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Low-Dose Salinomycin Targets Multidrug Resistance via P-Glycoprotein and NF-κB in Osteosarcoma Cells

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Abstract. Chemotherapy resistance in osteosarcoma remains a major clinical obstacle, largely driven by drug efflux mechanisms mediated by P-glycoprotein (ACBC1/ABCB1) and activation of NF-κB signaling (NFKB1). This study investigated the potential of Salinomycin to modulate these multidrug resistance–associated genes in U2OS osteosarcoma cells and explore its role in overcoming chemoresistance. A true experimental post-test-only control group design was used. U2OS cells were treated with varying concentrations of Salinomycin, doxorubicin, and their combination. Cell viability was assessed using MTT assay, while gene expression of ACBC1 and NFKB1 was quantified using qRT-PCR with SYBR Green chemistry. Relative expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method normalized to GAPDH. Salinomycin demonstrated dose-dependent cytotoxic effects in the low micromolar range. At lower concentrations, it significantly reduced NFKB1

expression, while also showing a tendency to downregulate ACBC1. However, intermediate concentrations showed variable effects, including a transient increase in NFKB1 expression. The combination treatment with doxorubicin produced only modest and non-significant changes in both resistance-related genes. Overall, low-dose Salinomycin exhibited a more consistent suppressive effect on NF-κB signaling, suggesting a potential role in sensitizing osteosarcoma cells to chemotherapy. In contrast, higher doses primarily enhanced cytotoxicity without clearly improving suppression of resistance markers. This study highlights a novel dual-action, dose-dependent regulatory effect of Salinomycin at the transcriptomic level, targeting both drug efflux (ACBC1/ABCB1) and survival signaling (NF-κB/NFKB1). These findings provide new insight into its potential as an adjunct agent in overcoming multidrug resistance in osteosarcoma and warrant further validation at the protein and functional levels.

Keywords: Multidrug resistance, NF-κB, osteosarcoma, P-Glycoprotein, salinomycin

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1. Introduction

Osteosarcoma is still a major global health issue, especially for children and adolescents between the ages of 10 and 19. Known for its aggressive and invasive nature, this high-grade malignancy has an estimated annual incidence of approximately 8 cases per million individuals [1,2]. While combined modalities of surgery and chemotherapy have improved the 5-year survival rate to around 70%, chemotherapy resistance remains a major challenge, occurring in approximately 25% of cases. Resistance often leads to a drastic decline in survival rates to 20%, accompanied by increased metastasis and recurrence [1,3,4]. The overexpression of P-glycoprotein (PGP), an ATP-binding cassette (ABC) transporter produced by the multidrug resistance 1 (MDR1) gene, is a major cause of this resistance. PGP mediates drug efflux, reducing intracellular concentrations of chemotherapeutic agents such as doxorubicin and contributing to poor treatment outcomes [3,5,6].

Recent research has focused on identifying agents capable of overcoming chemo-resistance and targeting cancer cells through novel mechanisms. Salinomycin, an anti-coccidial agent widely used in the livestock industry, has emerged as a promising candidate due to its potent anticancer activity. Studies have demonstrated its efficacy in various human cancers, including breast, lung, prostate, colorectal, and pancreatic cancers [7–12]. Notably, Salinomycin has been shown to inhibit ABC (ATP-binding Cassette) transporters in leukemia cells, suggesting its potential to counteract drug efflux mechanisms [13]. Structurally, Salinomycin is a 751-Da monocarboxylic polyether ionophore that facilitates the transport of potassium ions (K^+) across biological membranes [14,15]. Despite these findings, its effects on PGP expression in osteosarcoma cells remain poorly understood.

Chemotherapy resistance remains a major barrier in osteosarcoma treatment, largely driven by overexpression of P-glycoprotein (PGP) and activation of NF-κB–mediated survival signaling pathways. Although Salinomycin has been reported to exhibit anticancer and chemosensitizing properties, its precise regulatory effects on PGP-related drug efflux and NF-κB signaling in osteosarcoma cells are still not fully understood. In particular, previous studies have not clearly addressed whether Salinomycin exerts a dose-dependent dual effect on both transporter-mediated resistance and inflammatory survival pathways at the transcriptomic level.

