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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,