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Research Article

Salinomycin Suppresses PDGFRβ, MYC, and Notch Signaling in Human Medulloblastoma

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Abstract

Medulloblastoma (MB) is the most common childhood brain tumor. Despite improved therapy and management, approximately 30% of patients die of the disease. To search for a more effective therapeutic strategy, the effects of salinomycin were tested on cell proliferation, cell death, and cell cycle progression in human MB cell lines. The results demonstrated that salinomycin inhibits cell proliferation, induces cell death , and disrupts cell cycle progression in MB cells. Salinomycin was also tested on the expression levels of key genes involved in proliferation and survival signaling and revealed that salinomycin down-regulates the expression of PDGFRβ, MYC, p21 and Bcl-2 as well as up-regulates the expression of cyclin A. In addition, the results reveal that salinomycin suppresses the expression of *Hes1* and *Hes5* in MB cells. Our data shed light on the potential of using salinomycin as a novel therapeutic agent for patients with MB.

Keywords: Salinomycin; Medulloblastoma; PDGFRβ; MYC; Notch Signaling

Abbreviations

PDGFRβ: Beta-type Platelet-Derived Growth Factor Receptor; Bcl-2: B-cell Lymphoma 2; DLL1: Delta-Like 1 (Drosophila); Dll3: Delta-Like 3 (Drosophila); Hes1: Hairy And Enhancer Of Split 1 (Drosophila); Hes5: Hairy And Enhancer of Split 5 (Drosophila); Hey1: Hairy/Enhancer-of-Split Related with Yrpw Motif 1; Hey2: Hairy/Enhancer-of-Split Related with Yrpw Motif 2; Dtx1: Deltex Homolog 1 (Drosophila); Dtx2: Deltex Homolog 2 (Drosophila); MAML1: Mastermind-Like 1 (Drosophila); MAML2: Mastermind-Like 2 (Drosophila); MAML3: Mastermind-Like 3 (Drosophila); RBPJ: Recombination Signal Binding Protein for Immunoglobulin Kappa J Region; MTS: 3-(4, 5-Dimethylthiazol-2-Yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H- Tetrazolium, Inner Salt; DMSO: Dimethyl Sulfoxide

Introduction

Medulloblastoma (MB), an embryonal neuroepithelial tumor of the cerebellum, is the most common malignant brain tumor in children [1]. This highly invasive tumor has a tendency to disseminate throughout the central nervous system early in its course. Although, the medical treatment outcome for children with MB has improved over the past several decades, approximately one-third of patients with MB tumors remain incurable. Moreover, current medical treatments have associated toxicities that can cause significant disabilities in long-term survivors [2]. Thus, more effective drugs are needed for treating patients with MB.

Salinomycin is a 751 Da mono carboxylic polyether antibiotic which is widely used as an anti-coccidial drug. Recently, salinomycin has been found to reduce the proportion of breast cancer stem cells (CSC) by more than 100-fold compared to paclitaxel, a common drug used for breast cancer [3]. Cumulative findings strongly suggest that salinomycin is a selective killer of human CSC and an effective killer of multi-drug resistant human CSC-like cells [4-11]. The action mechanism of salinomycin in cancer and CSCs has been shown to modulate multiple signaling pathways including the Wnt, NF-κB, and p38 MAPK pathways [12-14].

MB cells have the potential to differentiate to neuronal and/or glial cells [15,16], indicating that MB cells are of stem cell origin. Growing evidence points toward the existence of a CSC-like population that may contribute to MB therapy resistance [17-19]. Notch signaling is critical for cell differentiation and proliferation and plays a fundamental role in MB initiation and progression by regulating downstream effectors, e.g., MYC [20-22]. The Notch pathway inhibitors, e.g., γ-secretase inhibitor MK-0752, suppress the cleavage of Notch, eliminate the stem cell like population [23-25], reduce cell proliferation and increase apoptosis [23-25], which implicates Notch signaling as a target that may constitute an additional promising treatment strategy for MB patients. In the present study, for the first time, we determined the anticancer effects of salinomycin in 3 MB cell lines. We also measured the effects of salinomycin on the expression of a few genes critical in cell proliferation, survival, and differentiation in MB cells.

