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Study of modified Salinomycin analogs on inhibition of triple negative breast cancer metastasis and invasion

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STUDY OF MODIFIED SALINOMYCIN ANALOGS ON INHIBITION OF TRIPLE NEGATIVE BREAST CANCER METASTASIS AND INVASION

by

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Dedication

I would like to dedicate this manuscript to my sister, Niloufar Malekmarzban, this would never have been possible without her.

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Abstract

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Triple negative breast cancer (TNBC) is a more aggressive type of breast cancer which contains faster growth, higher metastasis rate and worse prognosis. The main challenge toward treatment of this type of cancer is being heterogeneous. Recently, Salinomycin (SAL) has been considered as a potential agent for triple negative breast cancer treatment. However, chemical modification of SAL is needed to improve SAL selectivity to cations and decrease its cytotoxicity. The purpose of this study was to evaluate the effect of chemically modified analogs of SAL on the viability, metastasis, Wnt signaling pathway as well as angiogenesis of Triple negative breast cancer (TNBC) cell lines using MTT assay, Western blot and Invasion assay. Obtained results indicated that the tested compounds may have the potential to inhibit angiogenesis, metastasis and alter Wnt signaling pathway. However, more studies are needed to clarify their effects on CSCs, in vivo and moreover, their effects on both tumor and normal cells should be evaluated.

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Chapter 1

Introduction

Cancer is a life-threatening disease worldwide. Though the success of anti-cancer agents can't be ignored, the undesirable side effects of these agents are still considered as a big problem.

Breast cancer is the second fatal cancer in women and formed by accelerated malignant cell growth in the breast and can be developed as a result of other cancer metastasis. Some risk factors including obesity, alcohol and coffee consumption, diabetes, lack of physical exercise are involved in the development of breast cancer. Moreover, genetic mutations such as BRCA1, BRCA2, CCND1, ERBB2, FGFR1, MYC, PIK3CA, PTEN, GATA3, MAP3K1 are participating in initiation and progression of breast cancer. The histological classification of breast cancer is determined by molecular biomarkers including estrogen receptor (ER), progesterone receptor (PR) and ERBB2 receptor (HER2) (1).

Triple negative breast cancer (TNBC) is distinguished from the other types of breast cancer by not expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). 10-15% of all breast cancer is related to TNBC. TNBC tumors compared to the other types of breast cancer tumors contain higher proliferation index and mean tumor size as well as invasiveness.

Based on genomic profile, TNBC categorized into six subtypes, including, basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), mesenchymal stem-like (MSL),

immunomodulatory (IM), and luminal androgen receptor (LAR). Mutations of regulators of cell cycle and genes of DNA repair proteins have been observed in the BL1 subtype. The BL2 subtype is related to malfunctioning of signaling pathways, including, EGFR, MET, NGF, Wnt/β-catenin, IGF-1R. The M type is considered as a metaplastic type of TNBC due to overexpression of cell motility, cell differentiation, growth factor and migration-related signaling pathways. The characteristic of MSL subtype is related to decreased expression of proliferation genes and increased expression of genes associated with stemness. The IM subtype is defined by high activation of immune cell associated signaling pathways, including, Th1/Th2, NK cell, B cell receptor signaling, dendritic cell (DC) pathways as well as T cell receptor signaling CTLA4, IL2, IL7 pathways. The LAR subtype due to high expression of AR receptors is distinguished from the other types of TNBC responding to anti-AR therapy.

TNBC can be high grade or low grade that their characteristics are different. The low grade contains a lower proliferation rate than the high grade. The high grade or level lll is characterized with increased metastasis and demonstrated as large tumors. Moreover, TNBC is detectable when the tumor is in grade III (Li et al, 2020).

The major problem regarding the TNBC treatment is the heterogeneity in TNBC. Due to low or absent expression of receptors such as, ER, PR and HER2+, chemotherapy is considered as a main treatment strategy. The combination of chemotherapeutic agents such as anthracycline, alkylators and taxenes is recommended for early-stage TNBC.

Chemotherapy is considered as a first –line therapy for treatment of TNBC. However, based on the nature of TNBC containing molecular phenotype and

demonstrating no expression of PR, ER and HER2, chemotherapy is not very successful. Therefore, the survival time of TNBC is shorter compared to the other types of breast cancer. It seems that combinational therapy can be an effective approach to potentiate the effects of the current therapy. As a result, some of the patients with similar pathological situations may respond well to the current chemotherapeutic regimen, although some of them will relapse.

Considering that there is no standard treatment strategy for patients demonstrating high metastasis as well as relapse and the median overall survival of such patients have not been enhanced, novel treatments have been developed targeting the mutation and key signaling pathway are responsible for demonstrating malignancy or resistance to the chemotherapy.

PD-L1 expression in infiltrating immune cells especially T cells and binding to PD-1 results to suppress T cells. PD-L1 is highly expressed in TNBC tumors and consequently, it can be served as a therapeutic target in metastatic TNBC. The existence of TILs and the heterogeneous entity of TNBC provide the application of immunotherapy for TNBC treatment.

Currently, atezolizumab (Tecentriq) and pembrolizumab (Keytruda) as anti_PD-1 monoclonal antibodies have been approved by FDA. Tecentriq can be served in combination with nab-paclitaxel and Keytruda can be applied in combination with neoadjuvant chemotherapy for TNBC treatment (Won & Spruck, 2020).

Since TNBC is heterogeneous and the FDA approved agents are specific for certain populations, their administration has not been very successful regarding improving the median overall survival (OS) for mTNBC. Moreover, novel immune checkpoints are targeting PI3K/AKT/mTOR or AMPK pathways under investigation in early phase trials.

Olaparib has been approved by FDA for patients carrying the mutated BRCA1 and BRCA2. Following Olaparib treatment, patients have not demonystrated much side effects. Moreover, Talazoparib is FDA approved and able to enhance median progression-free survival (PFS) (Gupta et al., 2020).

Currently, Salinomycin (SAL) has been attended as an effective anti-cancer agent based on excessive studies reported anti-cancer and anti-CSCs activities of SAL. The anti-cancer function of SAL is attributed to interactions with multiple molecular targets. It has been reported that cell signaling pathways and targets including, Wnt, apoptosis, autophagy, MAPK, ER stress, PI3K/AKT/mTOR, AMPK, EMT, iron homeostasis, angiogenesis, Hedgehog, NF-κB and Notch, are affected by SAL. The advantages of SAL compared to the current anticancer treatments are being more selective and safer (Naujokat & Steinhart, 2012).

Studies have demonstrated that SAL is a potential agent against resistant cancer cells and does not cause severe side effects on human normal cells unlike the conventional chemotherapeutic agents (Dominik et al, 2009). SAL originally is an ionophore antibiotic traditionally applied as an anti_coccidial drug (Shuang et al., 2013).

Also, SAL shows anti-viral and anti-fungal function. Here, we have reviewed different aspects of SAL including, the structural characteristics, mechanism of action, anti-CSCs and anti-cancer activities, its effect on triple negative breast cancer cells as well as its modification to improve the potency.

SAL is an anti-biotic which was synthesized through the culture broth of Streptomyces albus strain no. 80614 by Miyazaki and colleagues in the early seventies. SAL demonstrated anti-coccidial effect on chicken and average body weight of them was increased following SAL treatment. Therefore, it is implied as the anicoccidial agent. Also, it is effective for controlling necrotic enteritis in chickens which is caused by clostridium perfringens which is harmful to humans consuming the diseased meat. SAL has been approved by the FDA (Miyazaki et al., 1974; Lindemann et al., 1985; Piperno et al., 2015).

It has been demonstrated that SAL and its derivatives exert high anti-microbial activity against Gram-positive bacteria, including methicillin-resistant Staphylococcus aureus, methicillin-resistant Staphylococcus epidermidis, and Mycobacterium tuberculosis, Bacillus subtilis, Micrococcus flavus, Sarcina lutea, some filamentous fungi, Plasmodium falciparum. However, it is unable to destroy Gram negative bacteria which is correlated with its impermeability to the outer cell membrane.

The problem due to antibiotic application is the development of resistance. Consequently, patients do not respond to the antibiotic. The formation of resistance results in prolonged infection course, higher mortality rate as well as formation of drug resistance of bacterial strains. It has been shown that the effect of SAL against Staphylococcus aureus and Staphylococcus epidermidis was łike the effect of ciprofloxacin. SAL exhibited

activity against some antibiotic-sensitive bacterial strains and some antibiotic-resistant pathogenic bacteria. It has been shown that SAL was effective against Staphylococcus aureus isolated from patients which were resistant to the activity of erythromycin, streptomycin, chloramphenicol, and tetracycline. Hence, it can be potent against some antibiotic-resistant bacteria and is able to solve the problem due to formation of resistance (Johansen et al., 2007; Stefasnka et al., 2015)

1.1. Mechanism of Action of Salinomycin

SAL is a monovalent ionophore; polyether ionophores are natural compounds containing lipid soluble structure assisting ion transporting via these molecules. These compounds have been focused on in recent years due to their therapeutic properties. Ionophores are used extensively to control coccidiosis. It seems that they exert such an effect through facilitate ion transport across hydrophobic membranes. Regarding their lipid-soluble structure, they protect ion charge from the surrounding environment through formation a protective structure in which ion is inserted inside the matrix. As a result, ions are protected from the interaction with surrounding molecules passing easily across membranes.

SAL (molecular formula, $C_4H_{70}O_{11}$) is a polyether antibiotic. Since the molecule contains monocarboxylic acid shows acidic property. SAL as an ionophore can facilitate the cation transport (K+, Na+, Ca2+ or Mg2+) through cell membranes of protozoa and gram-positive bacteria. The facilitated ion transportation across cell membranes causes the anticoccidial effect intensifying intracellular calcium to levels toxic to coccidian, through disrupting the osmotic balance.

This molecule is pentacyclic containing oxygen atoms which are placed inside the molecule and has capability to react with cations. The alkyl side chains are directed outside conferring the lipophilic characteristics to the molecule.

The antibacterial function of SAL is due to its complex formation with metal cations and transport them across lipid bilayers. SAL can form complex with mono- ($K^+ > Na^+ > Cs^+$) and divalent ($Sr^{2+} > Ca^{2+}, Mg^{2+}$) cations (15,16). The formation of the cage around the metal cations confers the lipophilic characteristic. SAL takes the cation on one side of the lipid bilayer, the solvate molecules one by one are replaced with its polar groups. By moving across the membrane, the complex releases cation on the other side. The mechanism of cation transport is dependent on the pH of environment. Three types of cation transport can be processed. If the environment is neutral or slightly alkaline, first the carboxyl group of ionophores becomes deprotonated (I-COO-) and binds the metal or proton (H^+) to give a neutral salt (I-COO-M^+) or a neutral ionophore in the acidic form (I-COOH). The cation transport is performed by the electroneutral process.

The second type called electrogenic process which is occurred in a non-alkaline environment. The polyether ionophore in its acidic condition (I-COOH-M^+) forms a complex and the cation is transported. The third type is called biomimetic that is performed through derivatives of polyether ionophores. The formed amide or ester prevents the carboxylic function of SAL (Piperno et al., 2015).

1.2. Anti-Fungal Activity

It has been observed that SAL displays moderate anti-fungal activity against Penicillium digitatum, Paecilomyces variotii, Candida albicans, and Saccharomyces

cerevisiae. Moreover, SAL in combination with polymyxin B can be served against yeasts and filamentous fungi through increasing the permeability of cells to polymyxin B (Westkey, 1982).

1.3. Anti-Viral Activity

The anti-viral activity of SAL was tested. It was observed that SAL can potentiate the antiviral effect of oseltamivir, the neuraminidase inhibitor. The inhibition of the proton channel function of viral matrix protein 2 and nuclear migration of viral nuclear protein by SAL caused to display the antiviral activity (Jang et al., 2018).

Chapter 2

Anti-Cancer Activity of Salinomycin

2.1. Anti-Cancer Stem Cells Function

Cancer stem cells (CSCs) include a subpopulation of tumor cells having ability to self-renew and tumor initiation capacity. Due to the existence of CSCs within a tumor, tumor contains heterogenous entity. Due to contain several intrinsic mechanisms, CSCs are resistant to current chemotherapeutic drugs and radio therapy. Moreover, therapy resistant CSC and, it is need for being targeted some intrinsic pathways by therapy-resistant CSCs. Several mechanisms including, expression of ATP-binding cassette (ABC) drug transporters, aberrant regulation of Wnt/β-catenin signaling, activation of the Hedgehog and Notch signaling pathways, having epithelial mesenchymal transition (EMT), elevated activity of aldehyde dehydrogenase 1 (ALDH1), intensified checkpoint activation and improved repair of DNA and oxidative damage, expression and activation of the Akt/PKB and ATR/CHK1 survival pathways, aberrant PI3 K/Akt/mTOR mediated signaling and loss of phosphatase and tensin homolog (PTEN), activation of NF-κB, are participated in formation of resistance. Several research has been performed in order to clarify the exerted anti-cancer action against CSCs and tumor cells. Stem cells are distinguished from their differentiated progeny by cell surface markers (immunophenotype) expression. These cell surface markers like CD44, CD24, CD29, CD90, CD133, epithelial-specific antigen (ESA) and aldehyde dehydrogenase1 (ALDH1) lead to differentiating CSCs from different tumor cells. Moreover, CSCs of different tumors contain distinct patterns of cell surface markers. For instance, CD133+ lineage is related to colon, brain, and long CSCs, CD44+/CD24-

/low and ALDH+ to breast, CD34+CD8- to leukemia, CD44+ to head and neck, CD90+ to liver and CD44+/ CD24+/ESA+ to pancreas CSCs (Yang et al., 2020).