This study aimed to evaluate the effects of Salinomycin, compared with doxorubicin and a control group, on PGP expression in the U2OS human osteosarcoma cell line. PGP expression was assessed at the transcript level through the ACBC1 gene, which encodes ATP-binding cassette sub-family B member 1 (MDR1). NF-κB pathway activity was evaluated indirectly through NFKB1 gene expression, which encodes the p50 subunit of the NF-κB transcription factor complex. Given the central role of NF-κB in regulating PGP expression and drug resistance signaling, simultaneous assessment of both markers provides a more comprehensive understanding of Salinomycin's potential molecular effects. The hypothesis of this study is that Salinomycin can modulate multidrug resistance in osteosarcoma cells by downregulating ACBC1 and suppressing NF-κB signaling, thereby potentially enhancing chemosensitivity. This work offers a moderate level of novelty by focusing on the dose-dependent and dual regulatory effects of Salinomycin on both drug efflux and survival pathways. However, its findings remain limited to transcriptomic analysis in a single cell line, and future studies incorporating protein-level validation and additional osteosarcoma models are needed to strengthen the translational relevance.

2. Methods

This study is a true experimental in vitro study with a post-test-only control group design. The research was conducted at the Center for Primate Studies, Research Institute, and Community Service, Bogor Agricultural University (PSSP LPPM-IPB). The study protocol was approved by the University of North Sumatra Faculty of Medicine's research ethics committee under approval number 70/KEPK/USU/2023. The research utilized U2OS cells derived from the U2OS cell line. These cells originate from osteosarcoma in the tibia bone of a 15-year-old female and were obtained from the American Tissue Culture Collection (ATCC).

2.1 Cell Culture

U2OS cells were maintained using cryopreservation methods at a temperature of -196°C using liquid nitrogen. The cells were then sub-cultured in T25 flasks to obtain cells for testing. Using growth media consisting of DMEM + 10% FBS, 2 mM L-Glutamine, 100 units/ml penicillin, and 100 µM streptomycin, cell culture was carried out in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). This culture was maintained in a 5% CO2 atmosphere at 37°C. U2OS cells were seeded at a density of 5000 cells per well (In the MTT assay, cells were grown in a 96-well plate, while for gene expression assays, cells were grown in a 6-well plate) and incubated for 24 hours in 100 µL of growth media (McCoy's 5A medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µM streptomycin). Upon reaching 70-80% confluence, various concentrations of Salinomycin were added to determine the IC50 dose. The same procedure was carried out to determine the IC50 dose of doxorubicin.

2.2 Inhibition of cell proliferation (IC50) and viability

The IC50 values of Salinomycin and doxorubicin were determined solely based on absorbance values obtained from the MTT assay. Absorbance was measured at 595 nm, and the percentage of inhibition was calculated by subtracting the absorbance of the sample from that of the untreated control, dividing the result by the control absorbance, and multiplying by 100. In contrast, comparisons of cell viability between treatment groups were performed using direct cell counting with a hemocytometer under a light microscope, providing an independent validation of treatment effects on viable cell numbers.

The cytotoxicity tests for both Salinomycin and doxorubicin were conducted using twelve concentrations: 0.000005, 0.0001, 0.0002, 0.0004, 0.0007, 0.0015, 0.0031, 0.0062, 0.0125,

0.025, 0.05, and 0.1 μM. This range was selected to construct a complete dose–response curve and accurately determine the IC50 values. The inclusion of ultra-low doses, including 5 pM Salinomycin, aimed to capture potential biphasic effects or paradoxical responses at minimal exposure levels. All concentrations are presented in Figures 1 and 2. The IC50 value for Salinomycin was determined using nonlinear regression analysis with the Hill equation (four-parameter logistic model), based on the dose–response relationship from the MTT assay. Curve fitting and analysis were performed using GraphPad Prism software (version 10.4). All experimental conditions were performed in triplicate using independent biological replicates (n = 3), where the data were expressed as mean ± standard deviation (SD).