Materials and Methods

Cell Culture and Chemicals

MB cell lines (Daoy and D283) were obtained from ATCC and D425 cells were a gift from Dr. Darell D. Bigner [26]. Daoy and D283 MB cells were maintained in minimum Essential Medium (MEM) (Cellgro) supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, and 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. D425 cells were maintained in improved MEM (Zinc Optioin 1 x) (GIBCO) supplemented with 10% FBS at 37°C with 5% CO_2. Salinomycin and propidium iodide (PI) were obtained from Sigma.

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Cell Proliferation Analysis

MB cells (Daoy 1x10^4/well, D283 5 x10^4/well, D425 1 x10^5/well) were placed in 96-well plates overnight. Salinomycin solution or identical volume of control (DMSO) was added to the appropriate wells. After 48 hours of treatment, a 20-µl of MTS solution (Promega) was added to each well. The cell number in each condition was determined by measuring the optical densities at 490 nm after 4-hour of incubation. The results were expressed as the percentages of control cultures.

Cell Death and Cell Cycle Analysis

MB cells (5 x10^5 /well) were placed in 6-well plates overnight. Salinomycin or identical volume of control was added to the appropriate wells. After 24 hours of treatment, the cells were stained with PI and analyzed for cell death and cell cycle distribution using the Cell Lab Quanta TM SC system (Beckman Coulter) followed by Flow Cytometry Analysis (FACS).

Western Blotting Analysis

MB cells were harvested at the 24-hour time point for protein by adding Tris-triton cell lysis buffer (1% Triton, 50 mM Tris-HCl pH 7.4, 10% glycerol, 150 mM NaCl) supplemented with protease inhibitor cocktail (Roche Applied Science) and phosphates inhibitor cocktail 100x (cell signaling). The protein samples were separated using a 10%-12% SDS-PAGE gel, and then transferred onto a nitrocellulose membrane. Immunoblots were probed with antibodies specific for cyclin A, Bcl-2, p-21 (Santa Cruz), PDGFRβ (Epitomic), and MYC (Cell signaling). β-actin (Sigma) served as a loading control. Signals of the specific proteins were detected by using the Immun-Star HRP peroxide Luminol/Enhancer kit (BIO–RAD) and recorded on KODAK Biomax light film.

RT-PCR Analysis

RNA was isolated from MB cells using the RNeasy Plus (Qiagen) by following the manufacturer's protocol. The quantity and purity of RNA were determined using a Nano Drop 1000 spectrophotometer (Thermo Scientific). 1 µg of total RNA was used to prepare cDNA using Super Script first-strand synthesis system (Invitrogen) by following the instructions provided by the manufacturer. Primers used in this study are listed in Table 1 and synthesized by Integrated DNA Technology. Semi-quantitative PCR was achieved by amplifying genes using an equal amount of cDNA and limited number of cycles. For detection of basal levels, 35 cycles were used for all genes except GAPDH. For detection the effects of salinomycin on gene expression, PCR conditions are listed in Table 1. PCR amplification was

Table 1: Primers and conditions used for the semi-quantitative RT-PCR analysis.