In 2009, Gupta et al. screened 16000 compounds to probe the chemicals preferentially eradicate CSCs. They observed that the most potent one was SAL having a more than 100-fold efficacy compared to paclitaxel. In this study, it was observed that the more CD44high/CD24low CSC-enriched subpopulations, the more sensitive to SAL. Notably, SAL was able to reduce the percentage of CD44high/CD24low breast cancer stem cells, however, paclitaxel elevated such population. Down-regulation of E-cadherin resulted in inducing EMT in HMLER-shEcad cells. Due to EMT, these cells are resistant to chemotherapeutic agents such as Paclitaxel, doxorubicin D, campthotecin and staurosporine. Treatment of SAL could eradicate HMLER-shEcad cells. Moreover, following treatment with SAL caused a decrease in the number of tumorspheres, whereas paclitaxel markedly enlarged the size of tumorsphere. In breast cancer, markers such as CD44, CD24 as well as high level of aldehyde dehydrogenase 1 (ALDH1) were used to isolate progenitor/CSCs cells. In addition, the other distinguishing feature of CSCs is their ability to form tumor sphere in low-adherence cultures in serum-free medium (Gupta et al., 2009).

Following the discovery of anti CSCs action of SAL by Gupta, several studies have been accomplished in order to reveal the potency of this agent against the other types of CSCs. It was noticed that SAL was able to decrease the percentage of breast CSCs through destroying ALDH1+ and SOX2-positive cells. SAL suppressed CD44+ expressing VCaP- and LNCaP-derived prostate CSCs. It has been shown that SAL was able to destroy

CD133+ colorectal and pancreatic CSCs. SAL was able to down regulate the genes of CSCs related to the poor prognosis tumor. The inhibitory action of SAL against HMLER-shEcad cells was prevention of tumorsphere formation, epithelial differentiation, tumor-seeding ability of these cells in xenograft mice. Moreover, SAL caused regression, differentiation necrosis and apoptosis of SUM159 tumors in xenograft mice. Taken together, it was concluded that SAL may serve as a potential agent to eradicate CSCs (Dewangan, Srivastava & Rath, 2017).

It was noticed that SAL could decrease the population of CD133 CSCs in HT29 and SW480 cells suppress the tumorsphere formation in CSCs population of HT29 and SW480 cells (Klose et al., 2019).

Studies have revealed that SAL is able to suppress tumorosphere formation in breast, lung, gastric, osteosarcoma, colorectal, and pancreatic CSCs. Guan-Nan Zhang et al. investigated the SAL effect on pancreatic CSCs. It was observed that SAL could be helpful in combination with other medicine such as Gemcitabine for destroying CSCs. One of the problems related to Gemcitabine is the formation of resistance to this medicine. In the study, it was noticed that combinational therapy could reduce the cell viability of CSCs (Zhang et al., 2011).

Kamlund, et al. observed SAL induced behavior effects in JIMT-1 breast cancer cells. SAL decreased the stemness through different mechanisms, including, increasing the $CD24^{+}$ population, increasing the differentiation by inducing more epithelial characteristics. Since SAL increased the level of E-cadherin, it could inhibit cell proliferation and cell migration (Kamlund et al., 2020).

It was shown that SAL can improve the efficacy of Oncostatin M (OSM) for eradicating liver stem cells. In this study, The CD133+ population of liver stem cells were treated with combination of Oncostatin with SAL. The results indicated that combinational therapy not only caused to decrease CD133 expression, but also inhibited the gene expression which are responsible for stemness. Combinational therapy reduced the levels of AFP and increased the amount of ALB. Also, treatment with SAL and OSM caused to induce cell death. The increased level of caspase-3 indicated that SAL induced the mitochondrial apoptosis pathway. Following SAL treatment, the levels of OCT4, NANOG, and c-MYC mRNA were decreased concluding that SAL was effective against the stem cell population. These genes are involved in maintaining the characteristics of stem cells as well as regulating the downstream genes which are responsible for differentiation and self-renewal (Fu et al., 2020).

Other studies showed that SAL eradicated CSCs in different human cancers. After discovery of anti-CSCs action of SAL, its anticancer properties were discovered. For example, it was observed that SAL could decrease the population of breast CSCs by eradicating ALDH1+ and SOX2-positive cells. In addition, SAL inhibited CD44+ expressing VCaP- and LNCaP-derived prostate CSCs and it decreased the number of CD133+ colorectal and pancreatic CSCs. Besides its significant anti-cancer activities, it has been noticed that SAL does not induce severe adverse effects on human normal cells like other chemotherapeutic agents (Naujokat & Steinhart, 2012).

2.2. Anti-Cancer Characteristics

The ionophoric characteristics enable SAL to bind selectively the metal ions and transport them through membranes. By disturbing the Na+/K+ balance and causing the release of Ca2+ into the cytosol, it induces the change of intracellular pH, increasing osmotic pressure, vacuolization, mitochondrial injuries, and cell death. Based on the acidic environment of cancer cells (Warburg effect), electrogenic transport mechanism can be applied effectively by ionophores, therefore they can exert their anticancer property (Zhang et al., 2020).

SAL shows its anti-cancer effect through different mechanisms in various cancer cells. It has been demonstrated that cells signaling pathways and targets including, Wnt, apoptosis, autophagy, MAPK, ER stress, PI3K/AKT/mTOR, AMPK, EMT, reducing activity of ABC transporters, iron homeostasis, angiogenesis, Hedgehog, NF-kB and Notch, are affected by SAL.

2.3. Salinomycin Effect on Wnt Signaling Pathway

One of the most important mechanisms that direct cell proliferation, cell polarity as well as fate determination throughout embryonic development and tissue homeostasis is signaling mediated by the Wnt family of secreted glycolipoproteins. Considering the critical role of Wnt signaling pathway, mutations of proteins involved in the pathway are correlated with human birth defects, cancer, and other diseases. It is distinguished that activation of Wnt signaling is linked to 50% of breast cancer patients and lowered overall survival. Wnt signaling has been examined intensively in triple negative breast cancer formation and progress. Although, increased levels of nuclear β-catenin were also detected

in other breast cancer types. Wnt proteins bind to the N-terminal extra-cellular cysteine-rich domain of Frizzled (Fz) receptor family which is seven transmembrane-span proteins and compose a family of G-protein coupled receptors (GPCRs). Also, co-receptors such as low-density-lipoprotein- protein5/6 (LRP5/6), receptor tyrosine kinase (RTK), and ROR2 are required for mediating Wnt signaling. Branches downstream of the Fz receptor are a canonical or Wnt/β-catenin dependent pathway and the non-canonical or β-catenin-independent pathway which includes the Planar Cell Polarity and the Wnt/Ca2+ pathway (Komiya & Habas, 2008; De, 2011).

2.4. The Canonical Wnt Pathway

The major characteristic of the canonical Wnt pathway is the aggregation of β-catenin and translocation into the nucleus. In the absence of Wnt signaling, β-catenin is targeted by a destruction complex which consists of Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase, 3 (GSK3) and casein kinase 1α (CK1α). In this complex, β-catenin is phosphorylated by Casein Kinase and ubiquinated by GSK3. Thereafter, it is proteolytically destructed by the proteosomal machinery. Binding of Wnt to its receptor complex including Fz and the LRP5/6 leads to prevent the destruction of β-catenin which is performed by APC/Axin/GSK3 complex disruption. Synthesis of Wnt ligands is a determining factor in Wnt signaling.

2.5. Wnt Signaling Pathway in Cancer Stem Cells

The prominent role of the Wnt pathway for cancer stem cells function has been clarified. One of the considerable characteristics of stem cells is prolonged lifespan because of activation of the TERT gene and maintain long telomeres. It has been described that

Wnt signaling is correlated to telomerase activity. Binding of βcatenin to TERT promoter region leads to prevent β-catenin destruction and activation of Wnt signaling pathway.

It has been shown that Co-activation of the NF-κB and Wnt signaling can convert normal intestinal cells into stem cells. Mutations of the adenomatous polyposis coli (APC) gene are correlated with colon cancer syndrome. Lgr5 as a marker of intestinal stem cells can cause tumor growth when APC is inactivated in these cells. In additionRAC1 is activated following APC gene deletion leading to both increase of ROS production and NF-κB signaling activation (Yang et al., 2016).

It has been clarified that TNBC is related to the aberrant Wnt signaling, including canonical and non-canonical pathways which are associated with tumorigenesis, metastasis as well as poor clinical outcomes. Since the accumulated β-catenin facilitates cell migration, colony formation, displaying stem-like features and tumorigenesis in mouse cancer models, the abnormal Wnt pathway is a considerable cause of TNBC tumorigenesis. Also, it has been demonstrated that Wnt/β-catenin signaling activity is more elevated in breast CSCs than the bulk tumor population (Pohl et al., 2017).

2.6. Salinomycin Inhibitory Action on Wnt Signaling Pathway

Researchers developed a fluorescent conjugate of SAL to observe the ionophore properties. By this method, they could discover cellular uptake and subcellular localization of SAL. To improve the characteristics of SAL, the conjugation of Whitehouse's nitrobenzoxadiazole (NBD) reporter and SAL was prepared and tested in breast cancer cell lines, including, JIMT-1, MCF-7 and HCC1937. The conjugation of SAL with NBD

reporter was ligated at the C20-hydroxyl group and contained some advantages including, small size, lack of reactivity (bio-orthogonality) as well as low polarity of NBD. Through subcellular imaging, it was noticed a cellular uptake of this conjugate and accumulation in the endoplasmic reticulum (ER). The accumulated compound inhibited Wnt signaling pathway through two mechanisms. First, it induced Ca^{2+} release from the ER into the cytosol and up-regulation of C/EBP homologous protein (CHOP). This protein in turn decreased the β-catenin expression and Wnt signaling inhibition. Second, increased cytosolic Ca^{2+} activated protein kinase C and consequently, it inhibited the Wnt pathway. Therefore, it was concluded that SAL was able to inhibit Wnt pathway (Huang et al., 2018).

Klose et al. investigated the SAL effect on stem cells primary tumor-initiating cells (TIC) isolated from human patients with colorectal liver metastasis or from human primary colon carcinoma. In this study, the effect of both SAL and combination of SAL with FOLFOX was tested.

It was noticed that SAL alone or in combination with FOLFOX can imply intensified antitumor activity compared to FOLFOX therapy in a patient-derived mouse xenograft model of colorectal cancer. It seemed that SAL exerts anti apoptotic effect on human colorectal cancer cells through formation of dysfunctional mitochondria and reactive oxygen species (ROS) as well as decreased cellular ATP production. Also, SAL caused down-regulation of superoxide dismutase-1 (SOD1) which is correlated with these effects. Consequently, SOD1 can be considered as a potential target of anti-cancer agent. It was observed that SAL induces elevated anti-cancer stem cell activity compared to 5-FU and oxaliplatin. The other behavior of SAL related to anti CSCs was prevention of tumor

growth. The treatment with combination of SAL, 5-fluorouracil and oxaliplatin, caused superior outcomes compared to SAL monotherapy. It was observed that SAL lowered the expression of Lgr5 which is the main gene that is responsible for stem cell hierarchy and maintenance of spheroid-derived colorectal cancer cells. Moreover, it inhibited the ALDH1 expression, which labels colorectal cancer stem cells (Klose at al., 2019).

Other studies showed that SAL can decrease the stemness in colorectal CSC HT29 and SW480 cells. It was observed that SAL prevented the formation of β-catenin/TCF4E transcriptional complex. In addition, SAL down regulated the Wnt target genes such as LGR5, CD44 and Sox2. In colorectal CSC xenografts, APCmin/+ mice, and patient-derived colorectal tumor xenografts, SAL caused to decrease the tumor growth and expression of LGR5 gene. Based on the observations, it was concluded that the inhibitory effect of SAL on the β-catenin/TCF complex can be a target for colorectal cancer treatment (Wang et al., 2019).

In accordance with this result, it was observed that SAL inhibited the Wnt pathway in HT29 and HCT116 cells. The inhibitory effect on Wnt pathway was through reducing the interaction of β-catenin and TCF4E in both HT29 and HCT116 cells and decreasing the expression of Wnt target inhibit stemness by prevention of LGR5 and CD44 expression (Wang, et al., 2020).

In pancreatic cancer cell lines, SAL may be effective through inhibition of Wnt signaling pathway and invasion. Human pancreatic cancer cell lines, including PANC-1, SW1990, and AsPC1 were treated with SAL. SAL exerted anti-invasion effect by up

regulation of Bax and E-cadherin, however, the anti-apoptotic effect was mediated through down regulation of Bcl-2 and PCNA. Moreover, the expression of Wnt/ βcatenin signaling-related proteins (b-catenin and p-GSK-3b) was suppressed. Treatment with SAL caused to decrease the expression of CD133 in AsPC-1 and SW1990 cells which is characteristic of pancreatic cancer cell lines (He et al., 2013).

The investigation of SAL effect on nasopharyngeal carcinoma cells (NPC) including, CNE-1, CNE-2 and CNE-2/DDP indicated that SAL induced apoptosis as well as inhibition of cell proliferation. The anti-neoplastic effect of SAL was the activation of caspase-3 and caspase-9, and reduction of mitochondrial membrane potential. SAL down regulated Bcl-2 and up regulated Bax. Moreover, SAL inhibited the ability for invasion. Since the results of western blot indicated that SAL reduced the expression both of LRP6 and βcatenin in a dose-dependent manner, it was concluded that inhibition of Wnt pathway by SAL was correlated with stimulation of apoptosis. Moreover, in vivo studies showed that SAL treatment caused decreased expression of beta catenin, inhibit tumor growth as well as elevated epithelial differentiation of tumor cells (Wu et al., 2014).

