

Effects of salinomycin and CGP37157 on head and neck squamous cell carcinoma cell lines *in vitro*

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Abstract. Surgery, radiation, chemotherapy or a combinations of these are all accepted modalities for the treatment of head and neck squamous cell carcinoma (HNSCC). Despite this, 40-60% of patients suffering from HNSCC develop loco-regional failure and/or distant metastases. Salinomycin has been demonstrated to be >100-fold more effective than paclitaxel at causing cancer stem cell death, therefore, it may offer an important improvement in cancer therapy. However, the toxicity of salinomycin is of concern. A possible solution may be the administration of additive drugs, which reduce the toxicity. By inhibiting the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger using the benzodiazepine derivate, CGP37157 (CGP), a significant reduction in salinomycin neuronal toxicity has been observed. This raises the question of whether CGP also inhibits the tumor toxicity of salinomycin. In the present study, the FaDu and HLaC79 C1 HNSCC cell lines were treated with salinomycin with or without CGP. Comparative viability assessments were performed using microscopy, a fluorescein diacetate assay, an MTT assay, a clonogenic assay and annexin V-propidium iodide staining. The expression levels of MDR-1 were monitored using reverse transcription-quantitative polymerase chain reaction. Salinomycin alone, and in combination with CGP, achieved a significant attenuation of cell viability and increased apoptosis in a dose-dependent manner. However, the tumor toxicity of salinomycin was not inhibited by CGP. The HLaC79 C1 cells were more sensitive to salinomycin, compared with the FaDu cells, with this sensitivity being due to high expression levels of MDR-1 by the HLaC79 C1 cells. In conclusion, CGP did not counteract the tumor toxicity of salinomycin *in vitro* and may be a promising drug in future

anticancer therapy. The results of the present study encourages further investigation of the toxicological aspects of salinomycin, particularly in human cells and animal models.

Introduction

In total, ~500,000 new cases of head and neck squamous cell carcinoma (HNSCC) are diagnosed annually worldwide (1). The majority of these patients suffer from locally advanced stage disease (2). The treatment options for head and neck cancer include surgery, radiation, chemotherapy or a combination of these modalities. Unresectable SCC formations of the head and neck are frequently treated with concurrent chemotherapy (3). However, 40-60% of patients suffering from HNSCC develop loco-regional failure and distant metastases (4). It has been suggested that this cancer treatment failure is due to the presence of a certain subpopulation of cancer cells, termed cancer stem cell-like cells or cancer initiating cells (CSCs) (5,6). Therefore, there is an increasing requirement for more cancer cell-specific drugs. Gupta *et al* (7) investigated salinomycin as a selective killer of CSCs in a high-throughput screening investigation. Salinomycin was the most effective agent at destroying CSCs out of a total of 16,000 compounds (7). Salinomycin is a 751 Dalton monocarboxylic polyether antibiotic, which is isolated from the bacterium *Streptomyces albus* (8). It has a broad spectrum of bioactivity, including antibacterial, antifungal, antiparasitic, antimalarial and tumor cell cytotoxicity (9). It is widely used in veterinary medicine as an antiprotozoal agent against coccidial parasites. Salinomycin is >100-fold more effective than paclitaxel at causing CSC death (7). However, the complex underlying mechanisms involved remain to be fully elucidated. A previous study by Riccioni *et al* (10) reported that salinomycin acts as a potent inhibitor of p-glycoprotein.

The potential clinical treatment options of salinomycin are restricted by neuronal, muscular and other toxicities (11). The former is mediated by an increased concentration of Na^+ , which leads to an increase in cytosolic Ca^{2+} concentration via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger ($\text{Na}^+/\text{Ca}^{2+}\text{-X}$) in the plasma membrane and the mitochondria (11). By inhibiting the mitochondrial $\text{Na}^+/\text{Ca}^{2+}\text{-X}$ using the benzodiazepine derivate, CGP37157 (CGP), Boehmerle and Endres (11) demonstrated a significant reduction in salinomycin neuronal toxicity. CGP may offer

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potential as a novel additive in treatment with salinomycin to reduce its toxicity and, thus enable future clinical use. The aim of the present study was to investigate whether CGP also inhibits the tumor toxicity of salinomycin.