2.3 Examination of gene expression using qRT-PCR

The examination of ACBC1 and NFKB1 gene expression was performed using the qRT-PCR method. U2OS cells were seeded in a 6-well tissue culture plate and exposed to doxorubicin, Salinomycin, and vehicle control under standardized culture conditions. Salinomycin concentrations were selected based on preliminary cytotoxicity screening (MTT assay) to represent low, sub-cytotoxic, and near-IC50 ranges, allowing evaluation of dose-dependent regulatory effects on resistance-related gene expression. Cells were then incubated for 48 hours following treatment. This 48-hour exposure period was chosen to capture early-to-intermediate transcriptional responses associated with drug-induced modulation of PGP- and NF-κB–related pathways, while minimizing secondary effects due to prolonged cell death or nutrient depletion. All experimental conditions were performed in biological triplicates, using independent cell culture preparations to ensure reproducibility across separate experimental runs. In addition, each qRT-PCR measurement was conducted in technical triplicates to reduce variability arising from pipetting and amplification procedures. This combined design strengthens the reliability of gene expression data by accounting for both biological variability and technical measurement error.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Reverse transcription was then performed using random pri-mers to generate complementary DNA (cDNA) for target gene amplification. The NanoDrop 2000 UV-Vis Spectrophotometer was used to measure the amount of extracted mRNA. The mRNA expression of each gene was evaluated using an RT-PCR device (IQ5 Multicolor Real-Time PCR Detection System, Biorad). Each reaction included 12.5 μl of 2 × SYBR Green RT-PCR reaction mix, 0.5 μl of iScript One-Step RT-PCR, 2.5 μl of nucle-ase-free water, 1 μl of primers for each gene, and 2.5 μl of RNA template. The following conditions were used for the reaction: reverse transcriptase activation at 50 °C for 10 minutes and reverse transcriptase inactivation at 95 °C for 5 minutes. For 40 cycles, the reaction was carried out at 95 °C for 10 seconds to denature the DNA, 52 °C for 20 seconds to anneal the primer, and 72 °C for 10 seconds to extend the DNA. The RT-PCR machine software set the baseline and threshold automatically. Every target gene was expressed in fold change and normalized to GAPDH. The 2-ΔΔCT technique was used to calculate the relative gene expression [16].

Table 1. Primer sequences used for PCR analysis

Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')
ACBC1	GCTGTCAAGGAAGCCAATGCCT	TGCAATGGCGATCCTCTGCTTC
NFKB1	TGC ACC TAG CTG CCA AAG AAG GA	TCT GCT CCT GCT GCT TTG AGA
GAPDH	CGG ATT GG TCG TAT TGG TCA	AGG TGT GAG GAC TGG

2.4 Statistical analysis

Data derived from the triplicates were presented as mean±standard deviation (SD). Distribution normality was carried out by using the Shapiro-Wilk test. Value was shown as

means ± standard deviations. Comparative statistics were carried out using a pair-wise t-test for the relative gene expression among the groups. The relationship between ACBC1 and NFKB1 gene expression was assessed using both Pearson's correlation coefficient and Spearman's rank correlation. P value < 0.05 was considered statistically significant (*P < 0.05). GraphPad Prism software (version 10.4) was employed to all statistical analyses.

3. Results and Discussion

3.1 Cytotoxicity of Drugs against U2Os cells

Figures 1 and 2 present the dose–response curves for Salinomycin and doxorubicin, respectively, with percent inhibition calculated relative to the untreated control (set at 0%). Based on the Hill equation, the IC50 value of Salinomycin is 0.011 µM, a concentration required to inhibit 50% of cell viability. As for doxorubicin, the IC50 was approximately 0.18 µM. At sub-IC50 concentrations of Salinomycin (e.g., 0.005 µM), some negative inhibition values were observed, indicating higher absorbance than the control, which may suggest increased cell metabolic activity or proliferation. This potential biphasic or hormetic effect has been reported in studies involving ionophores and should be considered in dose–response evaluation. However, the overall cytotoxic profile of Salinomycin remains evident at IC50 and higher doses.