One of major problems regarding the treatment of ovarian cancer is metastasis and subsequently, aggravation of malignancy. The anti-cancer effect of SAL was evaluated in EOC cells, including SK-OV-3 and A2780. Since, up regulation of GSK-3β and βcatenin was observed in these cells, it was concluded that Wnt pathway is participated in the cell progression. Moreover, the Wnt signaling pathway can regulate EMT as well as migration of EOC cells. It was observed that SAL inhibited the invasion as well as migratory ability of EOC cells. In addition, SAL inhibited the Wnt pathway through decrease of expression

of downstream effectors of the Wnt pathway, such as p-GSK-3β-ser9 (the inactivated GSK-3β), β-catenin and Slug as well as down regulation of target genes, such as Axin2 and CCND1. The increased expression of Slug has been observed in different invasive tumors. Wnt pathway through increasing the expression of β-catenin causes to elevate Slug expression and subsequently, activates EMT. Considering the inhibitory effect of SAL on Slug expression, it may inhibit EMT in EOC cells through suppression of Slug. Therefore, SAL inhibited EMT in EOC cells via blocking of Wnt pathway. Also, the results of western blot confirmed that SAL can upregulate the epithelial markers protein (E-cadherin and Keratin), whereas lowers the amount of the expression of mesenchymal markers (N-cadherin and vimentin). As a result, SAL could induce differentiation to the epithelial cells (Li et al., 2017).

2.7. Salinomycin Effect on Apoptosis

Apoptosis is defined as a programmed cell death. In this process the cell morphology is altered following cell shrinkage, nuclear fragmentation, chromatin condensation, DNA damage and mRNA decay. The apoptosis is started by disintegration of the inner mitochondrial transmembrane potential and membrane permeabilization. Then, chromatin condensation and nuclear fragmentation occur, and the apoptotic bodies are formed which are membrane-bound. The formed apoptotic bodies are ingested by macrophages. Apoptosis can be triggered by one of two mechanisms. In the intrinsic pathway, when cells sense stress, they undergo apoptosis. However, the extrinsic pathway happens in

response to signals from other cells. Uncontrolled apoptosis causes over proliferation of cells (Kaczanowski, 2016).

Studies have revealed that SAL activates apoptosis in different CSCs, however, the exact mechanism of anti-apoptotic effect of SAL is not clearly understood. It seems that difference in anti-apoptotic effect of SAL depends on the cell type.

The cell cycle is controlled by two classes of regulatory molecules including cyclins and cyclin-dependent kinases (CDKs). Levels of cyclins and CDK inhibitors at each cycle are regulated by the Skp, cullin, and F-box–containing (SCF) complex and the anaphase promoting complex (APC). Since cyclin D1 is a positive regulator of the G1- to S-phase transition, it is considered as a biomarker of cancer progression.

Cell cycle progression is prevented by two CDK inhibitors, p21Cip1 and p27Kip1. The proteasomal degradation of p27Kip1 is controlled by S-phase kinase-associated protein 2 (Skp2) which belongs to SCF E3 ligase complex. SKp2 causes degradation of tumor suppressor proteins such as p27Kip1. Over expression of Skp2 and down regulation of p27Kip1 is correlated with tumorigenesis.

The effect of SAL on various cancer cells was evaluated in order to clarify if it induces the induction or inhibition of apoptosis. One of performed study was testing the SAL effect on ovarian (OVCAR-8) cancer cell line. It has been observed that SAL could prevent cell growth and induce apoptosis through cell cycle arrest at G1. The apoptotic effect of SAL was related to inhibition of transcription 3 (Stat3) activity and subsequent down regulation of Stat3 target genes, including, cyclin D1, Skp2, and survivin. Following

degradation of Skp2, the p27Kip1 level was increased. It was observed that SAL induced down regulation of oncogenes correlated with cell cycle including, Stat3, cyclin D1, and Skp2, phospho-Akt and phosphor Bad as well as up regulation of caspase-3 activation and PARP cleavage. Based on these findings, it was concluded that SAL induced apoptosis in ovarian cancer cells (Koo et al., 2013).

Recently, it was demonstrated that SAL in combination with dasatinib (Das) (a Src kinase inhibitor) was able to induce apoptosis in breast cancer cell lines, including, MDA-MB-468, MDA-MB-231, and MCF-7. In this study, it was noticed that both SAL and Das down regulated several target genes correlated with STAT3, Wnt/β-catenin, and hedgehog cell signaling pathways.

SAL down regulated CCND1 gene which is a common downstream transcriptional target of the STAT3, Wnt/β-catenin, and hedgehog pathways. Moreover, SAL inhibited the estrogen-mediated S-phase entry pathway which is responsible for controlling the G1/S phase transition. The inhibitory mechanism of SAL was exerted through down regulation of some transcription factors such as E2F1, E2F2, and E2F7, which belong to E2F transcription factor family. Following the down regulation of E2F, the transcription of target genes which are needed for proceeding to the S-phase was deactivated. Based on the observation, it was concluded that SAL hindered cell cycle at the G1/S phase through the estrogenmediated S-phase entry pathway. Also, it was noticed that Das in combination with SAL can function better to arrest the estrogen-mediated S-phase entry pathway. Therefore, SAL can potentiate the anti-cancer effect of Das (Bellat et al., 2020).

The treatment of human PC-3 prostate cancer cells with SAL induced G2/M phage arrest and apoptosis. It was noticed that SAL treatment caused to down regulation of c-Myc, β-catenin and cyclin D1 and up regulation of GSK-3 β. It seemed that increase of GSK-3 β expression was related to inhibition of Wnt/ β-catenin pathway. Moreover, not only SAL decreased xenografts tumor size but also inhibited Wnt/ β-catenin pathway. The observation implied that inhibition of Wnt/ β-catenin pathway can be regarded as one of the anti-tumor effects of SAL. In these cells, SAL by blocking the Wnt pathway target and down regulation of c-Myc level triggered apoptosis. SAL decreased xenografts tumor size as well as inhibit Wnt/ β-catenin pathway; hence, SAL thorough Wnt/β-catenin pathway induced apoptosis in PC-3 cells. Moreover, SAL targeting CSCs derived from PC-3 cells to suppress tumor relapse (Zhang et al., 2017).

In one study, the effect of co-treatment of SAL with doxorubicin (DOX) or SAL and etoposide (ETO) was tested on HepG2, MCF-7 and MDA-MB-231 cells. The problem due to these is the development of resistance to medications.

It was observed that a single treatment with SAL, caused to elevate pH2AX protein levels and DNA foci formation. Since DNA foci formation of pH2AX was increased more following co-treatment of ETO with SAL, it may be possible that SAL enhanced DNA breakage and the sensitization of the cells to ETO. Since SAL led to elevate pre-G1 interval in the treated cells, the co-treatment of DOX with SAL showed that the cells responded better than monotherapy of cells with ETO.

It was observed that co-treatment induced to decrease the expression of cyclin D1, p16, p21, p27 and p53 and p21 are protective mechanisms against apoptosis. It was noticed that co-treatment with SAL reduced the expression of p21 and as a result it enhanced proteasome activity. Therefore, the down regulation of p21, was the apoptotic effect exerted by SAL. SAL can increase the amount of DNA damage as well as phosphorylation of p53, BRCA1, Chk1 and H2A histone family member X (H2AX). Therefore, SAL can overcome the developed resistance to the current medications (Kim et al., 2011).

Some studies have demonstrated that SAL can induce apoptosis through distinctive mechanisms. The SAL effect was evaluated on CD4+ T-cell, Molt-4 T-cell and Jurkat T-cell leukemia cells, MES-SA/Dx5 uterine sarcoma cells, as well as Namalwa Burkitt lymphoma cells. The results of immunoblot analysis indicated that SAL did not cause to increase the amount of p53 in the cells. Considering that SAL treatment did not cause to accumulate p53 and/or the cyclin-dependent kinase inhibitor p27 in Molt-4 cells, SAL did not induce cell cycle arrest. Using N-benzyloxycarbonyl-L-valyl-L-alanylL-aspartate fluoromethylketone (Z-VADfmk) as an inhibitor of caspase activation showed that it could not inhibit the apoptosis induced by SAL.

Flow cytometry analysis showed that SAL did not stimulate the expression of cells of CD95L. Taken together, the observation suggested that SAL may induce an apoptotic pathway which is not correlated with cell cycle arrest and its function is independent of tumor suppressor protein p53, induction of caspase, the proteasome and the CD95/CD95L system. Based on the finding, it seemed that SAL could overcome the formed resistance to apoptosis (Dominik et al., 2009).

It has been shown that SAL can induce apoptosis in breast cancer cell lines including MCF-7, T47D and MDA-MB-231. The obtained results confirmed that high concentrations of SAL (25 and 50 μM) caused G2 arrest, lower expression of survivin as well as induction of apoptosis. Following activation of apoptosis, caspase 3/7 and PARP cleavage were stimulated. Moreover, treatment of MDA-MB-231 cells with low concentrations of SAL (≤10 μM) resulted in induction of senescence. The markers of senescence including, SA-β-galactosidase activity, cell cycle arrest, changes in morphology, hyper acetylation of histone H3 and H4 as well as increased expression of p21, the cyclin-dependent kinase inhibitor were observed following treatment of MDAMB-231 cells with SAL. Following treatment of cells with SAL (5 μM), down regulation of the p27 was observed.

In breast cancer cells, survivin is highly expressed to protect cells against induced apoptosis by chemotherapeutic agents. In this study, it was observed that SAL can lead to down regulation of survivin and consequently can induce apoptosis. In this study, SAL caused to up regulate p21 and induce cell cycle arrest in MDA-MB-231 cells. It has been observed that up regulation of p21 is correlated with Histone hyper acetylation. In this study, low concentrations of SAL stimulated hyper acetylation of histone H3 and H4. Moreover, high concentrations of SAL induced apoptotic signaling pathway via activation of caspase 3/7 and cleavage of PARP (Al Dhaheri et al., 2013).

It has been demonstrated that SAL up regulated sarcoplasmic/endoplasmic ATPase reticulum Ca^{2+} transporting 3 (ATP2A3). ATP2A3 is in the ER membrane and has a role in Ca^{2+} transport; it suppresses Ca^{2+} release and induces ER stress. Therefore, ATP2A3 can

be a target for treatment with SAL.SAL applied anti-cancer effect through over expression of ATP2A3 and subsequently, stimulating ER stress in PC-3 and DU145 cells. The stimulated ER stress was correlated with ATP2A3-induced Ca^{2+} release and apoptosis in PC-3 cells (Zhang et al., 2019).

One exerted mechanism by SAL for inducing apoptosis was associated with increased concentrations of Na+ concentrations. It was observed that the enhanced Na^{+} concentrations caused to increase cytosolic Ca^{2+} through Na^{+}/Ca^{2+} exchangers (NCXs) in the plasma membrane and mitochondria of dorsal root ganglia as well as Schwann cells. Increased levels of Ca^{2+} stimulated calpain. The activated calpain triggered apoptosis by activation of caspases 12, 9 and 3 (Boehmerle & Endres, 2011).

The problem of acute promyelocytic leukemia (APL) treatment is related to drug toxicity and development of resistance to the current therapy. SAL treatment of APL cell lines suppressed cell proliferation, elevated Bax/Bcl-2 and cytochrome c expression as well as activated caspase-3 and -9 and stimulated cell apoptosis. It was observed that SAL decreased stemness through stimulation of differentiation of APL cells, mature morphological changes, elevated NBT-positive cell, and CD11b-positive cell percentages and enhanced CD11b and C/EBPβ levels. Moreover, SAL treatment caused to down regulate β-catenin and cyclin D1 and C-myc. The combinational therapy of SAL with IWR-1, an inhibitor of Wnt signaling, synergistically induced differentiation of APL cells (Zhao et al., 2018).

2.8. Salinomycin Effect on Autophagy and DNA Damage

Autophagy is a catabolic process in which double membraned vesicles are formed in order to deliver cellular proteins and organelles to the lysosome. Autophagy is controlled by different signals and cellular stresses. Autophagy can maintain the cell survival by inhibiting the accumulated damaged proteins and organelles and causes to limit the ROS production as well as providing the cell with nutrients and energy. Autophagy has multifarious roles regarding cancer cells. On the one hand, it functions as an anti-tumor agent by increasing genomic stability through reducing ROS production as well as destroying damaged mitochondria and degradation of oncoproteins. However, after formation of tumor, autophagy functions as an agent for cell survival. Studies have demonstrated that autophagy can be considered as a cell survival pathway, although elevated autophagy may lead to cell death. Autophagy confers cancer cell resistance to several chemotherapeutics leading to treatment failure. For example, autophagy induction leads to cause resistance to paclitaxel in ovarian cancer and resistance to cisplatin in ovarian and esophageal cancer. Autophagy induction due to an ER stress response in melanoma and lymphocytic leukemia cells causes resistance to cyclin dependent kinase (CDK) inhibitors. Considering the significant role of autophagy in resistance development to chemotherapy, it can be considered as a promising target in cancer treatment.

It has been clarified that autophagy has a critical role in both tumor cells responses to therapy and altering the environmental stimuli. In the tumor cells which have been exposed to chemotherapy with agents including etoposide, histone deacetylase inhibitors, vitamin D analogs, arsenic trioxide, temozolomide, tamoxifen, or radiation, the current

anticancer strategies are applied to induce autophagy. Therefore, autophagy has a dual role in cancer progression. Based on the condition, autophagy inhibition or stimulation may be effective in cancer therapy (Mulcahy Levy, Towers & Thorburn, 2017).