Materials and methods

Culture of the human carcinoma cell line. The FaDu HNSCC cell line (American Type Culture Collection, Manassas, VA, USA), which is derived from a human hypopharyngeal carcinoma, and HLaC79 clone 1 (C1), a paclitaxel resistant clone, were used in the present study. The HLaC79 C1 was generated in the Department of Otolaryngology, University of Wuerzburg (Wuerzburg, Germany) (12-14). The cells were grown in RPMI-1640 medium (Biochrom AG, Berlin, Germany), supplemented with 10% fetal calf serum (Linaris, Wertheim-Bettingen Germany), 100 U/ml penicillin, 100 µg/ml streptomycin, 1% sodium pyruvate (100 mM; Biochrom AG) and 1% non-essential amino acids (100-fold concentration; Biochrom AG), which was termed RPMI-expansion medium (RPMI-EM). The cells were cultured at 37°C with 5% CO₂ in culture flasks. The medium was replaced every other day and passaging was performed on reaching 70-80% confluence by trypsinization (0.25% trypsin; Gibco Life Technologies, Karlsruhe, Germany), washing with phosphate-buffered saline (PBS; Roche Diagnostics GmbH, Mannheim, Germany) and seeding into flasks or treatment wells. The subsequent experiments were performed using cells in the exponential growth phase.

The HNSCC cells were seeded at a density of 6x10³ cells/well and incubated for 24 h at 37°C. Subsequently, the cells were treated either with salinomycin (1, 5 and 10 µM; Sigma-Aldrich, Schnellendorf, Germany) or with salinomycin (1, 5 and 10 µM) + CGP (10 µM; Sigma-Aldrich) for 24 h, in order to assess the possible interaction between salinomycin and CGP. All experiments were performed in triplicate.

Cell morphology and fluorescein diacetate assay (FDA). The cells were cultured as a monolayer and in three-dimensional conditions (spheroids). Following the treatment with salinomycin, an FDA (5 mg/ml FDA in acetone; Sigma-Aldrich) was used in order to identify viable cells in the culture. Following treatment with salinomycin and salinomycin + CGP for 24 h, the cells (6x10³) were stained with a solution of 80 µg/ml fluorescein diacetate and 50 µg/ml ethidium bromide (Sigma-Aldrich). Subsequently, the cells were observed under a fluorescence microscope (Leica DMI 4000B Inverted Microscope; Leica Microsystems, Wetzlar, Germany). Living cells convert the non-fluorescent FDA into the green fluorescent compound, fluorescein, while dead cells exhibit red-orange staining in their nuclei.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and clonogenic assay. The MTT assay (Sigma-Aldrich) is a colorimetric staining method, according to Mosmann (15) and was used to study the viability of cells. The plates were incubated with 100 µl MTT (1 mg/ml), followed by 5 h incubation at 37°C with 5% CO₂. Following the removal of MTT, 100 µl isopropanol (Sigma-Aldrich) was added for 1 h at 37°C with 5% CO₂. The viability of the cells was quantified

by measuring the absorbance at 570 nm (Titertek Multiskan PLUS (MK II) ELISA-reader; Labsystems, Helsinki, Finland).

To determine the long-term effects of the treatment reagents, the cells were treated with salinomycin for 24 h at 37°C. Subsequently, the supernatant was removed and fresh medium was added. Following incubation for 14 days at 37°C the cell colonies were stained with crystal violet (0.4 g/l; Sigma-Aldrich) and counted using the Leica DMI 4000B Inverted Microscope (Leica Microsystems, Wetzlar, Germany).

Annexin V-propidium iodide (PI) staining. Apoptosis of the FaDu and HLaC79 C1 HNSCC cell lines was assessed using an Apoptosis Detection kit (BD Biosciences, Heidelberg, Germany). Following exposure to salinomycin or salinomycin + CGP for 24 h at different concentrations (1, 5 and 10 µM) at 37°C, the cells in suspension and the adherent cells were harvested and washed twice with cold PBS. The cells were resuspended in 1:10 binding buffer (Sigma-Aldrich), containing 0.1 M HEPES (pH 7.4), 1.4 M NaCl and 25 mM CaCl₂, at a concentration of 1x10⁶ cells/ml, and 100 µl aliquots of this cell suspension (1x10⁵ cells) were subsequently transferred to a 5 ml culture tube. Annexin V-APC (5 µl) and PI (5 µl) were added to each aliquot containing 1x10⁵ cells. Following incubation for 15 min in the dark at room temperature, the cells were resuspended in 400 µl 1:10 binding buffer. A FACSCanto flow cytometer (Beckton Dickinson, Heidelberg, Germany) was used to analyze the samples. PI staining is visible in cells with damaged membranes, as observed during necrosis.