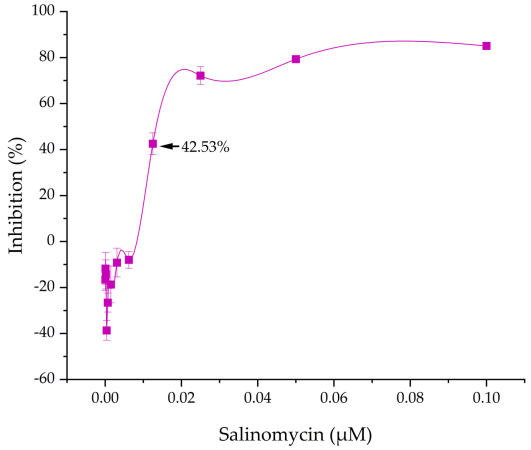

Figure 1. Percentage of U2OS inhibition against Salinomycin at various concentrations. Values represent mean ± SD (n = 3). Error bars indicate SD.

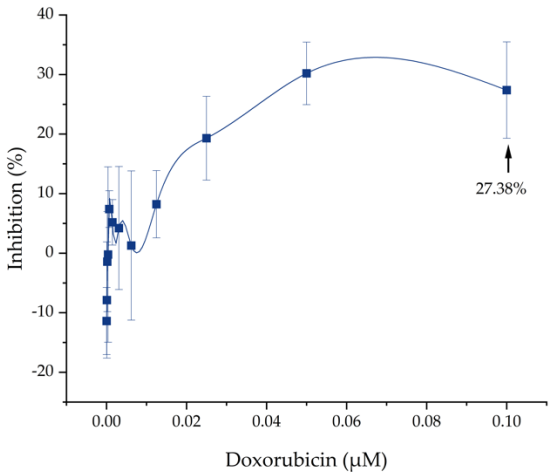

Figure 2. Percentage of U2OS inhibition against doxorubicin at various concentrations. Values represent mean ± SD (n = 3). Error bars indicate SD.

Figure 3a presents cell viability results obtained via hemocytometer counting. Alt-hough the overall pattern was consistent with the MTT assay, some discrepancies were observed, particularly at 0.025 µM Salinomycin, where hemocytometer-based viability was 2.20 ± 1.89%, while MTT showed a stronger inhibition. This variation likely reflects the different endpoints measured: MTT detects mitochondrial activity, whereas the he-mocytometer quantifies total viable cells. To allow for clearer comparison, all hemocytom-eter-based results were normalized against the control group (57.25 × 104 cells, set as 100%). The cell viabilities in percentages are presented in Figure 3b. The lowest viability was seen in the 0.025 µM Salinomycin group (1.25 × 104 cells; 2.20 ± 1.89%), followed by 0.005 µM (2.1 × 104 cells; 3.66 ± 0.80%) and 0.0125 µM (9.5 × 104 cells; 16.62 ± 2.78%). Co-treatment with doxorubicin and 0.0125 µM Salinomycin yielded 29.27 ± 1.16% viabil-ity, while doxorubicin alone reduced it to 19.25 ± 4.06%.

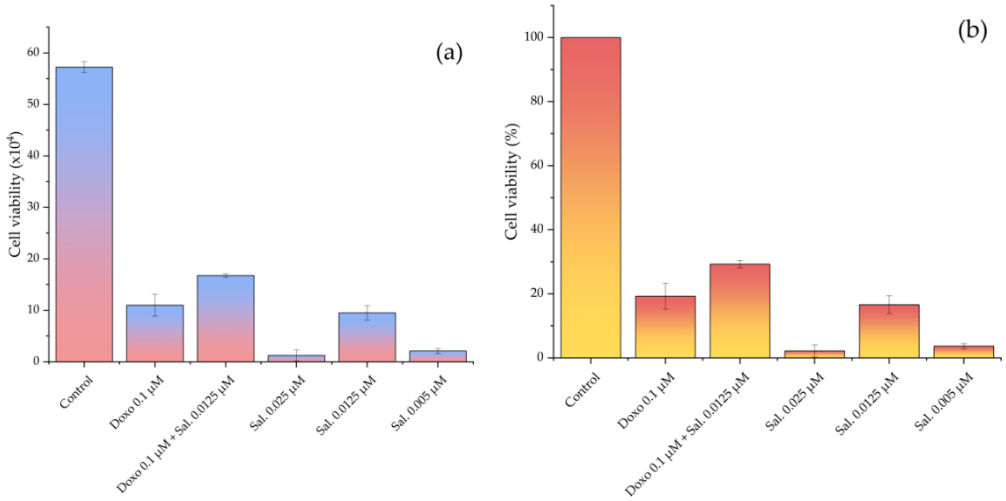

Figure 3. Cell viability (a) and its percentage (b) following the exposure to doxorubicin, Salinomycin, or their combination. Values represent mean ± SD (n = 3), with error bars indicating SD.