Several studies have examined the SAL effect on autophagy. SAL impact on autophagy was different in several studies and can be related to the type of tumor and cellular context. Since autophagy is a protective mechanism against apoptosis in some cancer cells, it has been suggested that inhibition of autophagy can lead to improve treatment of chemo-resistant cancer. In various cancer cells, depending on the cell type, SAL has different effects on autophagy.

For the first time, Wen Yue. et al. reported that through the inhibition of cathepsins lysosomal activity, SAL impeded the autophagy in cancer cells. Different breast cancer cell lines, such as HMLER, MCF-7, and HMLER CD24low/− and their CSCs and non-CSCs were used in this study. It was observed that SAL reduced autophagosome turnover and consequently prevented autophagic flux in both CSCs and non-CSCs. It was noticed that SAL caused to enlarge lysosomes and inhibited the degradation of mCherry-GFP-LC3. SAL inhibited the lysosomal hydrolyses especially cathepsin proteases and lysosomal function. Since autophagy is critical for BCSCs proliferation, the prevention of autophagy caused a decrease in BCSCs generation. Since autophagy is a protective mechanism against apoptosis, the inhibition caused to increase the apoptosis. It was noticed that SAL induced fall in autophagy flux in both ALDH+ and ALDH− populations, although, the level in ALDH+ population was more than in the ALDH- one. As a result, ALDH+ cells were more susceptible to autophagy inhibitory effect induced by SAL (Yue et al., 2013).

In one study, it was observed that SAL reduced the number of mitochondria with intact inner membrane potential. Following SAL treatment, the level of PGC1α protein expression, a major regulator for nuclear initiated mitochondrial biogenesis, was not changed, SAL inhibited mitochondria biogenesis. The damaged mitochondria can be eliminated by mitophagy or by contribution to the membrane form autophagosomes. Aberrant mitochondrial phenotypes and mitochondrial specific localization of autophagy marker LC3, showed that SAL treatment caused to induce mitophagy.

The damaged mitochondria can be eliminated by mitophagy or by contribution to the membrane form autophagosomes. Also, SAL treatment caused the consumption of total cellular ATP. ATP-depletion can be considered as a marker of apoptosis stimulation. The results of western blot confirmed that SAL treatment cause to increase the levels of caspase-3 and caspase-9 only in the cells containing hyperpolarized mitochondria. In addition, SAL induced the high expression of HMGB1 protein (necrotic marker) and as a result caused to activate necrosis (or necroptosis) (Jangamreddy et al., 2013).

In one study, the results of MTT assay confirmed that SAL reduced cell viability in breast cancer cells, including MCF-7 and MDA-MB-231cells. Since the response of breast cancer cells to SAL depends on their hormone independent status and chemoresistance, the rate of cell death induced by SAL was lower in MDA-MB-231 cells comparing to MCF-7 cells.

It was noticed that SAL treatment caused to up regulate pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 in a dose-dependent manner in MDA-MB-231 cells. Also, SAL treatment induced lower expression of caspase 3, however, increased concentrations of

SAL caused elevated cleavage of PARP. Moreover, it was observed that SAL induced autophagy in the treated cells. Whereas the rate of induced autophagy in MDA-MB-231 cells was more than MCF-7 cells. Treatment with autophagy inhibitor 3-MA, not only inhibited the induced autophagy by SAL, but also increased the apoptosis stimulated by SAL through caspase activation in MDA-MB-231 cells. Based on the finding, autophagy protected cells against the cytotoxic effects by SAL. ROS formation is involved in SAL induced mitochondrial apoptosis and autophagy. It was noticed that blockage of autophagy could elevate apoptosis by caspase activation and increase in ROS formation (Kim et al., 2017).

In most cases, SAL induced autophagy has a protective role against, however, in case of SW620 cells, the SAL induced autophagy was involved in triggering cell death (Verdoodt et al., 2012).

It has been demonstrated that the excessive generation of ROS (reactive forms of oxygen) induces apoptosis or necrosis by oxidative stress or DNA damage. In addition, ROS are participating in regulation of both apoptosis and autophagy. ROS-induced autophagy improves cell survival by promoting degradation of unnecessary components. ROS initiates PI3K/AKT/mTOR inactivation and MAPK activation during apoptosis and autophagy.

It has been suggested that inhibition of autophagy can lead to improved treatment of chemo-resistant cancer. Because autophagy is a protective mechanism against apoptosis in some cancer cells. In various cancer cells, depend on the cell type, SAL has different effects on autophagy. In one study, the SAL effect on apoptosis stimulation chemo-

resistant prostate cancer cells was tested. Hormone-independent and chemoresistant PC-3 cells and chemo-sensitive LNCaP cells were treated with SAL. Using MTT assay revealed that the chemo-resistant PC-3 cells were more resistant than LNCaP cells, since their apoptotic rates were lower.

Based on the difference between response of chemo-sensitive LNCaP cells and chemo-resistant PC-3 cells to SAL treatment, it was concluded that SAL can decrease autophagy in LNCaP cells and as a result, they are more sensitive to SAL than PC-3 cells.

Because SAL induced apoptosis, SAL through conversion of LC3 induced autophagy in the cells. Whereas induction of autophagy by SAL in LNCaP cells was lower than PC-3 cells. Treatment of cells with 3-MA suppressed autophagy. Subsequently, the rate of apoptosis induced by SAL was increased through activation of caspase-3 in both PC-3 and LNCaP cells. Based on the observation, it was concluded that autophagy is responsible for the resistance to apoptosis in prostate cancer cells (Zhang et al., 2017).

2.9. Salinomycin Effect on ROS Production and Autophagy

SAL can trigger ROS production and decline of mitochondrial membrane potential (MMP). It was observed that SAL induced both autophagy as well as apoptosis in U20S cells through formation of ROS. Consequently, following treatment with SAL, LC3II conversion and AVO accumulation was noticed. Moreover, U2OS cells were pretreated with N-acetyl-l-cysteine (NAC), a ROS inhibitor. Consequently, the reduced expression of LC3II and AVO accumulation was observed confirming the involvement of ROS in formation of autophagy by SAL. SAL up regulated caspase-3 as well as PARP indicating the role of SAL in apoptosis stimulation. Furthermore, upon treatment with 3 MA, the

increased expression of caspase-3 and PARP was detected showing that the induced autophagy protected the cells against cytotoxicity of SAL. The ROS production induced by SAL can inhibit PI3K/AKT/mTOR signaling and activate the AMP-activated protein kinase (AMPK) signaling. Besides, ROS can trigger mitogen-activated protein kinase (MAPK) signaling, including, C-jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinases (ERK). In addition, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is involved in autophagy formation induced by SAL (Kim et al., 2016).

2.10. Salinomycin Effect on MAPK Signaling Pathway

Mitogen-activated protein kinase (MAPK) family has a critical role in signaling cascades and conveys extracellular signals to intracellular targets. Considering this fact, MAPK signaling pathways manage important processes such as differentiation, stress response and cell proliferation (Guo et al., 1997).

It is believed that MAPK pathway is an initiator of autophagy. There are some ways for activation of MAPK pathway, one of them is the inactivation of MAPK-phosphatases. This is defined by oxidation of the cysteine which is positioned in the catalytic site which is mediated by ROS.

Another pathway is the activation of JNK pathway through apoptotic signal regulating kinase 1 (ASK1). This causes the phosphorylation of JNK and activation of the transcription of JUN. Subsequently, JUN activation can induce autophagy by triggering ATG7 or phosphorylation of BCL2 and results in dissociating BCL2 from Beclin-1. SAL can participate in autophagy activation through ROS formation and subsequently, JNK

activation and promotion of JUN transcription. As JNK suppression partially led to inhibit autophagy, it seemed that SAL induced other signaling pathways. It was observed that SAL can reduce the phosphorylation of AKT and mTOR in prostate cancer cells (Kim et al., 2017).

2.11. Salinomycin Effect on ER Stress

Aggregation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) results in formation of stress condition. ER stress triggers the unfolded protein response (UPR) which induces the inhibition of protein translation via phosphorylation of eukaryotic translation initiation factor 2A (EIF2A). Studies have revealed that autophagosomes can be formed by ER (Rashid et al., 2015).

It has been observed that SAL could increase the expression of proteins related to ER stress including phospho-EIF2A, ATF4, DDIT3 in human NSCLC cells. The endoplasmic reticulum (ER) can form the membrane of autophagosomes. ER through activation of the unfolded protein response (UPR), stimulates the inhibition of protein translation through phosphorylation of EIF2A. The phosphorylation of EIF2A selectively causes to initiate translation of transcription factors such as NUPR1, ATF4, and DDIT3 and the pseudo kinase TRIB3. SAL suppressed AKT1-MTOR through down regulation of phospho-AKT1 (p-AKT1), phospho-RPS6KB1 (p-RPS6KB1) and phospho-EIF4EBP1 (p-EIF4EBP1) and as a result, it induced autophagy (Li et al., 2013).

2.12. Salinomycin Effect on PI3K/AKT/mTOR Signaling Pathway

There are two types of mammalian target of rapamycin (mTOR) complexes, the mTOR complex 1 (mTORC1) and complex 2 (mTORC2). (mTORC1) is rapamycin

sensitive complex containing components including, mTOR, regulatory associated protein of mTOR (Raptor), G protein β-subunit-like protein (GβL) and DEP domain-containing mTOR-interacting protein (DEPTOR).

TSC2/TSC1 inhibition is mediated by AKT-mediated TSC2 phosphorylation resulting in activation of mTORC1. mTORC1 inhibition is happened under nutrient deprivation. It has been clarified that SAL terminates inhibitory phosphorylation of TSC2 by AKT.

Treatment of cells with 1 μM SAL following treatment with Triciribine, an Akt inhibitor, induced autophagy. The elevated amount of LC3-II confirmed autophagy stimulation. Although, treatment with 10 μM SAL did not alter LC3-II levels under the same condition. SAL treatment caused to inactivate mTORC1. Therefore, it seemed that ROS formation was involved in stimulation of autophagy. The activation of DNA-PKcs is involved in the formation of drug resistance. It has been clarified that DNA-PKcs has a critical role in regulating autophagy-associated proteins such as Beclin-1, which the up regulation is correlated with induction of autophagy. Since the inhibition of Beclin-1 caused to increase the cytotoxicity induced by SAL, autophagy induction is a protective mechanism (Zhen et al., 2016).

2.13. Salinomycin Effect on AMPK Signaling Pathway

AMPK activation participates in cellular energy metabolism, glucose and fatty acid uptake and consequently in cell energy homeostasis, cell mitosis, gene transcription and regulation of autophagy by its downstream kinases. AMPK is a positive regulator of autophagy functioning. Moreover, AMPK induces ULK1 complex phosphorylation and

triggers autophagy. Recently, it was observed that SAL induced AMPK activation in U2OS and MG-63 osteoblastoma cells.

Induction of autophagy by SAL is a protective mechanism against apoptosis in these cells. SAL activated both apoptosis and autophagy. It has been suggested that depending on intensity of the stress, AMPK can be anti-apoptotic or can induce apoptosis. It was noticed that SAL can activate AMPK which serves as anti-apoptotic agent in osteoblastoma cells. Also, AMPK suppression caused to increase cytotoxicity and apoptosis induced by SAL. It was understood that the ROS production participates in AMPK stimulation by SAL. Indeed, ROS is needed for both AMPK induced autophagy and SAL-induced apoptosis. ROS production played a role in activation of apoptosis and autophagy by SAL. Autophagy acts as a protective mechanism against the apoptosis induced by SAL. Based on the finding, AMPK-autophagy inhibition may be effective to increase sensitivity of cancer cells to SAL and can improve SAL function (Zhu et al., 2013).

Androgen receptor (AR) and mechanistic target of Rapamycin complex-1 (mTORC1) activities are two contributors in prostate cancer formation. The current therapies of castration sensitive prostate cancer were not successful due to the reoccurrence.

The PI3-kinase/AKT/mTORC1 pathway has a significant role in tumorigenesis especially in primary prostate cancer and mCRPC. The function of PI3-kinase/AKT axis is attributed to the serine-kinase activity of the mTORC1 complex. This causes to activate cell growth by multiple downstream effectors, increase protein synthesis and messenger

RNA translation under nutrient-rich conditions; prevent autophagy and induce lipid biogenesis.

The TSC2/TSC1 tuberous sclerosis complex is a downstream of AKT. It acts as a negative regulator of mTORC1 and prevents the GTPase activity of Rheb (Ras Homolog Enriched in Brain). Rheb functions as a positive regulator of mTORC1. AKT mediated TSC2 phosphorylation at serine-939 and threonine-1462 results in mTORC1 activation and inhibition of TSC2/TSC1. Prevention of mTORC1 leads to cell growth arrest under nutrient deprivation or oxidative stress which is triggered by AMPK. Phosphorylation of raptor at serine-722 and Ser-792, which is performed by AMPK causes to inactivate mTORC1. Due to phosphorylation of AR by HER family kinases and subsequent increase its stability and function, the PI3K pathway blockers were not successful regarding to inhibit mCRPC growth.

In one study, it was shown that SAL can suppress dual-acting AR and mTORC1, PTEN deficient castration-sensitive and castration-resistant prostate cancer in cultured and xenograft tumors. AR expressing LNCaP (castration-sensitive) and C4-2B (castration resistant) human prostate cancer cells were treated with SAL. It was observed that SAL suppressed cell proliferation and induced apoptosis. Since the p16 levels were not changed due to SAL treatment, it was concluded that SAL did not affect cellular senescence and inhibition of cell proliferation may be related to cytostasis. Based on the inhibition of AR and mTORC1 by SAL, it may be effective for treatment of advanced prostate cancer (Mirkheshti et al., 2016).