Total RNA extraction, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression levels of *MDR-1* in the FaDu and HLaC79 C1 cells were investigated using RT-qPCR. The total RNA was extracted from the cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The extracted total RNA was reverse transcribed into cDNA using the High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA). For the quantification of gene expression, a SYBR Green PCR Master Mix kit (Applied Biosystems) was used. The *MDR-1* primer was purchased from Applied Biosystems, with the following sequences: Forward 5'-AGAAAGCGAAGCAGTGGT TCA-3' and reverse 5'-CGAACTGTAGACAAACGATGAG CTA-3'. PCR was performed in duplicate using 100 ng cDNA per replicate on the StepOnePlus Real-Time PCR system (Applied Biosystems). The amplifications for gene quantification were as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. RNA levels were quantified using a photometer (BioPhotometer; Eppendorf, Hamburg Germany) and the expression levels of *GAPDH* were used as an internal control.

Statistical analysis. All data were analyzed by statistical analysis with GraphPad Prism software, version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). To investigate whether the cytotoxic effect of salinomycin was dose-dependent, Friedman's test was used. The Kruskal-Wallis test was conducted for all other tests to evaluate statistical significance.

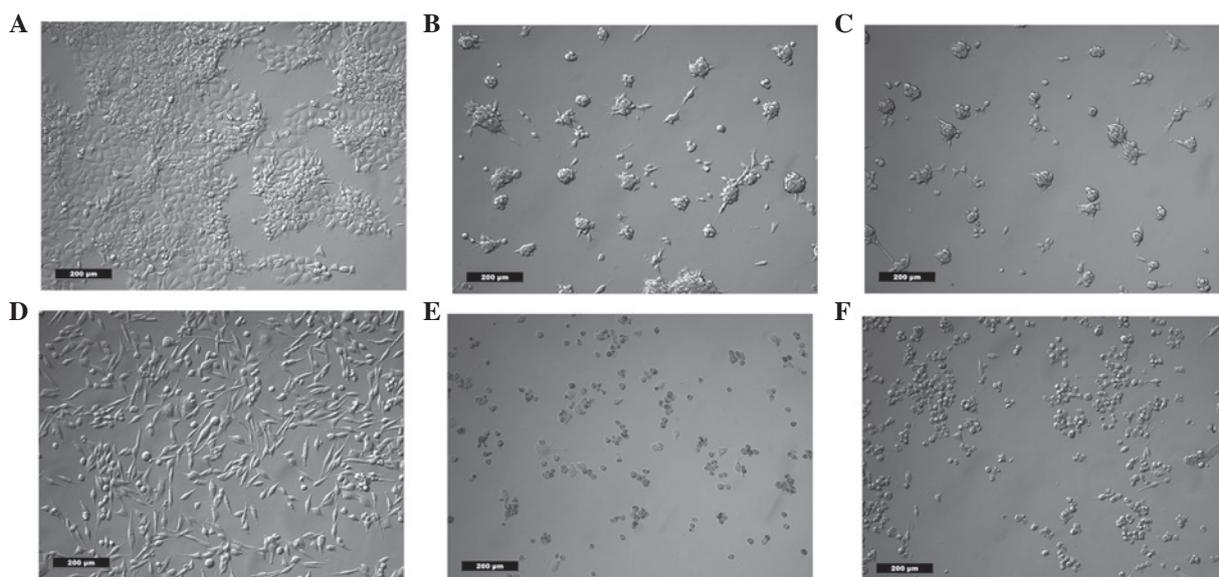


Figure 1. Microscopic analysis of head and neck squamous cell carcinoma cell lines treated with salinomycin or with salinomycin \pm CGP. Representative images of FaDu cells cultivated with (A) RPMI-EM, (B) $10 \mu\text{M}$ salinomycin and (C) $10 \mu\text{M}$ salinomycin \pm $10 \mu\text{M}$ CGP. Representative images of HLaC79 C1 cells cultivated with (D) RPMI-EM, (E) $10 \mu\text{M}$ salinomycin and (F) $10 \mu\text{M}$ salinomycin \pm $10 \mu\text{M}$ CGP. Magnification, $\times 100$. Significant attenuation of cell viability was observed following treatment with salinomycin. No differences were demonstrated between the cells treated with salinomycin and the cells treated with salinomycin \pm CGP. CGP, CGP37157; RPMI-EM, RPMI-expansion medium.

