



In Vitro Dose-Response Parameters of Salinomycin for Glioblastoma Cells

Glioblastoma Hücreleri İçin in Vitro Salinomisin Doz-Cevap Parametreleri

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Abstract

Salinomycin (SAL) has been reported previously to exhibit therapeutic activity in cancer. However, appropriate doses of SAL that potentiate cytotoxic effects to glioma cells are still unknown. In this study, in vitro anti-glioblastoma activity of salinomycin with the results of initial set of pharmacokinetics/pharmacodynamics parameters obtained from dose-response data, were evaluated. T98G human glioblastoma cells exposed to 5 μ M and 10 μ M SAL, showed significant cytotoxicity. Dose-response parameters of salinomycin (E_0 , E_{inP} , IC_{50} , HS, AUC, and GI_{50}) were determined which explores the in vitro preliminary assessment of salinomycin as an anti-cancer drug for targeting T98G glioblastoma cells.

Keywords: Cancer, Dose-response, Drug, Glioblastoma, Salinomycin

Öz

Salinomisin'in (SAL) kanser tedavisinde teröpatik aktiviteye sahip olduğu daha önceden rapor edilmiştir. Bununla beraber, uygun salinomisin dozlarının glioma hücrelerindeki sitotoksik etkisi halen bilinmemektedir. Bu çalışmada, salinomisin'in in vitro ortamda anti-glioblastoma aktivitesi, doz-cevap verilerinin analizi sonucu elde edilen farmakokinetik/farmakodinamik parametrelerin hesaplanması sonucunda incelenmiştir. 5 μ M ve 10 μ M dozlarında uygulanan SAL miktarlarının T98G insan glioblastoma hücrelerinde önemli derecede sitotoksik etki gösterdiği tespit edilmiştir. Salinomisin'in doz-cevap parametreleri (E_0 , E_{inP} , IC_{50} , HS, AUC, and GI_{50}) belirlenmiş ve in vitro öncül değerlendirmeler, salinomisin'in T98G glioblastoma hücrelerini hedefleyen anti-kanser ilacı olarak değerlendirilebileceğini ortaya çıkarmıştır.

Anahtar Kelimeler: Kanser, Doz-cevap, İlaç, Glioblastoma, Salinomisin

1. Introduction

Salinomycin ($C_{42}H_{70}O_{11}$), which is known as an antimicrobial drug, is a monocarboxylic polyether antibiotic isolated from *Streptomyces albus* strain (Zhou et al. 2013). Previously, it has been reported that salinomycin has 100-fold greater pharmacological effect than paclitaxel (an anti-breast cancer drug) (Gupta et al. 2009). After this discovery, which concludes salinomycin as an anti-cancer drug regarding to its pharmacological effect on cancer stem cells, there is an intensive interest on salinomycin and several in vitro and in vivo studies have been carried out in terms of evaluating the effects of salinomycin on various cell lines including for both cancer stem cells and cancer cells (Zhou et al. 2013). As far as our knowledge in literature, only few studies showed

the effects of salinomycin on brain tumor cells (Delwar et al. 2011, Calzolari et al. 2014). It has been reported that tumor cells (glioma DBTRG-05MG cell line) surviving to hydroxyurea or aphidicolin are slowly depleted by treatment with salinomycin (Delwar et al. 2011). Very recently, the effects of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), salinomycin and the combination of both agents in glioblastoma cell lines were investigated and the results demonstrated that salinomycin enhanced TRAIL-induced apoptosis, mainly by up-regulating the expression of TRAIL-R2 (Calzolari et al. 2014).

However, the effects of salinomycin on glioblastoma cells with regard to cell-drug interactions need to be explored in terms of effective doses and pharmacokinetic/pharmacodynamic parameters. Therefore, this study was motivated by the need to determine key dose-response parameters and effective doses of salinomycin as an anti-cancer drug for targeting T98G human glioblastoma cells.

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T98G glioblastoma viability was assessed by toxicology assay and the results were computed as the response data in dose-response curves.

2. Material and Methods

Salinomycin (SAL) (%98) from *Streptomyces albus* strain obtained from Sigma Aldrich Chemical Co. (Germany). Human glioblastoma cell-line T98G was kindly provided by Prof. Dr. Menemşe Gümüşderelioğlu from Hacettepe University, Turkey.

2.1. Cell Culture Studies

Cell culture studies were carried out with T98G, human glioblastoma cell-line at passage number of six. The cells were subcultured in flasks using Dulbecco's modified Eagle medium-F12 (DMEM-F12) (Sigma Co.) supplemented with 10 % (v/v) fetal bovine serum (FBS, Sigma Co.) and 1 % penicillin-streptomycin (Biological Industries, Ashrat, Israel).