3.2 Effect on ACBC1 Gene Expression

The effect of doxorubicin, salonomycin, or their combination on the ACBC1 gene expression in U2OS cells, normalized to GAPDH and calibrated to the control group, is presented in Figure 4a. Treatment with Salinomycin 0.005 µM slightly reduced ACBC1 expression (0.78 ± 0.07-fold), while Salinomycin 0.0125 µM increased expression to 2.25 ± 0.84-fold. A moderate rise was also observed with Salinomycin 0.025 µM (1.40 ± 1.92-fold). Doxorubicin alone elevated ACBC1 expression (2.24 ± 0.84-fold), whereas the combined treatment led to a decrease (0.49 ± 0.28-fold). However, post-hoc analysis using the LSD test indicated that most differences in ACBC1 expression were not statistically significant, including Salinomycin 0.0125 µM vs control ($p = 0.282$), doxorubicin vs control ($p = 0.283$), and doxorubicin + Salinomycin vs control ($p = 0.242$) (Table 2). This suggests that although ACBC1 expression varied across treatments, the changes were not statistically robust.

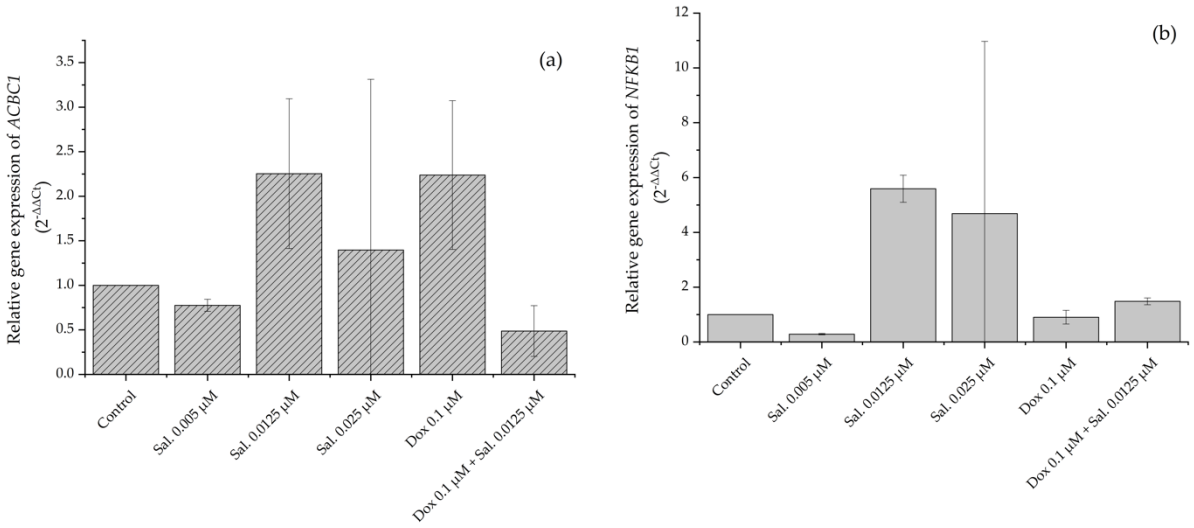

Figure 4. Relative gene expressions of ACBC1 (a) and NFKB1 (b) in U2OS cells following exposures to doxorubicin, Salinomycin, and their combination. Values represent mean ± SD (n = 3), with error bars indicating SD.