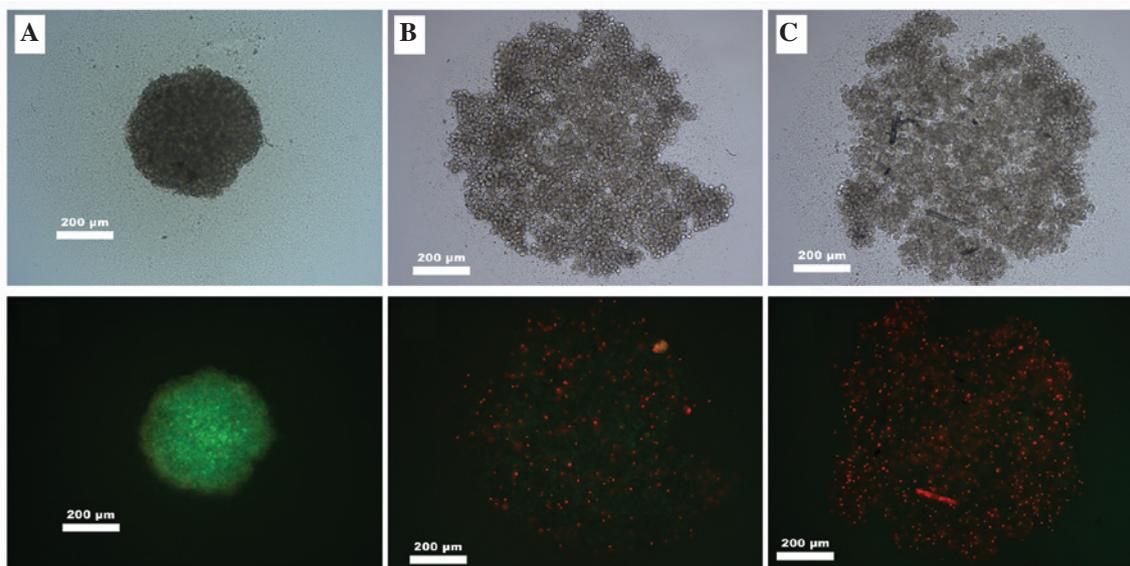


Figure 2. FaDu spheroids were assessed following treatment with salinomycin or salinomycin \pm CGP. Cells cultivated in RPMI-expansion medium were used as a control. A fluorescein diacetate assay was used to identify viable and dead cells. Viable cells retain the ability to convert the non-fluorescent fluorescein diacetate into a green fluorescent composite, whereas red-orange fluorescence is observed in the presence of nuclear membrane damage. (A) Untreated FaDu spheroid of viable cells, determined by green fluorescence. (B) FaDu spheroid treated with $10 \mu\text{M}$ salinomycin for 24 h and (C) FaDu spheroid treated with $10 \mu\text{M}$ salinomycin \pm CGP for 24 h exhibited dead cells, indicated by a red-orange appearance. The images are representative of the phase contrast (top row) and fluorescent microscopy (bottom row) images. CGP, CGP37157.

$P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell morphology and FDA. Following treatment with salinomycin or salinomycin + CGP, the morphology of the FaDu and HLaC79 C1 cells was assessed using an inverted microscope. No morphological differences were observed between the

cells treated with salinomycin and the cells treated with salinomycin + CGP (Fig. 1). In each group, the cells were rounded, suggesting they were undergoing apoptosis.

Following spheroid formation, the cells were treated with salinomycin or salinomycin + CGP. The cells were then stained with FDA solution. A significant attenuation of cell viability was observed following the FDA staining, with no weakening of salinomycin tumor toxicity caused by the addition of CGP (Fig. 2).

