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Preclinical drug metabolism and pharmacokinetics of salinomycin, a potential candidate for targeting human cancer stem cells

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ABSTRACT

There has been a search for new anticancer agents to treat cancer resistance throughout the globe. Salinomycin (SAL), a broad spectrum antibiotic and a coccidiostat has been found to counter tumour resistance and kill cancer stem cells with better efficacy than the existing chemotherapeutic agents; paclitaxel and doxorubicin. This refocused its importance for treatment of human cancers. In this study, we studied the *in vitro* drug metabolism and pharmacokinetic parameters of SAL. SAL undergoes rapid metabolism in liver microsomes and has a high intrinsic clearance. SAL metabolism is mainly mediated by CYP enzymes; CYP3A4 the major enzyme metabolising SAL. The percent plasma protein binding of SAL in human was significantly lower as compared to mouse and rat plasma. CYP inhibition was carried out by chemical inhibition and recombinant enzyme studies. SAL was found to be a moderate inhibitor of CYP2D6 as well as CYP3A4. As CYP3A4 was the major enzyme responsible for metabolism of SAL, *in vivo* pharmacokinetic study in rats was done to check the effect of concomitant administration of Ketoconazole (KTC) on SAL pharmacokinetics. KTC, being a selective CYP3A4 inhibitor increased the systemic exposure of SAL significantly to 7-fold in $AUC_{0-\alpha}$ and 3-fold increase in C_{max} of SAL in rats with concomitant KTC administration.

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1. Introduction

Salinomycin (SAL) has been used for more than 30 years as an effective anti-coccidial drug in poultry and is also fed to ruminants and pigs to improve nutrient absorption and feed efficiency. It is a 751 Da monocarboxylic polyether ionophore isolated from *Streptomyces albus*, constituting a large pentacyclic molecule with a unique tricyclic spiroketal ring system and an unsaturated six-membered ring. It is a lipophilic, anionic and weakly acidic compound with the molecular formula $C_{42}H_{70}O_{11}$ [1–3]. SAL has been shown to target Cancer Stem Cells (CSCs) in different types of human cancers, including gastric cancer [4], lung [5], osteosarcoma [6], colorectal cancer [3], squamous cell carcinoma (SCC) [7], and prostate CSCs [8] mainly by inhibiting wnt signalling pathway [9–13]. SAL is able to enhance the cytotoxic effects of conventional anti cancer drugs such as doxorubicin, gemcitabine, etoposide, paclitaxel, docetaxel, vinblastine, and trastuzumab, envisioning a central role for SAL-based combination therapies in the future treatment of cancer [10,14–17].

One important caveat for developing SAL as a clinical treatment for cancer is the paucity of preclinical metabolism and pharmacokinetic data. Determination of drug metabolism and pharmacokinetic (DMPK) parameters and effect of these parameters on the pharmacological effects of SAL will help researchers to design the appropriate dose and also predict the interaction with other drugs in the body. Since no scientific reports have been published as yet regarding the DMPK parameters of this drug, it becomes a pre requisite to perform these studies to minimize its toxicity as well as have a complete understanding of this drug's behaviour in therapeutic use. In the present study, *in vitro* metabolism of SAL was

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studied in liver microsomes and S9 fractions for metabolic stability and identification of CYP enzymes involved in the metabolism of SAL along with *in vitro* plasma protein binding affinity studies.

2. Materials and methods

Salinomycin (SAL) was received as a gift sample from Bauji GuangPvt. Ltd. (China). Potassium phosphate buffer (0.1 M, pH 7.4), Fluorogenic substrates 7-benzyloxy-trifluromethyl coumarin (BFC), 3-cyano-7-ethoxycoumarin (CEC), di-benzyl fluorescein (DBF), 7-methoxy 4-trifluromethyl coumarin (MFC), 3-[2-(N,N-diethyl-N-methyl-amino) ethyl]-7-methoxy-4 methyl coumarin (AMMC), microsomes (human, rat and mouse liver); cofactors, G6PDH, control protein, potassium phosphate buffer (0.5 M, pH 7.4) were obtained from BD Gentest Corp. (Woburn, MA), NADP salt, G6PD salt and G6PDH enzyme were purchased from Sisco research laboratories Pvt. Ltd. (Mumbai).