3.3 Effect on NFKB1 Gene Expression

Salinomycin 0.005 µM markedly suppressed NFKB1 expression (0.28 ± 0.03-fold), whereas Salinomycin 0.0125 µM induced a strong upregulation by 5.59 ± 0.49-fold (Figure 4b). A moderate increase was observed with Salinomycin 0.025 µM (4.68 ± 6.28-fold). doxorubicin alone had minimal effect (0.90 ± 0.25-fold), while its combination with sali-nomycin slightly enhanced NFKB1 expression by 1.48 ± 0.12-fold (Figure 4b). Results of the LSD analysis are presented in Table 2, where significant suppression was observed in Salinomycin 0.005 µM vs control (p = 0.016) and significant upregulation with Salinomycin 0.0125 µM vs control (p = 0.048). The difference between Salinomycin 0.005 µM and 0.0125 µM was also significant (p = 0.041), indicating a clear dose-dependent effect. These results suggest a biphasic (hormetic) response of NFKB1 to Salinomycin treatment.

Table 2. Statistical comparisons of relative expressions of ACBC1 and NFKB1 genes using a pair-wise t-test

Group 1	**Group 2**	**ACBC1**		**NFKB1**	
		***p*-value**	***t*-stat**	***p*-value**	***t*-stat**
Control	Sal. 0.005 µM	0.135	4.65	0.016*	40.51
Control	Sal. 0.0125 µM	0.282	-2.11	0.048*	-13.18
Control	Sal. 0.025 µM	0.819	-0.29	0.559	-0.83
Control	Dox 0.1 µM	0.283	-2.09	0.680	0.55
Control	Dox 0.1 µM + Sal. 0.0125 µM	0.238	2.55	0.114	-5.53
Sal. 0.005 µM	Sal. 0.0125 µM	0.242	-2.48	0.041*	-15.22
Sal. 0.005 µM	Sal. 0.025 µM	0.727	-0.46	0.503	-0.99
Sal. 0.005 µM	Dox 0.1 µM	0.242	-2.47	0.173	-3.50
Sal. 0.005 µM	Dox 0.1 µM + Sal. 0.0125 µM	0.378	1.39	0.039*	-13.46
Sal. 0.0125 µM	Sal. 0.025 µM	0.643	0.58	0.872	0.20
Sal. 0.0125 µM	Dox 0.1 µM	0.987	0.02	0.019*	12.01
Sal. 0.0125 µM	Dox 0.1 µM + Sal. 0.0125 µM	0.179	2.81	0.042*	11.44
Sal. 0.025 µM	Dox 0.1 µM	0.648	-0.57	0.551	0.85

Sal. 0.025 μM	Dox 0.1 μM + Sal. 0.0125 μM	0.623	0.66	0.602	0.72
Dox 0.1 μM	Dox 0.1 μM + Sal. 0.0125 μM	0.179	2.81	0.141	-2.95

3.4 Relationship between ACBC1 and NFKB1 Expressions

To examine the association between ACBC1 and NFKB1 gene expression, correlation analyses were performed using relative expression values (2-ΔΔCt) across all treatment groups. Pearson's correlation showed a moderate positive relationship ($r = 0.50$, $p = 0.315$), while Spearman's rank correlation also indicated a moderate monotonic trend ($\rho = 0.43$, $p = 0.397$). However, neither correlation reached statistical significance, suggesting that although ACBC1 and NFKB1 may share similar expression patterns in response to treatment. The relationship is not robustly supported with the current sample size.

Discussion

Cancer management poses numerous challenges, particularly in identifying effective anticancer agents. Many chemotherapeutic drugs commonly used in treatment regimens exhibit reduced efficacy due to the ability of cancer cells to maintain survival mechanisms, ultimately leading to drug resistance. One such mechanism involves the overexpression of P-glycoprotein (PGP) on the cell membrane. Also known as multidrug resistance protein 1 (MDR1), PGP actively pumps drugs out of the cell, thereby reducing their intracellular concentration and therapeutic efficacy. Increased expression of ACBC1 is strongly associated with reduced response to chemotherapy and enhanced cancer progression [17].

In addition to drug efflux mechanisms, resistance is further reinforced by interconnected signaling pathways that regulate cell survival and stress adaptation. Increased ACBC1 expression does not operate in isolation but is often associated with broader molecular networks that sustain chemoresistant phenotypes, including inflammatory and transcriptional regulators such as NF-κB. These coordinated pathways enable cancer cells to adapt dynamically to cytotoxic stress, contributing to treatment failure and disease recurrence. Therefore, targeting both efflux transporters like PGP and upstream regulatory pathways represents a more promising strategy to overcome multidrug resistance and improve therapeutic outcomes in cancer management [18] [18].