Gene	Primer Sequences (5'->3')	Sizes of PCR products (bp)	Annealing temperature (°C)	Cycle of amplification
NOTCH1	Forward: GACAGCCTCAACGGGTACAA Reverse: CACACGTAGCCACTGGTCAT	137	55	40
NOTCH2	Forward: GGAGCTACTGTGAGGAGCAA Reverse: GATTTCATACCCCGAGTGCC	238	55	35
NOTCH3	Forward: TGTCAACGAGTGTCTGTCGG Reverse: TTGACTCGGTCCTTGCAGAC	174	55	35
NOTCH4	Forward: AGTGAGAGCTCTGAGGGTCC Reverse: TGGGTCTGACCACTGAGACA	137	55	35
JAG1	Forward: GGCTGCAATAAGTTCTGCCG Reverse: CAGCCTTGTCGGCAAATAGC	131	55	35
JAG2	Forward: TGCAAAAACCTGATTGGCGG Reverse: CACACACTGGTACCCGTTCA	144	60	35
DLL1	Forward: ACCTCGCAACAGAAAACCCA Reverse: GTGTTCGTCACACACGAAGC	146	55	35
DLL3	Forward: CCGAGCTCGTCCGTAGATTG Reverse: AGGGTAGGGAAAAAGCAGGTG	165	55	35
DLL4	Forward: TTAAGCACTTCCAGGCGGTC Reverse: GATGAGCGAGAAGGTACCCG	170	55	35
HES1	Forward: AAGAAAGATAGCTCGCGGCA Reverse: CCTCGGTATTAACGCCCTCG	208	55	40
HES5	Forward: GAGAAAAACCGACTGCGGAA Reverse: TAGTCCTGGTGCAGGCTCTT	221	60	35
HEY1	Forward: TAATTGAGAAGCGCCGACGA Reverse: GCTTAGCAGATCCTTGCTCCA	108	60	35
HEY2	Forward: AGATGCTTCAGGCAACAGGG Reverse: GCGCAACTTCTGTTAGGCAC	102	55	35
DTX1	Forward: ACTTGAATGGTACTGGGCCG Reverse: CACATCCTCGGGATTCTTACTCTT	221	55	35
DTX2	Forward: AGATTTGCCCGGTTTTTGTTG Reverse: TCCGGCAGATCTTTTCTCTCTG	136	55	35
MAML1	Forward: CACGAGCAGAACTCCCTGTT Reverse: CAGGGACACTGGAAGGGTTC	103	55	40
MAML2	Forward: ACAACCCTATGATGCCACGG Reverse: CCCAGTTTGGTGCAGTTGTG	189	55	40
MAML3	Forward: ATAGGACCCTCCCAGAACCC Reverse: CCCTGGGCTTGGTTATGTGT	185	55	35
RBPJ	Forward: CAGTGCTGGATCTGGGAATCT Reverse: AATTTCCCAGGCGATGGAGC	188	61	35
NRARP	Forward: CACCAGGACATCGTGCTCTA Reverse: GTAGTTGGCGGGAAGGTACA	128	55	35
GAPDH	Forward: GAGTCAACGGATTTGGTCGT Reverse: TTGATTTTGGAGGGATCTCG	237	57	25