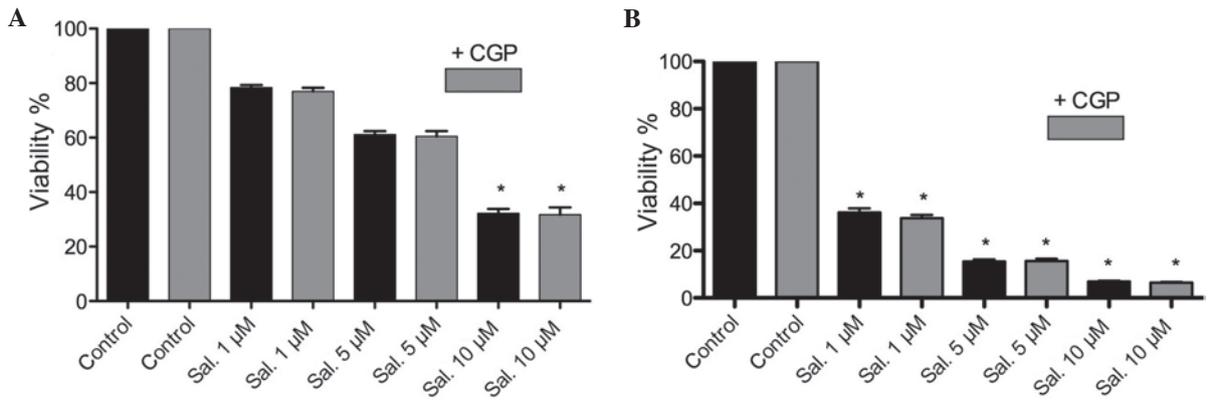


Figure 3. MTT assay of head and neck squamous cell carcinoma cell lines under different treatment conditions. The cells were treated with different concentrations of Sal or Sal + 10 μM CGP for 24 h. Following treatment for 24 h an MTT assay was performed, which revealed a significant attenuation of cell viability, in a dose-dependent manner. (A) FaDu cells exhibited a significant reduction in cell viability following treatment with 10 μM Sal. (B) HLaC79 C1 cells exhibited a significant attenuation in cell viability following treatment with 1 μM Sal. No inhibition of Sal tumor toxicity was observed following the addition of CGP. *P<0.05 vs. control. Data are expressed as the mean ± standard deviation of triplicate measurements. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sal, salinomycin; CGP, CGP37157.