In osteosarcoma, drug efflux mechanisms have been well documented and are known to induce resistance against commonly used chemotherapeutic agents such as doxorubicin, methotrexate, and cisplatin. Doxorubicin, although a key component in osteoarthritis treatment, can activate protein kinase B (Akt) signaling pathways upon prolonged use, which in turn upregulates ACBC1 expression on the cancer cell membrane and contributes to drug resistance [18].

In recent cancer research, PGP has become a critical target for therapeutic intervention, with a focus on identifying compounds capable of downregulating its expression. A study by Higuchi (2019) demonstrated that combining pioglitazone—a PPARγ agonist—with doxorubicin reversed resistance in osteosarcoma 143B cells. Similarly, curcumin (0.5–2 μM) was shown to decrease ACBC1 expression and overcome doxorubicin resistance in leukemia cells [19]. Verapamil, a calcium channel blocker, and cyclosporin have also been identified as PGP inhibitors; however, their clinical use is limited due to high-dose requirements and adverse effects such as immunosuppression [18]. Therefore, an ideal PGP inhibitor should possess high affinity and a favorable safety profile.

Chemoresistance in osteosarcoma is multifactorial, with PGP overexpression playing a pivotal role, especially in resistance to doxorubicin. While previous studies have shown that Salinomycin inhibits MDR via PGP in lymphoblastic lymphoma, its role in osteosarcoma had not been fully elucidated. NF-κB, a key regulator of apoptosis, also contributes significantly to tumor progression by influencing cell proliferation and survival. It regulates genes involved in the cell cycle (e.g., cyclin A, cyclin D1, and CDK6) and suppresses TNF-α-induced apoptosis

[20]. In cisplatin-resistant MCF7DDP cells, Salinomycin has been shown to prevent NF-κB nuclear translocation, thereby downregulating pro-survival proteins under NF-κB control [21]. NF-κB is known to regulate ACBC1 expression by binding to the MDR1 gene promoter and enhancing its transcription. In a previous study, a calcium channel blocker (tetrandrine), was reported to inhibit PGP expression by suppressing NF-κB activity, hence disrupting this transcriptional activation pathway [22] [18].

In the present study, the lowest NFKB1 expression was observed in the 0.005 μM Salinomycin group, with a statistical significance as compared to the control. When the concentration of Salinomycin was increased to 0.0125 μM, the expression increased significantly as compared to the control ($p = 0.048$). Nonetheless, in the present study, the change in expressions was observed not to correlate with that of ACBC1. This suggests independent or partially overlapping regulatory mechanisms.

Furthermore, the present study investigated the effects of Salinomycin at doses of 0.005 μM, 0.0111 μM, and 0.025 μM, compared to doxorubicin and a control group. Results from the MTT assay indicated that the IC50 for Salinomycin was 0.0111 μM. This suggests that Salinomycin effectively inhibits cell growth at low concentrations. A lower IC50 value indicates that less drug is required to achieve the desired therapeutic effect, which may translate to reduced systemic toxicity.

The differential effects observed at Salinomycin doses of 0.005 μM and 0.0125 μM indicate a dose-dependent mechanism. The lower dose (0.005 μM) reduced ACBC1 and NFKB1 expression, suggesting effective inhibition of molecular pathways involved in multidrug resistance. In contrast, the 0.0125 μM dose (approximating IC50) significantly inhibited cell proliferation but did not reduce ACBC1 expression, showing no significant difference from the control. At 0.025 μM, although a reduction in viability was observed, the gene expression results showed high standard deviations. This variability may reflect inconsistent cellular responses or technical variation at higher cytotoxic concentrations. This limits the interpretability of the expression data at that dose and suggests the need for validation with larger sample sizes or protein-level confirmation. Salinomycin at sub-IC50 levels may serve as a promising adjuvant to sensitize resistant osteosarcoma cells, while higher doses may be more suitable for direct tumor cytoreduction.