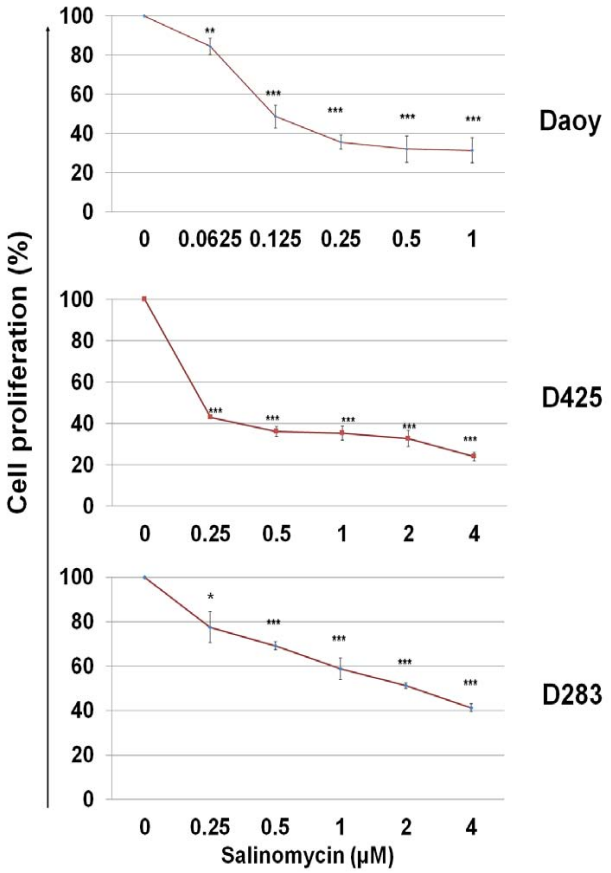

Figure 1: Effects of salinomycin on MB cell proliferation. Cells in complete medium were treated with salinomycin. After 48 hours, the number of viable cells in each well was determined using an MTS assay (Promega). The optical densities were measured at 490 nm. The results were calculated as the percentage against control cultures and presented as mean ± SD. The statistical differences were determined using paired student's t-test and performed using Minitab. * p<0.05, **p<0.01, and ***p<0.001.

performed using GoTaq Hot Start Colorless Master Mix (Promega) and MJ Mini Personal Thermal Cycler (Bio-Rad). The results were visualized by analyzing the samples using DNA gel.

Statistical Analysis

All quantitative data are represented as mean ± standard deviation (SD). Statistical tests were performed using the Minitab 16.1.1 software package. Comparisons between two groups were carried out using paired student's t test and $p < 0.05$ was considered as statistical significance.

Results

Salinomycin suppresses cell proliferation, and induces cell death and S/G_2 cell cycle arrest in MB cells

To assess the cytotoxicity of salinomycin on MB cells, Daoy, D425, and D283 cells were treated with salinomycin for 48 hours at indicated concentrations and then, the rates of cell proliferation were determined by using a MTS assay. As shown in Figure. 1, salinomycin suppressed MB cell proliferation in a dose-dependent manner. The IC50 of salinomycin are 0.1 μM, 0.25 μM, and 2 μM for Daoy, D425, and D283 cells, respectively (Figure. 1).

To further determine the effects of salinomycin on MB cells, Daoy, D425, and D283, cells were treated with salinomycin at the concentrations indicated in Figure. 2. After 24 hours of treatment, cells were analyzed for cell viability and cell cycle progression using PI staining followed by FACS analysis. As shown in Figure. 2, an increase in cell death (sub-G0) was observed in all three cell lines in response to salinomycin treatment. In addition, salinomycin induced Daoy cells arrest at S/G2 phases and D425 and D283 cells at G2 phase under low concentration. Under high concentration, with exception of D425 which showed little change, Daoy and D283 cells were both arrested at S phase (Figure. 2).

Salinomycin suppresses the expression of genes involved in MB proliferation and metastasis

MYC and PDGFRβ were previously reported to be involved in MB growth and metastasis [27-30]. To understand the action mechanism of salinomycin on MB, the effects of salinomycin were examined on the expression of MYC and PDGFRβ in MB cells. Our results showed that all three MB cell lines, Daoy, D283, and D425, expressed high levels of PDGFRβ and MYC. Notably, after treatment with salinomycin at the concentration indicated in Figure. 3 for 24 hours, we observed a markedly down-regulation of both MYC and PDGFRβ in all 3 tested MB cell lines (Figure. 3A).

Salinomycin treatment has differential effects on the expression levels of cyclin A, p21, and Bcl-2

To further elucidate the mechanism of salinomycin's action, the expression levels of key regulators of cell cycle progression and apoptosis were examined in response to salinomycin treatment by western blotting. The results showed that salinomycin treatment markedly increased cyclin A expression (Figure 3A). This effect could be due to the prolonged S/G2 phases. In addition, reduced anti-apoptotic protein p21 and the survival protein Bcl-2 in MB cells were observed in response to salinomycin treatment (Figure. 3B). The modulation of cyclin A and p21 may contribute to a reduction in cell proliferation and the down regulation of Bcl-2 and p21 may contribute to salinomycin induced cell death.