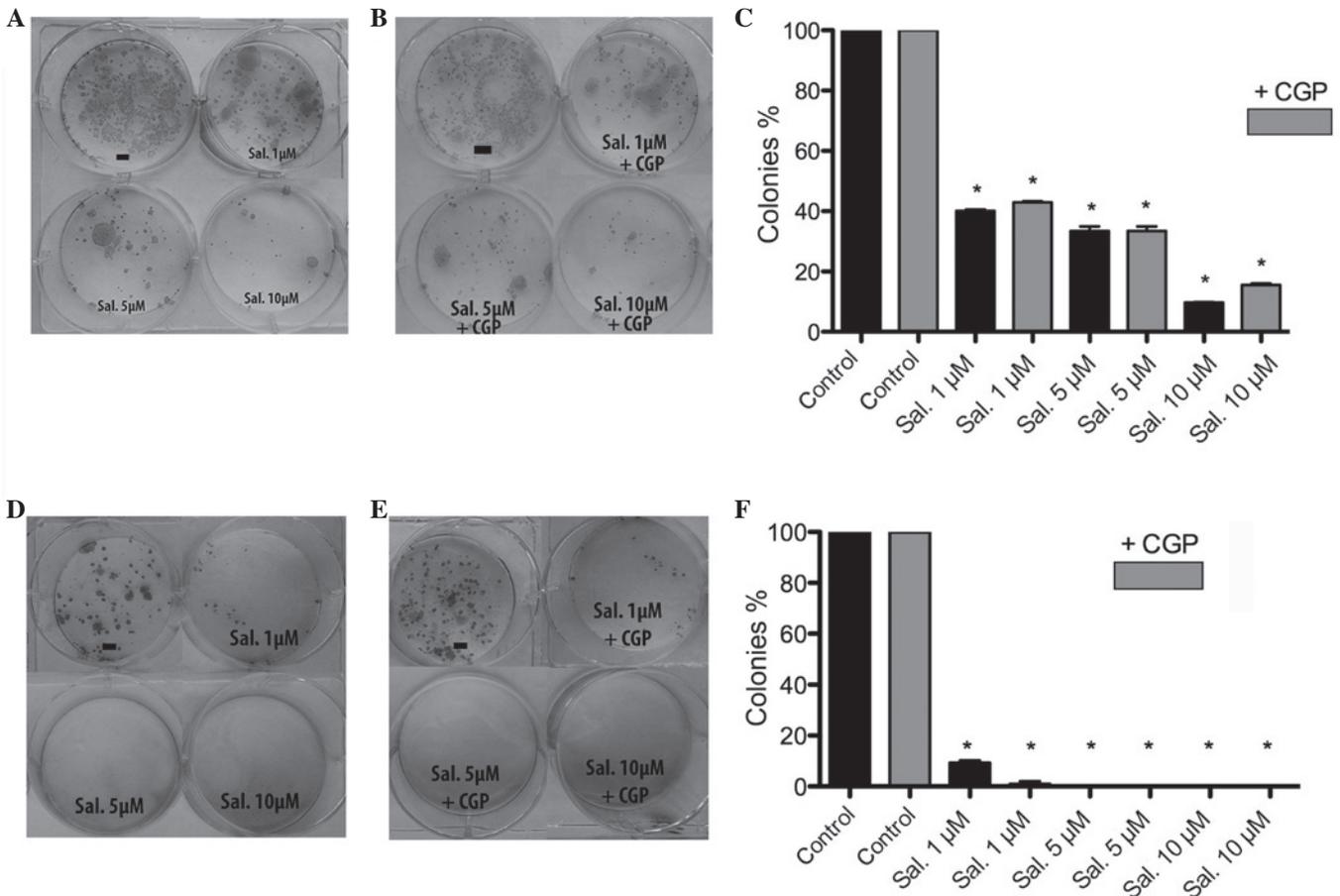


Figure 4. Colony assay of head and neck squamous cell carcinoma cell lines under different treatment conditions. The cells were treated with increasing concentrations of Sal or Sal ± 10 μM CGP for 24 h. Following treatment for 24 h, the medium was replaced. The cells were cultured for 14 days, following which the cell colonies were stained with crystal violet. The (A-C) FaDu and (D-F) HLaC79 C1 cells exhibited a significant reduction in colony formation. No inhibition of salinomycin tumor toxicity was observed following the addition of CGP. *P<0.05 vs. control. Data are expressed as the mean ± standard deviation of triplicate measurements. Sal, salinomycin; CGP, CGP37157; -, untreated control.

MTT assay and clonogenic assay. Following treatment with salinomycin or salinomycin + CGP, an MTT assay was performed. A significant (P<0.05) dose-dependent attenuation of cell viability was observed following treatment

with salinomycin. The combination of salinomycin and CGP revealed no inhibition of salinomycin tumor toxicity. However, a significant difference between the FaDu and HLaC79 C1 cells was observed in terms of the concentra-