2.2. Cell Seeding and Effective Dose Studies

In order to determine effective doses of salinomycin on T98G cells, cell culture studies were conducted in sterile 24-well tissue culture polystyrene (TCPS) dishes in stationary conditions, and then cells were exposed to SAL with different doses. In brief, T98G cell suspension at a density of 2×10^4 cells mL^{-1} was seeded in 24-well plates. After cells were completely attached (2 h), dose-response experiments were assessed with different concentrations of SAL inclusion into the cells. 0 μM SAL (control group A), 0.01 μM SAL (group B), 0.5 μM SAL (group C), 1 μM SAL (group D), 2.5 μM SAL (group E), 5 μM SAL (group F) and 10 μM SAL (group G) are added to each well and cell responses were evaluated in terms of cell viability.

2.3. Dose-Response Experiments With *in Vitro* Toxicology Assay and Cell Imaging

Cellular viability on groups (A-G) was assessed by Sulforhodamine B based *in vitro* toxicology assay kit (TOX6 kit, Sigma, Germany). After 24, 48, 72 and 96 h, cellular viabilities of cells on all groups were determined by using ELISA reader at 565 nm with reference to 690 nm and data were calculated according to the TOX6 kit supplier's instructions. The inhibitory rates of cells were performed by the toxicology assay and dose-response curves were generated for each selected time interval. Cell viability was evaluated by taking control group as reference. Cytoskeleton organizations and morphologies of cells, exposed to different

SAL doses (Groups A-G) were observed at the end of 96 h of incubation period. In brief, cells were rinsed twice with PBS, fixed in 2.5 % (v/v) glutaraldehyde in 0.1 M PBS (pH 7.4) for 10 min at 4 °C and permeabilized in 0.1 % Triton-X 100 for 5 min. Cell cytoskeletal filamentous actin (F-actin) was visualized by treating the cells with Alexa Fluor 488 phalloidin (Invitrogen, USA) for 20 min, and cell nuclei were counterstained with propidium iodide for 5 min. Samples were visualized using fluorescence microscope (Olympus, Japan).

2.4. Determination of Dose-Response Parameters

Dose-response curves were plotted for each time interval according to the cell survival data (viability) obtained from toxicology assay. Relative cell viability data ($y = N/N_c$), where the cell number N was measured in the presence of drug and N_c in a absence of drug as control, and relative cell growth data ($y^* = (N - N_0)/(N_c - N_0)$), where N_0 is the initial number of cells, were calculated and computed as the response data in dose-response curves 1 and 2, respectively. Curve fitting was performed by using nonlinear least squares regression in GraphPad Prism 6 and data were quantified by using a conventional logistical sigmoidal function which was described before (Fallahi-Sichani et al. 2013) as:

$$y = E_{inf} + \left(\frac{E_0 - E_{inf}}{1 + \left(\frac{D}{EC_{50}} \right)^{HS}} \right) \quad (1)$$

where; y is a response measure (relative viability/growth) at dose D (0.01, 0.5, 1, 2.5, 5 and 10 μM SAL), E_0 and E_{inf} are the top and bottom asymptotes of the response, EC_{50} is the concentration at half-maximal effect and equals to half-maximum inhibitory concentration (IC_{50}), and HS is a slope parameter analogous to the Hill coefficient (Hill 1910, Holford and Sheiner 1981, Fallahi-Sichani et al. 2013).

The pharmacokinetic parameters; IC_{50} : the concentration of drug at which response is half its theoretical maximum (the concentrations of drug result in 50% cell killing), and AUC: the area under the dose-response curve were determined from dose-response curve 1 (relative viability vs drug concentration), whereas half maximum growth inhibition (GI_{50}) parameters were determined from dose-response curve 2 (relative growth vs drug concentration). EC_{50} and IC_{50} are the measures of potency, E_{max} and E_{inf} are the measures of drug efficacy and AUC combines potency and efficacy of a drug (Fallahi-Sichani et al. 2013). Thus, the dose-response parameters of salinomycin (E_0 , E_{inf} , IC_{50} , HS , AUC, and GI_{50}) were determined by curve fitting to the cell viability and growth data.

2.5. Statistical Analysis

All data are expressed as means \pm standard deviations. Three similar experiments were done which were carried out in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) in conjunction with Tukey's post hoc test for multiple comparisons using Graph-Pad Instant (GraphPad Software) statistics program.