Suppression of Notch signaling by salinomycin in MB cells

To evaluate the effects of salinomycin on the expression levels of genes in Notch signaling in MB cells, we first determined the basal levels of genes in Notch signaling using RT-PCR analysis. As shown in Figure. 4A, all 3 cell lines expressed relatively high levels of *Notch 2, JAG1, MAML1-3, DLL3, Hes1, RBPJ1, NRARP*. The effects of salinomycin on the transcription of key genes in Notch signaling such as Notch receptors (e.g., *Notch 1* and *2*), Notch ligands (e.g., *JAG1* and *DLL1*), transcriptional co-activators for Notch signaling (e.g., *MAML 1-3*), and Notch signaling effectors (e.g., *Hes1, Hes5, and HEY1*) [31,32], were assessed. We observed an inhibition of Notch signaling by salinomycin in all tested cell lines that manifested suppression on transcription of *DLL1, MAML1, Hey1, Hes1, and Hes5* genes (Figure. 4B).

Discussion

In this study, we found that salinomycin at the concentration

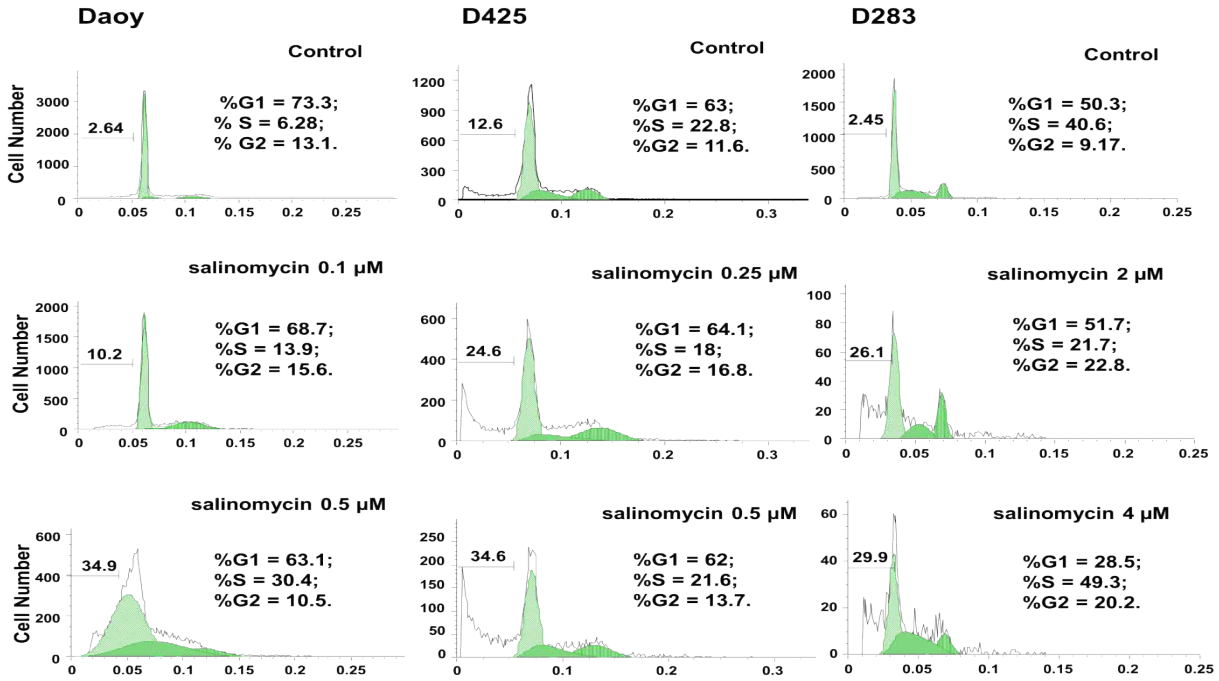

Figure 2: Salinomycin induces apoptosis and affects cell cycle progression in MB cell lines. Cells were treated with salinomycin for 24 hours and analyzed for cell cycle distribution and cell death. Cell cycle and cell death are analyzed independently using FlowJo. The cell death percentage is calculated against all cell population including apoptotic cells and live cells in cell cycle phases.

range of 0.25-4 μM significantly inhibits cell proliferation and induces cell death and cell cycle arrest. Through analyzing of changes in expression of genes and/or proteins that are involved in cell proliferation, cell death, and the Notch signaling pathway in response to salinomycin treatment, we reveal that salinomycin suppresses the expression of PDGFRβ, MYC, Bcl-2, p21 and some key effectors in the Notch signaling pathway (e.g., *Hes1*).

