions

Cytotoxicity of salinomycin was strongly dependent on the cell type, which confirms the potential of application of the drug as a selective anti-cancer agent. Although the modes of interactions with drugs influencing CYP3A4 were in accordance with literature data, their potency was lower than expected. That means that the differences in cytotoxicity cannot be explained by biotransformation alone. Our results confirmed the common opinion that primary hepatocytes could represent a valuable tool to study the impact of biotransformation on the cytotoxicity of the drug. Further research using microsomal and S9 fractions are needed for qualitative and quantitative analysis of metabolites of salinomycin.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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