3. Results

3.1. Cellular Viability and Dose-Response Parameters

Previously, it has been established that salinomycin has a considerable toxicity on breast cancer (Al Dhaheri et al. 2013), ovarian cancer (Kao et al. 2013), prostate cancer (Kim et al. 2011, Ketola et al. 2012) and brain cancer cells (Calzolari et al. 2014), where salinomycin induced cell death by apoptosis due to its severe toxicity for all those reported

tumor types. Therefore, it is critical to identify effective salinomycin concentration as an anti-cancer agent without using excess amount of it. In this study, T98G glioblastoma cells were incubated via increasing amounts of SAL (0.1 μ M to 10 μ M: groups B-G) and a toxicology assay was assessed with selected time intervals (24h to 96 h). 5 μ M and 10 μ M SAL doses were determined as the effective concentrations in terms of toxicity effect of SAL on T98G cells (data not shown). In terms of the changes in the filaments of cells, an incredible significant change in the morphologies of cells (exposed to 10 μ M SAL) was observed from Figure 1 which suggested cell apoptosis, since changes in the organization of the actin cytoskeleton indicate apoptotic signaling (Desouza et al. 2012). However, it is noteworthy that cells tried to resist for the SAL concentrations of 0.1 μ M, 0.5 μ M and 1 μ M.

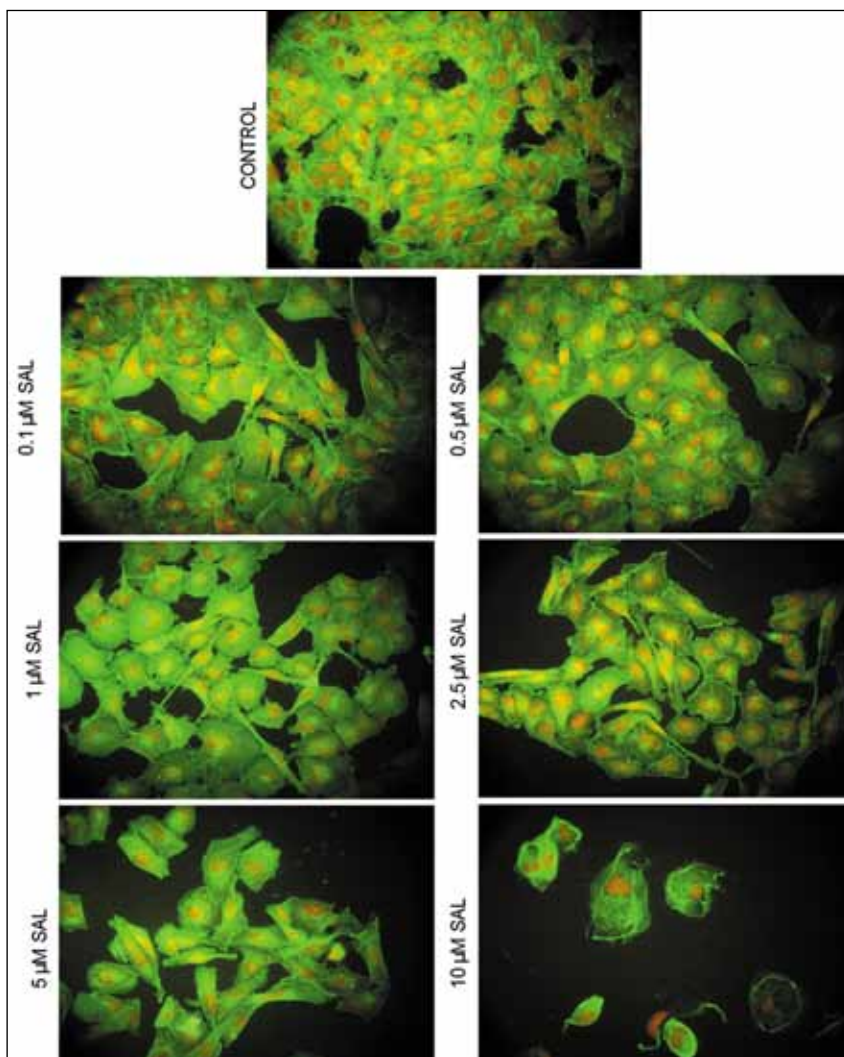


Figure 1. Fluorescence microscope images of T98G cells after 96 h of incubation. Magnification (20 X). Green and red areas indicate F-actin and nucleus of T98G cells, respectively.