Since the discovery that salinomycin has anti-CSC activity in breast cancer [3], several recent studies have shown that salinomycin possesses profound anti-cancer and anti-CSC activities in other cancer types *in vitro*, and *in vivo* xenografted mouse models, as well as pilot clinical studies in patients [33-35]. Nevertheless, the effects of salinomycin on MB cells have not been previously studied. In the present study, we demonstrate that salinomycin has profound cytotoxicity against human MB cells. This conclusion was supported by a dose-dependent increase of cell death (the sub-G0 population) and a significant reduction of cell proliferation upon salinomycin treatment. Cyclin A is required for DNA replication in both S and G2 phases [36], the up-regulated cyclin A levels might be due to the prolonged S/G2 phases by salinomycin treatment. In addition, the cell cycle arrest at S/G2 phases and up-regulation of cyclin A expression were well correlated with cell proliferation data.

Hes1 and *Hes 5* are critical effectors of the Notch signaling pathway which plays an important role in MB disease progression and patient survival. Fan et al. have reported that the Hes1 expression activated by Notch signaling is associated with significantly lower survival in MB patients [24]. Research from the same group also revealed that the blockade of the Notch pathway suppressed *Hes1* expression and can cause cell apoptosis, cell cycle exit, and differentiation in MB cells [23]. This research suggested a role of Notch signaling in MB CSC maintenance. Our data show that salinomycin downregulated the transcription of both *Hes1* and *DLL1*. This partially explained the effects of salinomycin on MB cell survial and indicated its role on MB CSC maintenance. In addition, *MAML1* which was previously shown as a coactivator to amplify the Notch induced transcription of *Hes1* was also inhibited by salinomycin in MB cells [37]. The suppression of the gene expression in Notch signaling may also partially explain the downregulated protein levels of p21 which is also a target gene of Notch signaling [38].

It has been noticed that MYC is a downstream target of canonical Wnt signaling [39]. Indeed, salinomycin blocks the phosphorylation of the Wnt co-receptor lipoprotein receptor related protein 6 (LRP6) and induces its degradation in Wnt-transfected HEK293 cells [13]. It is possible that the down-regulated MYC might be partially caused by salinomycin's impact on Wnt signaling. In this study, we have observed the suppression on Notch signaling by salinomycin in MB cells and MYC is also a target molecule of Notch signaling [40,41]. The possibility exists that salinomycin down-regulates MYC at least partially via Notch signaling. In addition, MYC is a downstream of PDGFR signaling [42,43]. Therefore, targeting these pathways simultaneously for MB should provide an effective strategy for the treatment of MB.

MYC is commonly deregulated in MB [44-46] and modulates multiple cellular events through alteration of the expression of a number of functionally important target genes [47]. Recent studies show that among the four subtypes of MB, the Group 3 MB chracterized with MYC overexpression indicates aggressive disease and poor prognosis [48]. Moreover, blocking MYC significantly

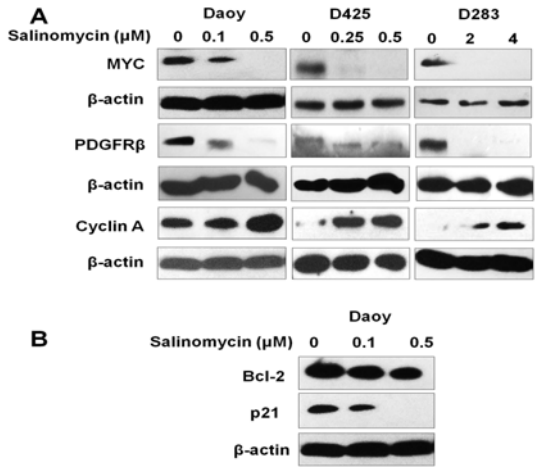

Figure 3: Effects of salinomycin on the expression levels of key regulators of cell proliferation, cell cycle progression and cell death. (A) Salinomycin down-regulates MYC and PDGFRβ, up-regulates cyclin A. (B) Salinomycin down-regulates the expression level of Bcl-2 and p21. Cells were treated with salinomycin for 24 hours at indicated concentrations and analyzed by Western blotting (β-actin was used as an internal loading control).