The tumor microenvironment has an important role in effectiveness of anti-cancer drugs. Hypoxia and accumulation of metabolites of glycolysis in tumor environment through acid by decreasing pH can affect pharmaco-kinetics of drugs including SAL. The effect of SAL on PC-3 cells was estimated under low glucose (0.75 g/L) and low serum (1% FBS) exposure at levels resemble to starvation condition. In these circumstances, it was observed that SAL was more effective at low glucose levels caused by starvation (0.75 g/L) (because glucose is the primary source for cancer cells).

Although SAL did not show such effect toward primary human fibroblasts. Also, glucose starvation was formed through using glucose analogues (2DG, 2FDG) that cannot enter glycolysis pathway. Similarly, this condition intensified the toxicity of SAL. Hypoxia together with starvation increased the SAL efficacy on PC3 cells. Therefore, increased oxidative phosphorylation through apoptosis induction may intensify the SAL effect. The obtained results of western blot indicated that SAL induced up regulation of caspase-3, -8 and-9 activity and apoptosis. Also, it was observed that SAL triggered autophagy through activation of AMPK pathway. The synergic induction of AMPK activity by SAL and starvation participated in enhancing cell death (Jangamreddy et al., 2015).

Since SAL mediates K+/H+ exchange across the inner mitochondrial membrane, it results in disruption of mitochondrial function. K+/H+ exchange causes inner mitochondrial membrane (IMM) hyperpolarization, mitochondrial matrix acidification and reduce respiration. The reduced respiration and elevated ROS produced by mitochondria led to ATP depletion. Moreover, the sensitivity of cells toward the induced mitochondrial respiratory inhibition, ATP depletion as well as cell death is correlated with cell type. Cells

such as CSC-like surviving pancreatic ductal adenocarcinoma cells (PDAC) are more dependent on mitochondrial respiration than on glycolysis. SAL through trigger mitochondrial membrane hyperpolarization exerted the anti-cancer effect on PDAC (Zhang et al., 2019).

On the other hand, in one study it has been shown that SAL can function as an antioxidant protecting cells against the severe oxidative stress. It has been distinguished that Arachidonic acid (AA, a ω-6 polyunsaturated fatty acid) is released from the membrane under oxidative stress. The released arachidonic acid triggers apoptosis. Consequently, AA is involved in over production of ROS and mitochondrial damage. Furthermore, AA together with iron can decrease cell viability and causes cell death. Considering the role of AA in induction of oxidative stress, HepG2 cells were treated with a combination of AA and iron. Thereafter, these were treated with SAL. The results of MTT indicated that SAL may serve as antioxidant and protect the cells against the cytotoxic effects induced by AA plus iron. Therefore, treatment with SAL caused to inhibit ROS production and recovered the mitochondrial damage such as de regulation of mitochondrial membrane potential (MMP) due to AA plus iron. The obtained results of western blot revealed that following SAL treatment, the expression of LC-3B and p62 which are involved in autophagy was increased. Also, SAL treatment caused phosphorylation of proteins involved in AMPK pathway, including, AMPK, ACC and LKB1.Activation of AMPK and autophagy by SAL inhibited apoptosis induced by AA plus iron. Therefore, SAL can serve as a potential agent to protect hepatic cells against oxidative stress (Kim et al., 2018).

Recently, the effect of SAL in combination with 17-allylamino-17-demethoxygeldanamycin (17-AAG) has been investigated on MDA-MB-231 cells. The mechanism of action of 17-AAG is inhibiting the heat shock protein Hsp90. HSP90 is overexpressed in cancer cells leading to stabilizing muted proteins and preventing them from degradation by proteasomes. Since the muted proteins are included in proliferation and survival, HSP90 helps to maintain cancer cells. AAG-17 acts as a competitive inhibitor of Hsp90 causing the degradation of muted proteins by the ubiquitin-proteasome pathway. It was noticed that SAL in combination with AAG-17 synergically induced apoptosis through down regulation of caspase 3 and Bcl-2 and up regulation of Bax and cleaved caspase-3. On the other hand, the combination therapy inhibited autophagy, which was indicated by decreased levels of LC3, Beclin1 and, P62. It seemed that there was a correlation with elevated ROS production and apoptosis as well as autophagy. The combinational therapy caused to increase the ROS production which was responsible for activation of JNK pathway.

The pathway through up regulation of pro-apoptotic proteins like BAX caused the release of cytochrome c and improved apoptosis. The inhibition of autophagy was related to Beclin 1 inducing the release of cytochrome c. therefore, it caused to activate apoptosis and suppress autophagy. Since in more cases induced autophagy is considered as a protective mechanism against induced apoptosis, the prevention of autophagy aimed to stimulate apoptosis further in the tested cells (He et al., 2022).

In one study, the SAL effect on caspase–dependent and independent cell death pathways was investigated in colon cancer cell lines, including, (RKO, SW480 and

SW620) and breast cancer cell lines, including (MCF-7, T47D, and MDA–MB -453). It was observed that SAL induced cell death as well as reduced colony formation and enriched sub-G1 phase cells in RKO, SW480, SW620, MCF-7, MDA-MB-453 and T47D in a dose dependent manner. Also, it was noticed that cell viability was decreased more in colorectal cancer cells compared to breast cancer cells; therefore, the colorectal cancer cells were more sensitive to SAL.

It was indicated that SAL induced autophagy through ROS production as well as activation of the JNK pathway. A difference in response to SAL regarding caspase activation can be due to their stem cell properties. Since SW620 compared to SW480 cells, have more expression of CD133/CD44/CD24, they contain more stem cells.

Although the absence of caspase activity was observed in SW620 cells, the high sensitivity of SW620 to SAL is correlated with this feature. this study, it has been suggested that autophagy is responsible for caspase-independent cell death. The appearance of vacuoles in caspase 3-negative MCF-7 cells following SAL treatment was observed proving this claim. In addition, SAL induced up regulation of Beclin-1 and ATG7 which are autophagy proteins. SAL by increase of NO2 production stimulated autophagy. In turn ROS activated JNK pathway as well as transcription factor JUN (Verdoodt et al., 2012).

The effect of SAL on human hepatocellular carcinoma (HCC) cells including HepG2 and Huh7 was tested. The results showed that SAL inhibited autophagic flux and led to both accumulation of dysfunctional mitochondria and ROS production in HCC cells. It was observed that SAL activated apoptosis in both HepG2 and Huh7 cells. Also, this effect was more in HepG2 compared to Huh cells. Since treatment with SAL induced dose

dependent increase of Annexin-V+ cells, SAL induced apoptosis in a dose dependent manner. The results of western blot showed that SAL caused to up regulate LC3-ll. As a result, SAL treatment induced accumulation of LC3-II which is an indicator of decrease in autophagic flux. It was concluded that SAL treatment inhibited autophagic flux which led to induced apoptosis in HCC cells. Since following SAL treatment caused to accumulate dysfunctional mitochondria as well as increase ROS production, SAL disrupted the mitochondria function.

The withdrawal of dysfunctional mitochondria and decrease in pro-apoptotic signals including release of cytochrome C is considered as a part of the pro-survival mechanism of autophagy. Because the changes in mitochondrial mass and ROS production are the outcomes of suppression of autophagic flux, autophagy inhibition is a cause for apoptosis of HCC cells (Kim et al., 2018).

2.14. Salinomycin Effect on EMT Process

The epithelial-mesenchymal transition (EMT) is a process enabling epithelial cells to acquire migratory and invasive property to become mesenchymal stem cells. In this process, epithelial cells lose both cell polarity and cell-cell adhesion and consequently, these can differentiate into different cell types. EMT is responsible for the initiation of metastasis in cancer progression. Studies have shown that EMT causes invasion which initiates metastasis. Loss of E-cadherin is considered as a fundamental cause of EMT. Initially, cancer cells in a primary tumor lose cell adhesion due to repression of E-cadherin and detach from the basement membrane and enter the bloodstream. After leaving the bloodstream, these cells can form macroscopic metastasis in different sites. Re-expression

of E-cadherin enables cells to retrieve epithelial phenotype, the process is called mesenchymal-epithelial transition (MET). Therefore, both EMT and MET are critical for the initiation and completion of invasion-metastasis cascade (Sciacovelli & Frezza, 2017).

The expression of extracellular markers such as, fibronectin and matrix metalloproteinases and intracellular markers, including, vimentin and E-cadherin is involved in the EMT-MET-related differentiation status. Performed studies have proposed that one of the anti-cancer activities of SAL is related to inhibit migratory and invasive potential of cancer cells.

Anoikis is a type of programmed cell death which is happened by detachment of anchorage-dependent cells from the surrounding extracellular matrix (ECM). Lack of normal cell–matrix interactions due to detachment of cells from the ECM is the main cause of anoikis. Although, cancer cells may escape from anoikis in the invasion process and disseminate to other organs. STAT3 is an important factor in anoikis resistance and the over expression of STAT3 is observed in cancer cells. It was observed that SAL through induction of caspase-3 and caspase-8 activation and PARP cleavage, activated anoikis-sensitivity. Moreover, SAL down regulated MMP-9 and MMP-2 mRNA levels and therefore inhibited cell migration and invasion. In this study, SAL by prevention of the mamospheres formation, reduced the CD44+/CD24- stem-like population in MDA-MB-231 cells. It was noticeable that SAL suppressed STAT3 phosphorylation and interleukin-6 (IL-6) expression which is responsible for STAT3 activation. Therefore, SAL through down regulation of STAT3 caused to induce anoikis (An et al., 2015).

The inherent tumor resistance to gemcitabine is a major problem regarding pancreas cancer treatment. It is believed that the resistance is correlated with different mechanisms including expression of EMT phenotype in cancer cells.

In one study, SAL was delivered to Human pancreatic cancer (AsPC-1) through polyethylene glycol-b-poly lactic acid (PEG-b-PLA) polymeric micelles (PMs). Aiding of polyethylene glycol-b-poly lactic acid (PEG-b-PLA) polymeric micelles (PMs) solved the problem due to the insolubility of SAL. The MTT assay confirmed that SAL reduced cell viability. Moreover, it was observed that SAL suppressed EMT in these cells. SAL caused the decrease expression of CDH1 and up regulation of Snail. Since Snail is a repressor of E-cadherin, the up regulation could justify the inhibitory role of SAL in EMT. In addition, SAL decreased the expression of ZEB1, a negative regulator of CDH1 and activator of EMT, as well as Vimentin, a marker of EMT. Hence, SAL prevented metastasis in AsPC-1 through EMT suppression (Daman et al., 2015).

Treatment of bladder cancer cell line, T24, with SAL resulted in decreasing the metastasis and invasion. The effect was achieved by up regulation of E-cadherin and down regulation of Vimentin (Qu et al., 2015).

The exerted inhibitory mechanism by SAL was correlated with two factors: SAL prevented transforming growth factor-β1 (TGF-β1)-induced EMT phenotypic transition and up regulation of some signaling molecules which are participated in Smad (pSmad2/3 and Snail1) and non-Smad (β-catenin and p-p38 MAPK) signals in MCF-7 cells. These two signals regulate the activation of EMT collectively (Zhang et al., 2016).

Considering the metformin effect on EMT prevention and cancer cell growth, it can decrease the risk of cancer in patients with diabetic type 2. It was observed that SAL in combination with metformin, inhibited TGFβ-induced EMT and EMT-induced cell migration in the two lung cancer cell lines, including, non-small cell A549 and HCC4006 (Koeck et al., 2016).

SAL in combination with conventional chemotherapeutic drugs is able to suppress invasion and migration of cancer cells. The highly metastatic ability of primary mantle cell lymphoma (MCL) is correlated with up regulation of ZEB1 and activation of Wnt signaling. SAL inhibited metastasis by suppression of Wnt signaling pathway and down regulation of ZEB1. As a result, the efficacy of gemcitabine, doxorubin and cytarabine was increased in MCL cells (Sanchez-Tillo et al., 2014).

It was observed that SAL could prevent invasion of metastatic renal cell carcinoma (RCC). The effect was exerted through increased expression of E-cadherin and down regulation of N-cadherin, Snail and MMP-2 in these cells. In addition, SAL induced DNA breaking and up regulation of pro-apoptotic biomarkers such as cleaved caspase3/9 and cleaved PARP1 and down regulated anti-apoptotic biomarkers like survivin. As a result, SAL is able to induce apoptosis in RCC cells. SAL treatment causes to inhibit the sphere formation ability and expression of stemness biomarkers such as CD105, ALDH1 and CD44. Due to multiple anti-tumor activities against RCC, SAL can be a potential agent for RCC treatment (Liu et al., 2016).

It has been shown that SAL can prevent tumor growth through stimulation of macrophages. SAL through M1 macrophage polarization can prevent tumor growth as well

as pulmonary metastasis of 4T1 (murine breast cancer) cells. Macrophages as a part of immune system are activated by depolarization. Under different stimuli, they can polarize into (M1-type) or alternative activated phenotype (M2-type). M1-type macrophages can prevent the cell growth of surrounding cells and cause to destroy the contiguous tissue, however, M2-type results in enhancing cell proliferation of contiguous cells. There are populations of M1 and M2 type macrophages in growing tumors contributing to progression and metastasis of tumors. It has been proposed that transformation into a higher ratio of M1/M2 may be involved in tumor regression. Therefore, increase of M1/M2 populations can be served as a promising agent for inhibition of tumor growth and this study demonstrated that SAL is able to polarize macrophages.