Regarding to early phases of drug development, pharmacokinetics is needed in order to interpret the magnitudes of the therapeutic and/or toxic responses according to given the doses (Holford and Sheiner 1981). Moreover, following a single dose, the magnitude of the drug effect (side effects, toxicity) which declines with time should be considered. In deed, individualized dose adjustment and improved clinical efficacy is achieved by pharmacokinetics and pharmacodynamics guided dosing which can reduce the risk of toxicity (Holford and Sheiner 1981, Harvey 2008). Thus, in this study, key dose-response parameters of salinomycin were evaluated with T98G glioblastoma cell line *in vitro*. Dose-response curves were plotted according to the time-dependent relative viability data obtained from T98G cells

and curve fitting was applied by nonlinear regression approximated by four-parameter logistic (4PL) model and a sigmoidal curve was fitted. For each time interval, best-fitted values of IC_{50} , HS, AUC, and GI_{50} dose-response parameters for each time interval were calculated from Figure 7a and Figure 7b and results were demonstrated in Table 1. As shown in Table 1, as incubation time of T98G cells is passed from 24 h to 72 h, IC_{50} values are decreased which suggests the potency of salinomycin increases up to 72 h. After 72 h, IC_{50} value suddenly increased which may be probably due to the reduction of the SAL effect on T98G cells. At the end of 24 h, a considerable high amount of (NA value, %95 confidence intervals) IC_{50} value was determined which indicates salinomycin has a limited effect on T98G

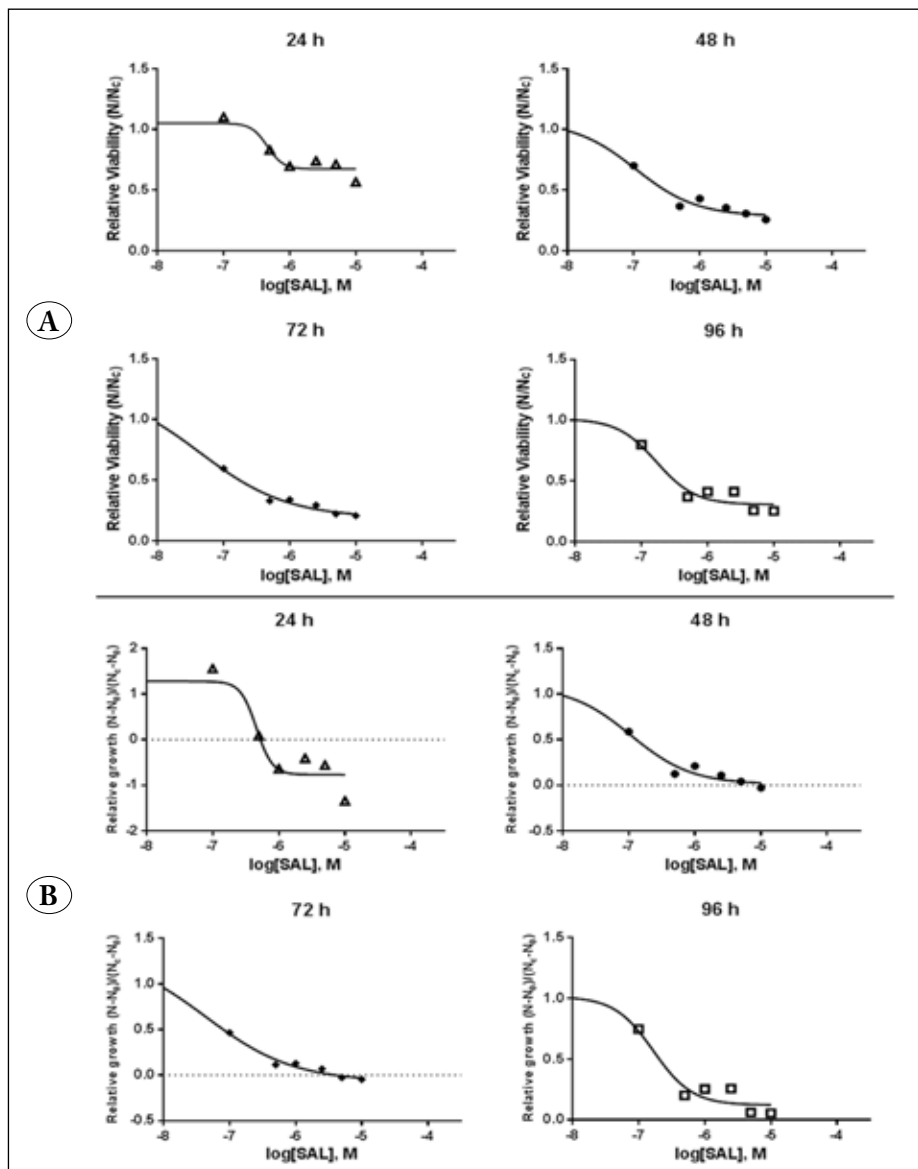


Figure 2. Dose-response curves of T98G cells according to curve fitting to (A) the cell survival data and (B) the relative cell growth data for salinomycin at different time frames.