reduces MB cell growth [49]. In addition to MYC, high levels of PDGFRβ have also been correlated with an aggressive phenotype of MB [50]. In this study, by showing that salinomycin could suppress the expression of MYC and PDGFRβ at the same time, our results uncover a new mechanistic aspect of salinomycin's potent anti-cancer effects and highlight the value of salinomycin as a very promising drug for treating MB [51].

Conclusion

Our study demonstrates that salinomycin exhibits cytotoxic effects in human MB cells. Our data reveal that the treatment with salinomycin is effective in inhibiting MB cell proliferation and cell cycle progression and inducing cell death. We also show that MB cells have altered multiple signaling pathways after salinomycin treatment. The down-regulation of PDGFRβ and MYC and the suppression of Notch signaling pathway are likely the contributing factors to salinomycin's cytotoxic effects. Taken together, this study suggests that salinomycin is a potential effective therapeutical agent for MB and warrants further investigation.

Acknowledgements

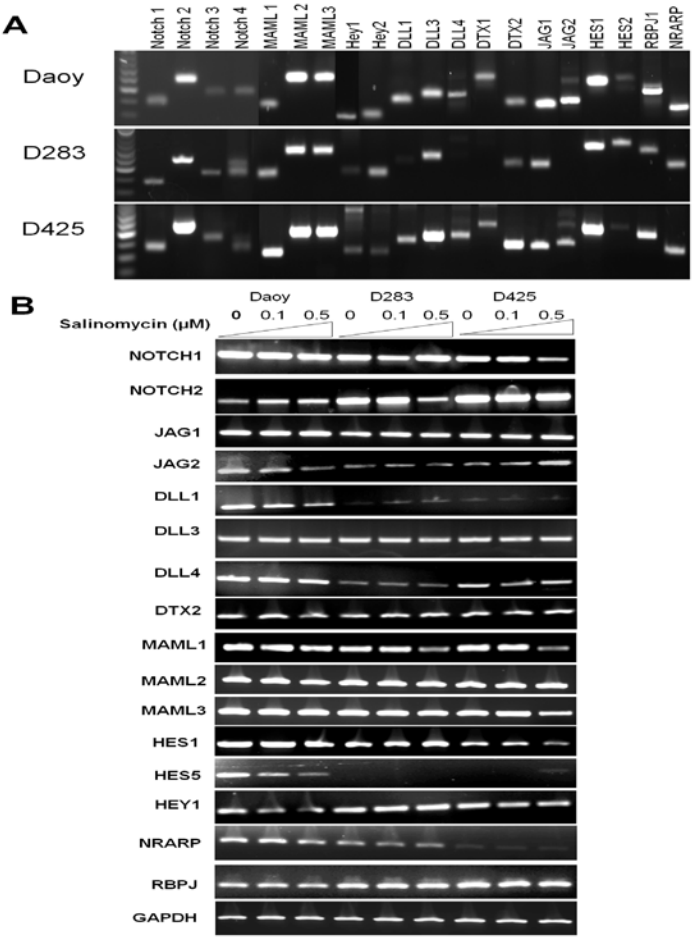

Figure 4: Salinomycin modulates Notch signaling in MB cells. (A) The basal levels of genes in Notch signaling in the three MB cells lines. (B) Salinomycin down-regulated the mRNA levels of key components in Notch signaling in MB cells. Cells were treated with salinomycin for 24 hours at indicated concentrations and analyzed by RT-PCR analysis (β-actin was used as an internal loading control).

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Conflicts of Interest

The author(s) confirm that this article content has no conflicts of interest.

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