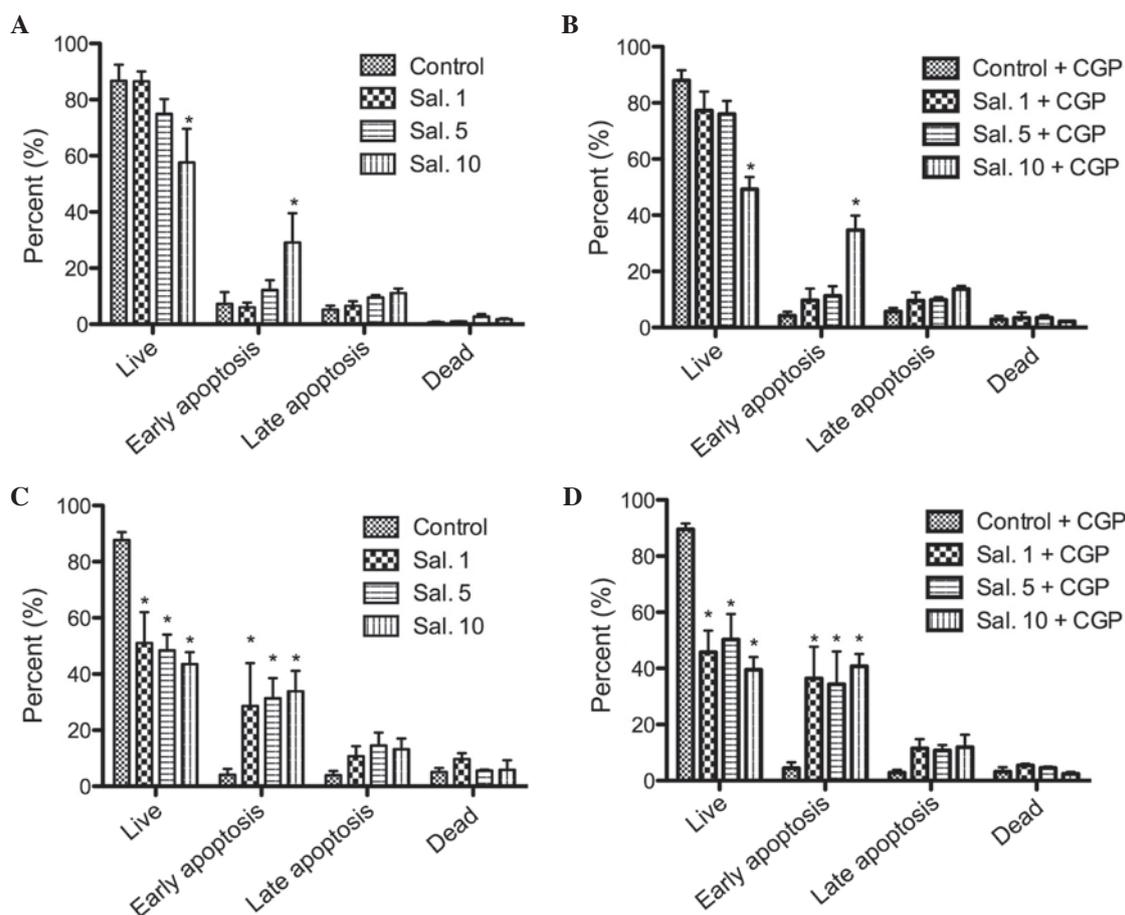


Figure 5. Annexin V-PI assay of apoptosis. The results of annexin V-PI staining revealed a dose-dependent increase of early apoptosis following treatment of the (A) FaDu cells with Sal. Following the addition of (B) CGP, the tumor toxicity of Sal was not counteracted. The increase of HLaC79 C1 apoptosis following treatment with (C) Sal or (D) Sal + CGP. No significant increases in late apoptosis or cell necrosis were observed following treatment with Sal at high concentrations. * $P < 0.05$ vs. control. Data are expressed as the mean \pm standard deviation of triplicate measurements. Sal, salinomycin; CGP, CGP37157; PI, propidium iodide.

tion of salinomycin ($P < 0.05$). The half maximal inhibitory concentration (IC_{50}) of salinomycin in the FaDu cells was calculated to be between 5 and 10 μM , whereas the IC_{50} of the HLaC79 C1 cells was calculated to be between 0.5 and 1 μM . The MTT assay revealed no counteraction of salinomycin tumor toxicity following the addition of CGP. Assessment of colony formation revealed the inhibition of colony formation following treatment with salinomycin, which was not inhibited by the addition of CGP. The HLaC79 C1 cells reacted more sensitively to salinomycin, compared with the FaDu cells (Figs. 3 and 4).

Annexin V-PI staining. The annexin V-PI staining was performed to confirm the results of the MTT assay and to differentiate between cell apoptosis and cell necrosis. No increase in the number of apoptotic cells was observed following treatment with CGP. A dose-dependent cell apoptosis response was observed following treatment with salinomycin. The HLaC79 C1 cells were more sensitive to salinomycin, compared with the FaDu cells (Fig. 5). The rate of early apoptosis increased significantly ($P < 0.05$) in a dose-dependent manner in the FaDu cells. No improvements in the rates of late apoptosis and necrosis were observed as the concentration of salinomycin increased (Fig. 5). The

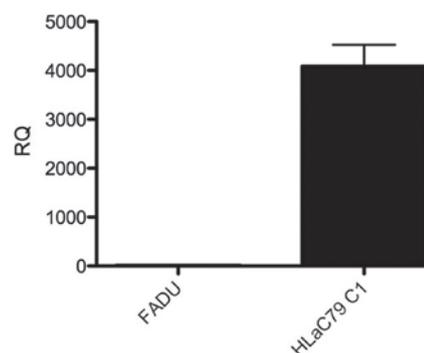


Figure 6. Expression levels of MDR-1 were assessed using reverse transcription-quantitative polymerase chain reaction. The HLaC79 C1 cells exhibited high expression levels of MDR-1, whereas no MDR-1 was expressed in the FaDu cells. Data are expressed as the mean \pm standard deviation of triplicate measurements. RQ, relative quantity.

addition of CGP did not counteract the tumor toxicity of salinomycin (data not shown).

RT-qPCR. The expression levels of MDR-1 in the FaDu and HLaC79 C1 cancer cell lines were assessed using RT-qPCR. The HLaC79 C1 cell line was observed to exhibit positive

expression of MDR-1, however no expression of MDR-1 was detected in the FaDu cells (Fig. 6).