It was clarified that effective dose of SAL is required for inducing M1-type of RAW264.7 (murine macrophage) cells was (30 nM) and the dose was not related to stimulate proliferation, mamosphere formation and migration of tumor cells. The results of western blot indicated that treatment of RAW264.7 cells with SAL caused to increase the amount of pSTAT1, whereas pSTAT3 expression was reduced. STAT1/3 participated in macrophage polarization. STAT1 contains antitumor activity, however, STAT3 enhances tumorigenesis. IL-4 has a prominent role in enhancing cytokines and as a result causes M2 activation. Furthermore, it was observed that treatment with SAL prompted to impair IL-4 induced M2 differentiation. As a result, SAL caused to promote the M1 type and decrease M2 type. Obtained results of in vivo study revealed that SAL (50 μg/kg) resulted in induction of M1 macrophage polarization in tumor as well as elevating the ratio of M1/M2 in female BALB/c mice (Shen et al., 2021).

SAL can inhibit invasion and metastasis in human non-small cell lung cancer cells (NSCLC), including LNM35 and A549. Also, it could induce the expression of NAG-1. NAG-1 is regarded as a tumor suppressor by inducing apoptosis. Moreover, it can inhibit the production of cytokine by macrophages. Arafat, et al, showed that treatment of LNM35 and A549 cells with different doses of SAL (0.1-50 mM) caused to inhibit cell migration as well as invasion in both of cells.

In this study, it has been noticed that SAL treatment stimulated a dose dependent expression of NAG-1. NAG-1 was involved in suppression of invasion of treated cells. Also, NAG-1 induced apoptosis through p53 up regulation. P53 caused to arrest cell cycle (Arafat et al., 2013).

It has been shown that SAL inhibited invasion through prevention of AMPK/SIRT pathway. SIRT1 enhances EMT, cancer cell survival through inactivation of p53 activation of MYC. In NSCLC such as A549 and H460 cells, TGF-β1 is involved in EMT stimulation. It was observed that SAL induced AMPK which was responsible for preventing invasion through down regulation of MMP or decreasing the EMT-related markers. In this study, treatment of cells with SAL resulted in increasing of E-cadhein expression, whereas SAL treatment induced decreasing of N-cadherin as well as vimentin expression. Therefore, SAL reversed the consequences of TGF-β1-induced EMT. SIRT1 as a histone deacetylase contributes to inflammation, metabolism, cell proliferation, apoptosis, and senescence. SIRT1 enhances EMT, cancer cell survival through inactivation of p53 activation of MYC. Results of western blot indicated that SAL inhibited the TGF-β1 induced SIRT1 expression, reduced AMPK phosphorylated, MMP2 as well as MMP-9.

AMPK is activated by phosphorylation and SAL by decreasing the levels of AMPK phosphorylated caused to its inactivation. Also, it was noticed that inhibition or AMPK caused MMP-2 and MMP-9 expression induced by TGF-β1. Based on the results, it seemed that AMPK was contributed to suppression of MMP-2 and MMP-9 induced by TGF-β1 (Hwang et al., 2021).

It was observed that SAL could inhibit invasion of prostate cancer cells, including CWR22rv1 and LNCaP cells through targeting CD44. CD44 is correlated with poor prognosis of carcinoma through regulating mesenchymal phenotype induced by TGFβ. TGF-β1 contributes to maintaining the stemness as well as mesenchymal phenotype of cancer cells. It was shown that CD44+ stem-like cells are initiators of invasion induced by TGFβ1-CD44 signaling. Also, dedifferentiation of prostate cancer cells has a major role in the development of EMT. In this study, treatment with SAL caused a decrease in CD44 and invasion. Thereafter, the expression of E-Cadherin was increased, whereas vimentin expression was decreased. Moreover, SAL treatment resulted in decreasing cancer stem cell markers including, c-Met, Sox2, Oct-4. Accordingly, SAL led to decrease invasion of CWR22rv1 and LNCaP cells (Shang et al., 2015).

SAL could inhibit EMT by preventing ARK5 expression in cholangiocarcinoma cells. In this study, cholangiocarcinoma cell lines, including RBE and Huh-28 cells, were treated with combination of doxorubicin with SAL. Since cholangiocarcinoma cells develop resistance to the current therapies including doxorubicin, the cells were treated with combination of doxorubicin with SAL. The formation of resistance to doxorubicin triggered EMT. Following treatment, it was observed that E-cadherin was up regulated and

CD133 expression was decreased. Indeed, SAL reversed the EMT due to doxorubicin resistance. ARK5 by regulating of EMT participates in stimulation of invasion as well as migration. Treatment of cells with doxorubicin caused enhance ARK5 expression. However, combinational therapy resulted in down regulating of ARK5. The decreased level of ARK5 was correlated with regulating EMT marker expressions such as E-cadherin and vimentin (Yu et al., 2017).

SAL in combination with gefitinib (GEF) could induce cell death in colon cancer cells. The combination therapy can cause loss of mitochondrial membrane potential (MMP) and lysosomal membrane potential (LMP). The problem regarding the use GEF is developing resistance to this medicine. It has been revealed that the resistance can be formed following altered expression and activity of downstream components of EGFR including, Ras and Raf. Also, the mutations which trigger the activation of activation of genes such as, KR, AS, NRAS, and BRAF genes, as well as amplification of MET and HER2 genes are considered as causes of formed resistance to GEF therapy.

In this study, colon cancer cells including SW1116, LOVO, HCT-116, SW480 and HT29 cells were treated with SAL together with GEF. It was observed that the treatment resulted in increasing ROS production in a time-dependent manner. ROS caused to disintegrate lysosomal membrane. Also, SAL in combination with GEF caused induced apoptosis through mitochondrial dysfunction. Following mitochondria damage, caspase-9 and -3 were activated. The results of western blot revealed that combinational therapy elevated the caspase-3 and -9, cathepsin B and D expression which confirmed the loss of LMP and MMP following the treatment. Apoptosis was initiated following the

translocation of cathepsin B and D and loss of MMP. Consequently, cytochrome c translocated into cytoplasm and activated caspase-3 dependent apoptosis. Also, SAL with GEF could increase the mitochondrial permeability which is involved in the loss of LMP. Furthermore, treatment of SW1116 and HCT-116 cells with Necrostatin 1 (Nec1) and Necrostatin 5 (Nec5) which are inhibitors of necrotic cell death, showed that they could not halt the cytotoxicity induced by combination of SAL with GEF and therefore, the induced cell death by combinational therapy was not correlated with induction of necrosis. Taken together, SAL could overcome developed resistance to GEF which unlike other anti-colon cancer agents were not related to inhibit EGFR pathway (Zou et al., 2017).

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive, highly metastatic and resistance to chemotherapy. It has been demonstrated that SAL was able to treat PDAC through inhibition of metastasis and induce apoptosis. The structure of actin cytoskeleton determines cell migration. Actin stress fibers are composed of actin filaments which are held together by proteins including, α-actinins and fascin. Following treatment with SAL, a loss of actin stress fibers was observed. The dis integrity was related to dislocation of fascin induced by SAL. Therefore, SAL was able to inhibit invasion by disintegration of actin stress fibers. of However, down regulation of facsin was not observed indicating that SAL altered the function of fascin but did not affect its expression.

Therefore, the disassembly of actin stress fibers as well as dislocation of fascin induced by SAL were involved in inhibition of migration. The disruption of actin stress fibers is RhoA/ROCK dependent. RhoA is a GTPase protein and disrupting the signaling cascade may lower migration of cancer cells. Contractility of stress fibers are mediated by

ROCK which phosphorylates the myosin light chain (MLC). The phosphorylated MLC modulates the contractility of stress fibers. Rac serves as a regulator of the fascin interaction with kinase C (PKC). It seemed that SAL by activating Rac caused to increase ROS production and decrease Rho activity. The decreased activity of Rho caused to disassemble of actin stress and inhibit migration (Schenk et al., 2015).

In one study, colon cancer cell lines, including HT29 and SW480 were treated with combination of SAL and MST-312 which is a telomerase inhibitor hindering growth of metastatic tumor cells. Following treatment, the cellular stress and apoptotic proteins were examined. It was noticed that Akt is activated to overcome the stress induced by SAL. Also, SAL decreased phosphorylation of Hsp27 providing thermo-tolerance. Therefore, SAL weakens thermo-tolerance. Since the treatment caused DNA damage, following treatment the levels of both Chk1 and p53 were increased. Telomerase is up regulated in colorectal tumors.

Since telomerase is responsible for the maintenance of telomeres, its activation is observed in many cancer cells. The activation of telomerase is correlated with metastasis. Moreover, it has been shown that human telomerase reverse transcriptase (hTERT) through stimulation of EMT can prompt stemness and cancer metastasis. In this study, SAL decreased the expression of telomerase and hTERT through inhibition of STAT3 and STAT1. Therefore, SAL inhibits EMT process in colorectal cancer which is mediated by STAT3 (Chaung et al., 2017).

In one study, liver cancer cells (MHCC97H) were treated with SAL. It was observed that treatment with SAL could inhibit the migration of cells through lowering

phosphorylation of FAK. Focal adhesion kinase (FAK) plays a critical role in cell migration and invasion. Therefore, FAK-deficient cells contain decreased ability for cell motility and as a result, have increased focal adhesion. Cancer cells having de regulated FAK demonstrate both increased migration and invasion. In this study, it was observed that SAL reduced LCSC migration by decreasing phosphorylation of FAK and expression of ERK1/2, MMP-2 as well as MMP-9. In addition, it was observed that SAL increased stiffness through FAK-ERK1/2 pathway. SAL induced F-actin formation and consequently enhanced stiffness. Cytoskeleton, which is composed of actin filaments, microtubules, intermediate filaments as well as nuclear skeleton proteins form cell stiffness. In line with other studies in which it was shown that the increase of actin formation caused to elevate stiffness, it was demonstrated that SAL by increasing actin resulted in enhancing stiffness and inhibition of invasion (Sun et al., 2017).

In one study, treatment with SAL enhanced the cytotoxicity induced by Doxorubicin in hepatocellular carcinoma cells (HHC). Doxorubicin, an anthracycline based drug, is extensively used for the treatment of cancer. Although, long-term treatment with this agent caused to develop resistance. In this study, HHC cells, including HuH-7, HepG2, SNU-449 as well as SNU-389 were treated with combination of SAL and Doxorubicin. After 48h of treatment, it was observed that the combinational therapy reduced cell viability. One of the factors which is involved in development of resistance is EMT and expression of EMT markers. Doxorubicin significantly decreased the expression of E-cadherin as well as enhanced the expression of Vimentin. The results of western blot indicated that SAL caused to reverse the expression of EMT markers induced by

doxorubicin. In addition, the ability of cell invasion and migration was diminished following treatment with SAL. Moreover, SAL activated FOXO3a. FOXO3a is a protein which its inhibition is correlated with drug resistance as well as EMT stimulation.

SAL failed to reverse the EMT in HCC cells following knockdown of FOXO3a. The phosphorylation of FOXO3a is regulated by PI3K/ AKT pathway and the pathway is considered as the most critical regulator of FOXO3a post-translational modifications and manages FOXO3a subcellular localization. Doxorubicin increased AKT expression and inactivated FOXO3a by its retention in the cytoplasm. Moreover, Doxorubicin caused to activate Wnt/β-catenin signaling and EMT. The β-catenin/TCF4 complex through activation of ZEB1 transcription was involved in metastasis. Moreover, β-catenin by switching from TCF4 to FoxO3a contributed to proliferation and apoptosis of HCC cells. In this study, SAL activated FOXO3a as well as reduced the association of the β-catenin/TCF4 and suppressed TCF4 target genes, including, CyclinD1, ZEB1 and c-Myc. The activation of FOXO3a suppressed Wnt signaling pathway (Zhou et al., 2015).

In one study, the effect of SAL in combination with LBH569 as a histone deacetylase inhibitor (HDAC) was tested on HCC1937 cells (triple negative cells with characteristics of ALDH-positive cells and ability to self-renewal). It was noticed that the combination therapy demonstrated synergic anti-cancer effect. Following treatment, DNA fragmentation and increased levels of cleaved PARP indicating apoptosis stimulation. Also, p21 and Ki67 levels were decreased implying cell cycle arrest. The anti-apoptotic and cell cycle arrest was more potent following the combination therapy compared to either drug alone. It was observed that the combination therapy was able to increase the

expression of E-cadherin and decrease the levels of Vimentin, N-Cadherin as well as SLUG. Since the EMT has a role in stemness, the therapy through prevention of EMT leading to decrease the Stemness (Kai et al., 2015).

2.15. Salinomycin Effect on Homeostasis of Iron

Iron has a dual role in cells. Iron has a pivotal role in cell proliferation, DNA stability as well as cell cycle regulation. Iron functions as a cofactor for important enzymes involved in DNA metabolism, such as DNA repair enzymes (helicases, nucleases, glycosylases, demethylases) and ribonucleotide reductase. Accordingly, consequences of iron deficiency are related to DNA damage, cell cycle arrest and programmed cell death. On the other hand, iron is involved in tumorogenesis. It participates in regulation of tumor microenvironment as well as metastasis through Wnt pathway. Considering the role of iron in cancer cells, it can be considered as a potential therapy. In one study, the cytotoxicity of SAL was attributed to inducing ferroptosis in cancer cells. SAL through chemoselective oxidation followed by a stereoselective reductive amination at C20 was modified. HMLERCD24low/CD44 high CSCw were targeted by the modified form of SAL, AMP5 (ironomycin). Since AMP5 contained the positive charge, it showed selectivity for iron. It was observed that AMP5 was accumulated in lysosome and interacted with iron (II).

Due to AMP5 competence with the translocation of the iron into cytosol, the cellular iron was sequestered in lysosomes. The accumulation of iron in lysosomes induced degradation of ferritin in lysosomes. The extra amount of iron resulted in des regulation of cysteine and carboxyl proteases such as, cathepsins and activation of autophagic flux. Accumulated soluble redox-active iron caused to produce ROS and peroxidation of lipid

belongs to lysosomal membrane. Following inhibition of cathepsin B in HMLER CD24low, the induced ROS production by AMP5 was decreased indicating the correlation of the lysosomal degradation of ferritin with ROS production (Mai et al., 2017).