cells. Although potency (IC_{50} or EC_{50}), defines cytotoxic activity, at the midpoint of the dose-response curve is considered as the most important difference between the drug and the resistant cells (Holford and Sheiner 1981, Harvey 2008, Fallahi-Sichani et al. 2013), the concentration of drug needed to inhibit growth measured as GI_{50} , defines growth inhibition activity, is also considered as significant parameters for especially in vitro tumor models (Harvey 2008, Kuo et al. 2009, Palmeira et al. 2012, Fallahi-Sichani et al. 2013, Vainstein et al. 2013). Table 1 demonstrates that from 24 h to 72 h of incubation of T98G cells, GI_{50} values were first decreased and then suddenly increased. This result indicated that salinomycin has the highest level of activity in terms of cellular growth inhibition at 72 h of incubation, After 96 h, salinomycin began to lose its activity. Hill slope (HS) parameters, defined as the steepness of the dose-response curves, determined and demonstrated in Table 1 for different time intervals. Steeper dose-response curves for 24 h and 96 h of incubation showed $HS > 1$, while shallow curves (for 48 h and 72 h of incubation) demonstrated $HS < 1$ values. Hill slope parameter, which shows how fast the response increases as the dose increases, also gives an idea of the accuracy of the response. If the slope is too shallow, it suggests a greater chance of overlap between desired effects and undesired effects. If slope is too steep and the maximum response is not therapeutic (toxic) then it may be very hard to achieve a dose for a particular wanted response, and not also have an unwanted response. Our results suggested that at 24 h and 96 h of incubation, the response of SAL is not stable, the maximum response may not be therapeutic and, desired and undesired effects are unpredictable. The AUC, also known as the activity area (Fallahi-Sichani et al. 2013), has been correlated to therapeutic efficacy or toxicity for several different chemotherapeutic agents. Moreover, AUC is defined as the combination of potency and efficacy into a single parameter (Fallahi-Sichani et al. 2013). The AUC

parameters demonstrated in Table 1, showed a similar trend as in potency and E_{inf} (bottom asymptotes) values. From 24 h to 72 h of incubation period of cells, the values first decreased, and then an increment was detected at 96 h of incubation (Table 1).

4. Toxicity Challenges and Discussion

Salinomycin offers a promising treatment due to its anti-cancer effects (e.g. inhibits the migration of breast and colon cancer cells (Kopp et al. 2014)); however, potential toxicities should be under control since it has relatively few side effects in normal cells (Zhou et al. 2013). Several studies demonstrated the neurotoxic side effects of salinomycin (van der Linde-Sipman et al. 1999, Story and Doube 2004, Boehmerle and Endres 2011). Therefore, efficient strategies should be developed in order to prevent side effects of salinomycin. Efforts on the development of a less cytotoxic drug for clinical uses are achieved by nanotechnological approaches (Aydın 2014). Very recently, we have studied salinomycin encapsulated nanoparticles for brain tumor targeting (Aydın et al. 2016) by using the effective doses/concentrations of salinomycin suggested in this study. Dose-response parameters obtained from dose-response curves for each time interval are correlated to therapeutic efficacy or toxicity of SAL in T98G cells. Pharmacokinetic and/or pharmacodynamic parameters obtained from the dose-response data indicated preliminary results suggesting anti-glioblastoma activities of SAL. However, it is noteworthy that future *in vivo* and clinical studies should be conducted in terms of evaluating effective doses of SAL as anti-cancer agent for glioblastoma cells. This in vitro study focused on the effective doses and the toxicity of SAL on glioblastoma cells as an anti-cancer drug. But it should be noted that clinical studies, demonstrate drug stability and biosafety, are warranted to fully understand the impact of this potential drug.

Table 1. Time-dependent dose-response parameters obtained from viability data of *in vitro* culture of T98G cells.

Best-fit values	24 h	48 h	72 h	96 h
Top (E_0)	1.008	1.000	1.001	1.000
Bottom (E_{inf})	0.676	0.334	0.248	0.332
HillSlope (HS)	-3.617	-0.9437	-0.6537	-1.478
$EC_{50}=IC_{50}$ (μM)	-	0.299	0.175	0.323
AUC	1.604	0.8352	0.6936	0.8678
GI_{50} (μM)	0.401	0.131	0.081	0.206
R^2	0.889	0.963	0.981	0.950

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