Discussion

The antitumor activity of salinomycin has been demonstrated in different types of CSCs, including breast CSCs (7), leukemia stem cells (16) and pancreatic CSCs (17). The mechanisms involved in salinomycin-induced cancer stem cell death remain to be fully elucidated. Lu *et al* (18) identified salinomycin as an inhibitor of the Wnt/ β -catenin signaling pathway. Salinomycin also inhibits the phosphorylation of Wnt-induced lipoprotein receptor related protein 6 (LRP6). This causes degradation of LRP6 and leads to the apoptosis of CSCs (18). In a previous study, Arafat *et al* (19) demonstrated that salinomycin caused concentration- and time-dependent reductions in the viability of HNSCC cell lines through a caspase 3/7-associated cell death pathway (19). In the present study, salinomycin induced dose-dependent apoptosis in the HNSCC cells. The sensitivities of the two cell lines differed significantly, with the HLaC79 C1 cells being more sensitive to salinomycin, compared with the FaDu cells. At a concentration of 1 μ M, cell death was observed in ~60% of the HLaC79 C1 cells, which is a salinomycin concentration ~10 times lower than the concentration required to achieve the same effect in the FaDu cells. The divergence in sensitivity between each cell line was also demonstrated in the annexin and colony assays. Following the addition of CGP, no alterations in salinomycin tumor toxicity were observed. One reason may be due to the expression of an MDR-1 receptor. Riccioni *et al* (10) demonstrated that salinomycin acted as a potent inhibitor of MDR-1. This was achieved through a conformational change in the adenosine triphosphate transporter (10). The present study revealed, using RT-qPCR, that MDR-1 was highly expressed in the HLaC79 C1 cells.

Overexpression of MDR-1 in cancer cells is associated with a poor clinical outcome due to its resistance to a majority of the drugs used (20), therefore, salinomycin may be a promising candidate in these cases. The major obstacle to using salinomycin is its cytotoxicity. The mechanism of salinomycin in dealing with rumen microflora and coccidia is well known (21), however, the toxic effect in mammals remains to be fully elucidated. Notably, the toxic effects of salinomycin depend on the species, for example, it exhibits low toxicity in poultry and cattle, and high toxicity in horses and dogs (9). Although the available data on the toxicity of salinomycin in humans is limited, there is one case report on human poisoning by salinomycin (22). In this case, a farmer accidentally ingested salinomycin at an unknown concentration, which resulted in life-threatening neuropathia, rhabdomyolysis and hospitalization for 6 weeks. The concentration of salinomycin in the plasma remained undetermined, however, it was estimated that 1 mg/kg body weight was ingested. In our previous study (23), the effects of salinomycin on human mesenchymal stem cells (hMSCs) was investigated. The essential functional properties of hMSC were unaffected by treatment with salinomycin, however, dose-dependent cytotoxicity effects were observed (23). Minati *et al* (24) examined the effects of salinomycin on alkali cation transport and membrane functions in rat liver mitochondria, the results of which demonstrated

that salinomycin inhibits mitochondrial functions by acting as a mobile carrier for alkali cations through membranes (24). Boehmerle and Endres (11) reported that salinomycin neuronal toxicity is mediated by an increase in the concentration of Na⁺, which causes an increase in cytosolic Ca²⁺ concentration via Na⁺/Ca²⁺-X in the plasma membrane and mitochondria (11). This effect was significantly inhibited following the addition of the CGP benzodiazepine derivate.

Additives in tumor therapy are required to reduce the toxicity of anticancer drugs. Human serum albumin-thioredoxin-1 has been used as an effective additive for preventing cisplatin nephrotoxicity (25). Kojouharov *et al* (26) demonstrated the reduction of 5-fluorouracil (5-FU) toxicity by the toll-like receptor 5 (TLR5) agonist, entolimod, in an *in vivo* model. TLR5 did not reduce the antitumor efficacy of 5-FU, however, the major potential concern of using entolimod was its protective/regenerative effects, which potentially reduced the antitumor efficacy of the chemotherapy (26). The most important fact for additives in tumor therapy is that they do not interact with the anticancer drug. In the present study, CGP did not counteract the tumor toxicity of salinomycin, nor did it exhibit any antitumor activity.

In conclusion, salinomycin may be a promising drug in future anticancer therapy, with CGP as a potential additive to reduce its toxicity. However, further investigations are required to examine the toxicological aspects of salinomycin in human cells. Healthy organs, which express MDR-1, including the liver, kidney, small intestine and colon, may be highly sensitive to salinomycin. Therefore, toxicological investigations of healthy cells, healthy cells expressing MDR-1, and animal experiments are required to identify critical organs in salinomycin treatment.

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