2.16. Overcoming Drug Resistance

Based on the ionophore activity, SAL acts as an efflux pump p-glycoprotein inhibitor leading to overcome the drug resistance in cancer cell lines. One of the exerted mechanisms by SAL is correlated with increasing DNA damage and lowering the levels of CDKN1A/p21 protein. As a result, the cancer cells become sensitive to both radiation and cytostatic drugs like etoposide or doxorubicin and undergo apoptosis (Kim et al., 2011).

ABC transporters are transmembrane macromolecules. They translocate substrates across cell membranes using ATP. Due to pump drugs out of the cells, ABC transporters are involved in the development of cancer cells resistance to the current therapies. In this process, ABC transporters are overexpressed and lead to developing multidrug resistance (MDR). By drug efflux, higher concentrations of the drugs are needed in order to destroy the cancer cells. SAL due to contain hydrophobic structure can be embedded in the cytoplasmic and mitochondrial membrane and act as a transmembrane K+ ionophore. Based on this fact, SAL cannot be a substrate of ABC transporters. SAL can prevent ABC transporter-mediated multi-drug resistance in KG-1a cells. These cells, due to expression of ABC transporters develop multi drug resistance. SAL treatment induces apoptosis in these cells by interference with ABC transporters (Fuchs et al., 2010).

Tamoxifen is widely used for the treatment of hormone-receptor-positive breast cancer. It has a dual role in endocrine therapy, as it acts as an agonist in bone tissue and is

efficacious in prevention of osteoporosis. In addition, it can function as an antagonist for prevention of tumor growth in estrogen dependent breast cancer. However, since there are three mechanisms that cause resistance formation to this medicine, the treatment of hormone-receptor-positive breast cancer is problematic. These mechanisms include overexpression of multidrug resistance protein 1 (MDR-1), the existence of CSCs as well as the expression of estrogen receptor α (ERα) by several Receptor Tyrosine Kinases (RTKs). Based on this fact, there is need to auxiliary therapy in order to enhance the efficacy of tamoxifen.

Sommer, et al. demonstrated that SAL can be effective to overcome the tamoxifen resistance through inhibition of activation of ERα. It was observed that due to the combinational therapy, the amount of PARP-1 was increased. The combinational therapy led to reduced ERα expression. In hormone receptor positive breast cancer, ERα is activated by Egfr-family members which confers the resistance mechanism to the current therapy. Resistance to tamoxifen caused to increase RTKs expression and Her2 and Her3 expression, however, the combinational therapy decreased RTKs expression, Erk phosphorylation and the expression of Egfr-family members. Increased Egfr expression causes to develop resistance to tamoxifen. SAL treatment caused to increase Ca^{2+} levels, endocytosis and consequently, stimulated autophagy (Sommer et al., 2016).

Manmuan et al. showed that SAL overcame the tamoxifen resistance in MCF-7 cells by reduction of ER co-activator; amplified breast 1 (AIB1) mRNA and protein. SAL enhanced the tamoxifen cytotoxicity by elevating caspase 3/7 activation. AIB1 is a nuclear receptor co-activator which its increased expression is associated with malignancy of breast

tumors, poor prognosis and development of tamoxifen resistance. It has been noticed that SAL decreased the expression of AIB1 mRNA and protein (Manmuan, Sakunrangsit & Ketchart, 2017).

The overexpression of cyclin D1 (CCND1) gene is correlated with malignancy and tamoxifen resistance. It was observed that SAL down regulated cyclin D1. Also, high expression of c-myc has been observed in biopsies of metastatic lesions of breast cancer. It was noticed that SAL lowered the expression c-myc. Both doxorubicin (DOX) and etoposide (ETO) are applied greatly in cancer treatment. The exerted mechanism by DOX and ETO against cancer cells is inducing apoptosis through DNA breakage by inhibition of topoisomerases. In order to overcome the formed resistance to these therapies, Hs578T and MDA-MB231 cells were treated with combination of SAL with DOX or ETO. It was noticed that SAL caused cell-cycle arrest and increased DNA damage. SAL exerted these effects by increasing phosphorylation of tumor-suppressor protein p53, DNA damaging protein pH2AX and DNA foci formation as well as lowering cyclin D1 and anti-apoptotic protein 21 level. In Hs578T and MDA-MB231 cells, SAL induced cell death by down regulation of cyclin D1. Therefore, SAL can be utilized to overcome the resistance to either DOX or ETO (Kim et al., 2011).

2.17. Salinomycin Effect on Angiogenesis

Angiogenesis is a determining factor in tumor progression and tumor metastasis. It is believed that angiogenesis plays different biological functions through binding of vascular endothelial growth factor (VEGF), a pro-angiogenic cytokine, to the receptor tyrosine kinases (RTKs) like VEGFR1 (Flt-1), VEGFR2 (KDR/ Flk-1), and VEGFR3.

VEGFR2 has a critical role in transducing angiogenic signals. VEGFR2 acts as an inducer of STAT3. As a result, it causes to proliferate and survival of tumor cells. Following activation, STAT3 translocates into the nucleus and results in expression of genes participating in survival (e.g., BCL-2, BCL-xl), cell proliferation (e.g., cyclinD1), angiogenesis (VEGF) and cell proliferation (e.g., cyclinD1). The abnormal expression in the VEGFR2 signaling pathway leads to proliferation, angiogenesis, metastasis, cell differentiation, inflammation, and apoptosis. Currently, several compounds have been used for targeting VEFFR2. However, the application of these due to produce side effects and even augment metastasis is problematic. Therefore, there is need to develop other VEGFR2 inhibitors in order to diminish the adverse effects.

Recently, SAL has been evaluated as an inhibitory agent of angiogenesis. Li, et al. showed that SAL could suppress angiogenesis and the consequences such as cell proliferation, migration as well as capillary structure formation in gastric cancer cells. In this study, SAL acted through competitive inhibition of VEGFR2. SAL can attach to the binding site of VEGFR2. The binding of SAL at the binding site can suppress the binding of ATP to VEGFR2 and results in inhibiting the activity. SAL decreased the proliferation of endothelial cells which is induced by VEGF. By inhibition of binding of VEGF to VEGFR2 which is mediated through SAL, other pathway such as migration was inhibited. SAL prevented the phosphorylation of VEGFR2 and downstream STAT3 and prevented the translocation of STAT3 and consequently, STAT3 DNA binding activity. By inhibition of STAT3 activity, the activity of downstream molecules like Bcl-2 and Bcl-xL was decreased and the expression of pro-caspase-3 was enhanced. As a result, the survival and

growth of tested cells was inhibited. Based on the finding, it was concluded that SAL is able to suppress vascularization. Moreover, it was revealed that SAL could diminish the paracrine secretion of VEGF from tumor cells indirectly (Li et al., 2016).

In one study, the SAL effect on angiogenesis was studied in Human Umbilical Vein Endothelial Cells (HUVECs) and breast cancer cells including MCF-7 and MDA-MB231 cells.

The obtained results demonstrated that SAL prevented invasion, cell proliferation and migration. SAL suppressed endothelial tubulogenesis by expression of CD31 in HUVEC. Moreover, the binding of HIF-1α transcription factor DNA to HRE sequence was reduced in HUVEC, MDA-MB-231 and MCF-7 cells following treatment with SAL. The in vivo test showed that SAL caused to inhibit neovascularization in the chick chorioallantoic membrane and the Matrigel plug implanted mice model. These results confirmed that SAL can be a potential agent for preventing angiogenesis for breast cancer treatment (Dewangan et al., 2019).

Yan-Ling Bi, et al. showed that SAL can inhibit angiogenesis through blocking the VEGFVEGFR2-AKT signaling pathway in the U251 human glioma cell line and human umbilical vein endothelial cells (HUVECs). SAL through down regulation of VEGF in HUVECs, reduced the phosphorylation of VERGR2 and AKT. Also, SAL inhibited the phosphorylation of FAK which is a kinase and participates in PI3K activation. As a result, suppression of FAK resulted in inhibition of metastasis through down regulation of matrix metalloproteinases and VEGF (Bi et al., 2017).

Kras et al. have shown that SAL was able to alter VEGF signaling pathway. It inhibited angiogenesis through down regulation of expression of VEGF-A and VEGFR-2. Moreover, SAL treatment resulted in down regulation of VEGF-B indicating the cell death. Since the induced ROS production by SAL caused to increase stress oxidative, the decreased level of VEGF-B could not protect the cells against the intensified ROS and led to cell death. The decreased expression of VEGFR-1 induced by SAL was very critical as VEGFR-1 participated in metastasis. Also, SAL down regulated VEGF-C, VEGF-D, VEGFR-2, and VEGFR-3. In the process of lymphangiogenesis the vascular endothelial growth factor family (VEGF-A, -B, -C, -D) and their receptors with tyrosine kinase activity (VEGFR-1,-2,-3) participated. Therefore, SAL inhibited this process (Kras et al., 2020).

2.18. Salinomycin Effect on Hedgehog Pathway

The Hedgehog (Hh) pathway has a critical role in embryonic development, stem cell renewal as well as tissue regeneration. The Hh protein family which is consisted of Sonic (SHH), Indian (IHH) and Desert (DHH) Hedgehog, stimulates a signaling cascade through binding to the twelve-transmembrane (TM) receptors Patched1 and/or Patched2 (PTCH1 and PTCH2) leading to inhibit the seven-TM protein Smoothened (SMO) expression. Inhibition of SMO expression causes to activate the gliomaassociated oncogene (GLI) transcription factors (GLI1, GLI2 and GLI3) and subsequently triggers the expression of downstream target genes. Following activation of GLI1 by GLI2 and GLI3, it functions as a transcriptional activator.

The crosstalk between Hh and other oncogenic pathways can induce both GLI1 and GLI2. The activation of GLI1 proteins can be happened through different ways: the

crosstalk between Hh and other oncogenic pathways can induce both GLI1 and GLI2; following posttranslational modifications; non-canonical Hh pathway which causes upregulation of the GLI proteins through Hh ligand-independent mechanism. It has been demonstrated that the canonical Hh pathway is involved in tumorigenesis and progression of different cancers (Gupta, Takebe & Lorusso, 2010).

It has been shown that both ligand-dependent activation of Hh signaling as well as SHH or IHH up regulation is correlated with different cancers. Abnormal upregulation of SHH has been observed in breast cancer. Non-canonical Hh signaling pathway through effect on angiogenesis, migration as well as activation of small Rho GTPases participates in the tumorogenesis. It has been clarified that canonical and non-canonical Hh signaling pathway is involved in breast cancer pathology, progression and particularly in tumorigenesis of triple negative breast cancer. Moreover, SHH overexpression is correlated with enhanced lymphatic invasion, metastasis and is related to angiogenesis. In many cancers including, breast cancer, NF-κB is a positive regulator of SSH. GLI1 and GLI2 target genes contribute to proliferation, migration, invasion, angiogenesis as well as osteolytic metastasis of breast cancer. Moreover, overexpression of GL1 is observed in triple negative breast cancer compared to the other types of breast cancer and to the normal breast cells. Over expression of SSH through up regulation of pro-angiogenic transcription participates in formation of vascularized tumors. In many cancers including, breast cancer, NF-κB is a positive regulator of SSH. GLI1 and GLI2 target genes contribute to proliferation, migration, invasion, angiogenesis as well as osteolytic metastasis of breast cancer. Noticeably, the higher expression of GLI1 is observed in triple negative breast

cancer compared to the other types of breast cancer and to the normal breast cells. One of stimulating factors for up regulation of GL1 and GL2 is estrogen. It seems that estrogen stimulates Hh pathway through up regulation of SHH and GLI1. It has been found that TNBC has a high proportion of basal-like progenitors and expresses GLI1. Studies showed that in the CSCs cells, PTCH1, GLI1, and GLI2 are highly expressed and in differentiated state of cells, they undergo down regulation. Moreover, it has been revealed that Hh signaling participates in EMT in breast and other cancers. Activation of GLI1 through both canonical and non-canonical pathway increases EMT (Riobo-Del Galdo et al., 2019).

He et al. studied the SAL effect on hedgehog in MCF-7 mammosphere cells. MCF-7 MS cells contained BCSC properties including enhanced ability to colony-forming, tumorigenicity and invasion and they are more resistant to paclitaxel than SAL. Since following SAL treatment, the expression of PTCH, SMO, Gli1 as well as Gli2 was decreased in MCF-7 MS cells but not in MCF-7 cells, it was concluded that Hh signaling is more activated in these cells and confer the resistance to the therapies. In addition, it was observed that SAL, but not paclitaxel, was able to suppress cell proliferation, induce apoptosis and reduce the ability to migration. These effects are correlated with down regulation of expression of c-Myc, Bcl-2 and Snail genes which are down stream of Hh pathway. Moreover, SAL decreased the volume of xenograft tumor more than paclitaxel and paclitaxel did not change the expression of PTCH, SMO, Gli1 and Gli2. Considering that treatment of MCF-7 MS cells with SAL, the expression of Wnt1, pLRP6, β-catenin as well as Axin2 was decreased and p-β-catenin expression was enhanced in MCF-7 MS cells, SAL caused to inhibit Wnt pathway. However, paclitaxel treatment did not alter the

expression of these genes. Since, the SAL inhibitory effect on the Wnt pathway was weaker than that on the Hh pathway, the resistance of MCF-7 MS cells to drugs was attributed to Hh signaling rather than Wnt pathway (He et al., 2015).