A key limitation of this study is the use of NFKB1 mRNA expression as an indirect proxy for NF-κB pathway activity. Although NFKB1 encodes the p50 subunit of the NF-κB complex, its transcriptional upregulation does not necessarily correspond to functional pathway activation. In fact, p50 can form homodimers that act as transcriptional repressors, and true NF-κB activation is primarily determined by post-translational events, particularly the phosphorylation and nuclear translocation of the p65 (RELA) subunit. Therefore, changes in NFKB1 expression alone cannot distinguish between canonical activation, compensatory transcriptional feedback, or inhibitory dimer formation. This mechanistic limitation highlights the need for future studies to include protein-level assessments, such as p65 nuclear localization assays and NF-κB DNA-binding activity measurements, to accurately define pathway activation. Another important limitation is the exclusive use of a single osteosarcoma cell line (U2OS), which limits biological generalizability. Validation across additional osteosarcoma models, including drug-resistant derivatives and primary tumor samples, is necessary to confirm the consistency of the observed effects.

The observed increase in PGP (ACBC1) expression at higher salinomycin concentrations may reflect a hormetic stress response, where sublethal or cytotoxic stress induces adaptive survival mechanisms, including upregulation of drug efflux transporters. Mechanistically, this may be linked to NF-κB–independent stress signaling pathways such as ROS-mediated activation of compensatory transcription factors, suggesting that high-dose salinomycin may inadvertently trigger cellular defense programs rather than suppress them. This dose-dependent shift underscores the importance of identifying a therapeutic window where salinomycin

maximally suppresses resistance pathways without inducing adaptive counter-regulation. To further clarify its chemosensitizing potential, future studies should evaluate salinomycin in combination and pretreatment regimens with doxorubicin, with particular attention to shifts in dose–response curves and reversal of resistance phenotypes. Additionally, the use of doxorubicin-resistant osteosarcoma models will be critical to determine whether salinomycin can overcome clinically relevant multidrug resistance mechanisms under conditions that better reflect tumor evolution and therapy-induced selection pressure.

4. Conclusion

Findings from the present study indicate that Salinomycin exerts a dose-dependent effect on resistance-related pathways in U2OS osteosarcoma cells. At low concentration, Salinomycin was associated with suppression of NF-κB mRNA expression and a modest reduction in ACBC1 expression, suggesting a potential role in modulating key signaling mechanisms linked to multidrug resistance. In contrast, a higher concentration, approximating the IC50, primarily induced cytotoxic effects without a consistent inhibitory impact on ACBC1 expression, indicating that cytotoxicity and resistance modulation may occur through partially distinct mechanisms. These results support a dual-action profile of Salinomycin, where lower doses preferentially influence transcriptional regulation of survival and efflux-related pathways, while higher doses mainly affect cell viability. A positive association between ACBC1 and NF-κB expression was observed; however, this relationship did not reach statistical significance, suggesting that their regulatory linkage in this model requires further clarification. The novelty of this study lies in demonstrating a dose-dependent dissociation between cytotoxic effects and resistance gene modulation of Salinomycin in osteosarcoma cells at the transcriptomic level. This provides preliminary evidence that therapeutic efficacy may depend not only on dose intensity but also on targeted pathway modulation. However, these findings are limited to a single in vitro cell line and mRNA-level analysis. Therefore, further validation at the protein level (e.g., PGP and NF-κB activity assays), as well as functional drug-efflux studies, is required to confirm biological relevance. In addition, extending the investigation to in vivo osteosarcoma models and additional cell lines would strengthen translational applicability. Future research should also focus on evaluating Salinomycin in combination with doxorubicin to determine whether it can effectively shift dose– esponse relationships and reverse chemoresistance phenotypes. A structured validation roadmap from transcriptomic findings to protein expression, functional assays, and in vivo models is essential to confirm its potential as an adjunct therapeutic strategy in overcoming multidrug resistance in osteosarcoma.

Declaration of AI and AI assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT in order to assist in language refinement, grammar checking, sentence restructuring, and improving the clarity of academic writing. After using this tool/service, the author(s) carefully reviewed and edited the content as needed and take full responsibility for the publication's content.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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