2.1. Methodology

2.1.1. In-vitro studies

2.1.1.1. Metabolic stability assay in liver microsomes (human, rat and mouse) and liver S9 fraction (human, rat and mouse). SAL (10 μM) was incubated with a mixture comprised of microsomes or liver S9 fraction (supernatant fraction obtained from liver homogenate by centrifuging at 9000g for 20 min in a suitable medium). Microsomes and liver S9 fractions equivalent to protein concentration of 0.5 mg/mL were added to phosphate buffer 0.1 M (pH 7.4), NADPH regenerating system (NRS) which contains 10 mM Magnesium chloride, 2.5 mM NADP salt, 25 mM G6PD salt, 0.5 units/mL G6PDH enzyme in a total volume of 1 mL). The control, standard/test compounds were added to the incubation mixture and further incubated for 10 min at 37° C. Aliquots of the incubation solution were withdrawn at different time points after the 10 min incubation time and the reaction was stopped using quenching solution (acetone: ethanol: acetic acid: 1 μM niflumic acid (79:20:0.9:0.1) at 0, 6, 12, 15, 24 and 30 min. In order to confirm the metabolism of SAL by CYP enzymes, SAL was incubated with microsomes in presence 1-amino benzotriazole (1-ABT) which is a non specific CYP inhibitor at concentrations of 50 μM, 100 μM, 200 μM and 400 μM. The concentration of parent compound in all the reaction mixtures was determined by LCMS and calculated in terms of area ratio with internal standard at each sampling point. *In vitro* rate constant (k) and intrinsic clearance were calculated using the following formula:

$$\begin{aligned}&\text{Clearance (mL/min/g liver)}\\&= \text{K(min)} \times [(\text{incubation volume(mL)/microsomal or}\\&\quad \text{S9 protein in incubation(mg)}] \times [\text{microsomal or}\\&\quad \text{S9 protein(mg)}]/\text{liver weight(g)}].\end{aligned}$$

Measured $CL_{IN\ VITRO,INT}$ values were extrapolated to the intact liver using a scaling factor L_{S9} (120.7 mg/g liver) and $L_{microsomal}$ (52.5 mg/g liver) for S9 and microsomal fractions respectively [18,19].

2.1.1.2. Identification of CYP enzymes involved in the metabolism of SAL

A. Chemical inhibition study: The experiment with known chemical inhibitors of CYP isoforms were performed at similar SAL concentration (10 μM) as in liver microsomal incubations. All the inhibitors were co-incubated with the substrate (SAL) at 37° C.

B. Recombinant enzyme study: Incubations with human recombinant P450s were also performed using the same conditions described herein for human liver microsomes, except that the mixture contained 20 pmol CYP (CYP3A4, CYP2C9, CYP2C19 supersomes) or 50 pmol CYP (CYP1A2, CYP2D6 supersomes) with SAL at concentration same as in liver microsomal incubations. The concentration of parent compound (SAL) was determined in terms of area ratio with internal standard for each sampling point considering concentration at zero min to be 100% determined by LCMS analysis.

2.1.1.3. Plasma protein binding assay. Warfarin and SAL 10 mM stock solutions were prepared in DMSO. 5 μM of Warfarin and SAL were incubated in plasma for 10 min at 37 °C (final concentration of DMSO 0.1%). Suitable quantity of the above mixture was taken in ultracentrifuge tubes and centrifuged at 41,000 rpm for 4.30 h at 4° C. After centrifugation, the middle layer plasma supernatants were collected and drug concentration was estimated by LCMS Waters UPLC attached to triple quadrupole mass detector from ABSCIEX. A calibration curve of standard as well as test drug was prepared to estimate the unknown concentration in the unbound fraction of plasma.

$$\text{Percent of drug bound to plasma} = 100 \times \left(1 - \frac{C_{uf}}{C_p}\right)$$

Where,C_{uf} = concentration of standard/test compound in ultra-filtrate.

C_p = initial concentration of standard/test comp in plasma.

2.1.1.4. Fluorescent based CYP inhibition assay. Fluorogenic CYP inhibition studies were conducted at 37 °C in 96-well flat bottomed black polystyrene plates. Enzyme mixture containing recombinant CYP, control protein, substrate, potassium phosphate buffer (0.1 M, pH 7.4) and MilliQ water was prepared; pre incubated for 10 min at 37 °C. SAL (10 μM) in DMSO (0.1%) was combined with enzyme-substrate solution and the reaction was initiated by addition of NRS. A positive control experiment, i.e., with standard inhibitor was performed simultaneously. The reaction was stopped by addition of stop solution (acetone: ethanol: acetic acid: 1 μM niflumic acid (79:20:0.9:0.1)). The fluorescence was detected as per the emission wavelengths of various substrates by fluorimetric plate reader (BioTek, USA). The percentage inhibition of test compound and positive control in comparison to no inhibitor control was determined using the following equation in Excel fit.

$$\begin{aligned}&\text{Percent inhibition} = 100\\&\quad - \frac{\text{average blank corrected fluorescence of test compound}}{\text{average blank corrected fluorescence of no inhibitor}} \times 100\end{aligned}$$

2.1.1.5. CYP inhibition assay for SAL in HLM using probe substrates. The inhibitory effects of SAL on CYP activity was examined by using 10 μM phenytoin (CYP1A2), 1 μM diclofenac (CYP2C9), 5 μM S-mephenytoin (CYP2C19), 0.3 μM dextromethorphan (CYP2D6), 1 μM nifedipine (CYP3A4) as CYP probe substrate (which is metabolized to a measurable product) using human liver microsomes (HLM). SAL was co-incubated with these CYP marker substrates from 0.1 μM to 100 μM with HLM before the reaction was

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