2.19. Salinomycin Effect on NF-κB Pathway

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex functions as regulator of DNA transcription, release of cytokine and cell survival. Inflammation has been identified as an indicator of cancer and NF-kB controls chronic inflammation which is correlated with cancer. It has been found that de regulation of NF-κB is associated with different types of cancer. Overexpression of NF-κB in cancer cells induces the expression of genes related to cell proliferation and anti-apoptotic genes. The activation of NF-κB stimulates the expression of pro-proliferative and anti-apoptotic genes. Moreover, NF-κB modulates some signaling pathways, such as those involving interferon regulatory factors, NRF2, STAT3, AP1, Notch, p53 and WNT-β-catenin. Since NF-κB and inflammation affect cellular metabolic changes, the acquisition of stemness characteristics, invasion, angiogenesis, metastasis, development of therapy resistance, NF-κB has been considered as one of therapeutic target (Xia et al., 2014).

One of the problems related to treatment of ovarian cancer is chemo resistance of the gynecologic cancer cells. Cisplatin is widely used for different solid tumors, although its application has some disadvantages due to severe side effects and development of drug resistance. It has been proposed that some molecular targets including, BCL2, cyclin D1 and NF-κB are involved in acquiring cisplatin resistance. NF-κB is involved in resistance formation to therapy by expression of anti-apoptotic factors like BCL-2 family members

(BCL-xL, BCL-2) and the IAP family (XIAP and cFLIP). Moreover, the induced over expression of survivin by NF-κB is the other cause of resistance development.

Tyiagi and Patro showed that treatment of MCF-7 resistant to cisplatin with SAL down regulated survivin, BCL-2 and XIAP proteins. In addition, this effect was intensified in the presence of a NF-κB specific inhibitor. Therefore, it seemed that SAL by inhibition of NF-κB pathway can overcome the cisplatin resistant breast cancer (Tyagia & Patro, 2019).

The effect of SAL on sensitization of the cisplatin-resistant ovarian cancer cells was investigated. In this study, SAL caused the down regulation of Akt, NF-κB proteins in A2780 and A2780cis cells, therefore, SAL can be efficacious to inhibit the Akt/NF-kB signaling pathway. Since siRNA Akt impaired the NF-κB function, it was proposed that Akt participates in apoptosis induction by SAL. The cisplatin resistance of these cells can be inhibited by SAL through inactivation of Akt/ NF-κB signaling pathway. AKt by induction of NF-κB involves chemo resistance. Since SAL down regulated Bcl-2 and enhanced the caspase-3 and PARP protein cleavage, it was concluded that it was able to suppress cell growth, partially inhibited survival factors as well as suppressed NF-κB through Akt/ NF-κB pathway. Consequently, it could stimulate apoptosis. In addition, it was noticed that SAL could increase cell numbers in the sub G1 phase of the cell cycle. However, the number of cells in G2/M phase. This implied that SAL induced an apoptotic pathway without cell cycle arrest. The cytoplasmic histone associated DNA fragments related to apoptotic cells were detected. Therefore, it seemed that SAL induces early-stage

apoptosis. Considering these effects, SAL can be an efficacious agent for treatment of cisplatin resistant ovarian cancer (Parajuli et al., 2013).

In one study, the effect of SAL on malignant (VCaP, LNCaP, PC-3, DU 145) and nonmalignant (RWPE-1, EP156T and PrEC) prostate cells were studied. It was observed that SAL inhibited cancer cell growth, especially VcaP cells. However, it did not affect non-malignant prostate epithelial cells. It was noticed that SAL treatment did not elevate the function of caspase 3 and 7. Therefore, SAL decreased the cell growth without induction of apoptosis. It was observed that treatment with SAL caused a decrease in the CSCs expressing ALDH and CD44. The CSC population containing ALDH and CD24 demonstrate the increased ROS production. It was noticed that SAL down regulated the prostate cancer oncogenes, including MYC, AR and ERG, which function as antioxidants. Moreover, treatment with SAL decreased NF-κB activity. Since NF-κB pathway mediates viability, redox control, tumourigenesis and metastasis of prostate cancer cells, its reduced activity was correlated with decrease in antioxidant defense and migration of tested cells (Ketola et al., 2012).

It has been demonstrated that co-treatment with SAL and SFN (isothiocyanate) was able to induce apoptosis as well as inhibit growth of colon cancer cells, including Caco2 and CX-1 cells. Since following combinational therapy, the expression of PI3K, Akt and p-Akt was reduced, therefore combinational therapy inhibited PI3K/Akt pathway. Also, it was noticed that the treatment through increase p53 expression and decrease Bcl-2 expression was able to suppress downstream of PI3K/Akt signaling pathway.

Following Bax levels were increased. The elevation of Bax induced the translocation of Bax from the cytosol to mitochondria and efflux of cytochrome c from mitochondria. This triggered the caspase activity and cleaved PARP. Accordingly, the amount of cleaved PARP was increased following treatment. Considering the observations, through inactivation of the PI3K/Akt pathway, SAL together with SFN can stimulate apoptosis (Liu et al., 2020).

2.20. Salinomycin Effect on Notch Pathway

The Notch pathway is a highly conserved signaling pathway in most animals. The pathway through cell-cell contacts triggers a response to different stimuli. Mammals contain four types of Notch receptors, including, NOTCH1, NOTCH2, NOTCH3, and NOTCH4, which are single-pass transmembrane receptor proteins. The activation of Notch pathway is mediated by proteolytic cleavage events creating the intracellular, activated form of NOTCH.

The deregulated expression of Notch pathway genes is correlated with different malignancies. Notch activity through regulating other signaling pathways is involved in proliferation, cell survival, invasion as well as metastasis of cancer cells. For example, by regulating mTOR/Akt and NF-κB pathway, Notch induces apoptosis in breast cancer cells. Notch pathway contributes to increase the expression of Snail, Slug, Twist, Zeb1, and Zeb2 and as a result it activates EMT and metastasis in breast, pa2ncreatic, prostate and colon cancers (Tamagnone et al., 2018).

It was observed that SAL suppressed Notch signaling pathway in Medulloblastoma (MB) cells. Since SAL increased the sub population of G0 and expression of Cyclin A, it

caused to block the cell division cycle. This is explained by induction of cell cycle arrest at S/G2 phases by SAL, the expression of cyclin A was increased due to the prolonged S/G2 phases. SAL inhibited the expression of PDGFRβ, MYC, Bcl-2, p21 and some significant effectors in the Notch signaling pathway. It was revealed that SAL reduced the expression of Hes1 and DLL1 which are the regulators of Notch pathway. Moreover, SAL suppressed the expression of MAML1 which serves as a transcriptional coactivator in the Notch pathway (Zhou et al., 2014).

2.21. Effect of Salinomycin on TNBC

One of the candidate compounds in order to overcome chemotherapy resistance in TNBC cells is SAL. The effect of SAL and in combination with other compounds has been evaluated.

Recently, the effect of SAL in combination with Das (Src inhibitor) has been investigated on MDA-MB-231 and MDA-MB-468 cells which are triple negative cell lines. It was noticed that the combination was able to inhibit three pathways such as, STAT3, Wnt/βcatenin and hedgehog cell signaling pathways through down regulation of genes were involved in these pathways. The involved genes which were suppressed following the combination therapy included, BIRC5, CCND1, MCL1, MYC, AXIN2, MMP7, BMP4, BMP5. Moreover, the combination suppressed the estrogen-mediated S-phase entry pathway and as a result, they arrested cell cycle at the G1/S phase. In addition, it was observed that combination therapy caused to suppress EGR1 protein which is a regulator of BRCA1 pathway. It has been clarified that the pathway is involved in

tumorigenesis. Indeed, the combination therapy inhibited the formation of complex B which participated in BRCA1 pathway.

In one study, the effect of ester derivatives of SAL was tested on MDA-MB-231 cells. It was observed that the SAL derivatives induced cell cycle arrest and apoptosis. The induced apoptosis seemed that was due to the increased release of Ca^{2+} by a stressed endoplasmic reticulum (ER). The ER stress is caused to activate UPR protein in order to alleviate the damage of ER stress.

Following the ER stress, the level of IRE1α protein was increased and bonded to protein kinase-like ER kinase (PERK). Since the amount of pospho-eIF2α (Ser51) protein which is the main downstream effector of the PERK-dependent UPR signaling pathway was increased, it was concluded that this pathway was activated. On the other hand, the prolonged activation of UPR caused induced apoptosis which was confirmed by the decreased levels of caspase-3 and -9. Moreover, the increased amount of 8-OxoG indicated the DNA damage following treatment with SAL esters (Kuran et al., 2021).

In one study, the effect of SAL in combination with LBH569 as a histone deacetylase inhibitor (HDAC) was tested on HCC1937 cells (triple negative cells with characteristics of ALDH-positive cells and ability to self-renewal). It was noticed that the combination therapy demonstrated synergic anticancer effect.

Following treatment, DNA fragmentation and increased levels of cleaved PARP indicating apoptosis stimulation. Also, p21 and Ki67 levels were decreased implying cell cycle arrest. The antiapoptotic and cell cycle arrest was more potent following the combination therapy compared to the either drug alone. It was observed that the

combination therapy was able to increase the expression of E-cadherin and decrease the levels of Vimentin, N-Cadherin as well as SLUG. Since the EMT has a role in stemness, the therapy through prevention of EMT leading to decrease the stemness (Kai et al., 2015).

It has been observed that SAL sensitized MDA-MB-231 cells to anoikis. The increased level of PARP cleavage as well as activation of caspase-3 and 8 implying the induction of anoikis by SAL. Moreover, following exposure of cells to IL-6, the level of STAT-3 was increased. It was noticed that SAL suppressed the expression of STAT3 and phopho-STAT3. The down regulation of downstream target genes of STAT3 including Cyclin D1, MMP-2 and MMP-9 testified the inhibition of STAT3 activation. Therefore, SAL was able to inhibit MDA-MB-231 cell migration and invasion. Moreover, SAL suppressed the mamosphere formation in CSC population expressing the ALDH marker. The inhibitory effect exerted by SAL was correlated with inhibition of STAT3 expression. Since the SAT3 activation induces the ALDH1 activity, the suppression of STAT3 could affect the ALDH1 activity (An et al., 2015).

Chapter 3

Salinomycin Delivery

SAL has been studied extensively in cancer treatment and eradication of CSCs, however, there is some problem related to effectively deliver the drug to the tumors due to poor solubility, cytotoxicity following systemic administration as well as unwanted pharmacokinetic profile. For solving the problem due to low solubility, some techniques, including, salt formation, solubilization, chemical modification, application of nanocarriers like liposomes and polymer-, silica- and carbon-based NPs and NCs have been used.

Nanocrystallization can be effective in order to enhance pharmacokinetics of drugs. This helps to minimize the application of excipients enhancing the bioavailability and solubility of drugs. NCs are crystalline bunches of drugs which contain no excipient material with range of (10 to 1000 nm) size measure. The problem due to use of excipients is possibility of certain incompatibilities which cause to drug –excipient interaction. Excipients may cause the formation of molecular complexes and enhance the rate of chemical degradation.

NCs have advantages such as enhancing dissolution rate velocity and improved bioavailability. Nanocrystalization helps to stabilize Chemically labile drugs.

One approach which has been studied in order to treat colon cancer using combination of inhibitors of oxidative phosphorylation with inhibitors of DNA replication. The combinational therapy can target the quiescent and oxidative colon cancer cells and can help to improve the effects of medicines.

However, the obstacle due to the method is related to their high toxicity to normal cells, uncontrolled clearance as well as impermeability to tumors. In order to overcome this problem, nanomedicines have been examined. They have capability to improve drug circulation time and diminish the side effects associated with the drugs.

In one study, the combination of SAL and SN38, as an inhibitor of topoisomerase, was encapsulated in LNC. The outcomes of LNC formation were related to increased stability and efficacy of two molecules and decreasing the side of SAL. One side effect following treatment with SAL is hemolysis. The increased stability was due to the increased permeation and retention effect (EPR) OF LNC. Colon cancer cells, HTC 116, containing heterogeneous cell populations were targeted.

Since LNC contained high specific surface areas, they improved the solubility of SAL. Also, the acute toxicity as well as hemolysis induced by SAL was prevented due to LNCs. The cell shape was changed to the differentiated form following treatment. The LNCs efficiently targeted CSCs and led to suppress spheroid growth and decrease colospheres size. The combinational therapy stimulated the differentiation of CSCs. The cell death induced by SAL was related to induce ferroptosis. It was observed that following treatment with LNCs, micropinocytosis was activated resulting in taking up the LNCs. On the other hand, the activation of micropinocytosis has been observed in RAS mutated tumors. HCT 116 cell lines are included in the cells which demonstrate RAS mutation. Considering the existence of RAS mutation in HC 116 cells and activation of micropinocytosis, it was concluded that SAL induced ferroptosis (Tsakiris et al., 2020).

In one study, Nano formulations which were prepared by SAL, chlorine e6 and vitamin E have been applied. The role of vitamin E acetate was aggregation and stabilization of nanoparticles. Moreover, vitamin E was used to help the binding of Ce6 and SAL to keratin. Vitamin E due to contain hydrophobic and hydrophilic domains, facilitate the role of keratin for a wide range of drug classes.

Keratin has some advantages for being used as a carrier of SAL and the PS chlorin e6 (Ce6). SAL/Ce6@kVE kNPs were examined against MDA-MB-231 and MCF-7 cells. Obtained results demonstrated that the unlike MCF-7 cells, the underlying mechanism of induced cell death was apoptosis in MDA-MB-231, however, the apoptosis was not activated in MCF-7 cells. This was in line with the results of cytotoxicity and ROS production test in MCF-7 cells. The amount of produced ROS was lowered in MCF-7 compared to MDA-MB-231 cells. It was observed that kNPs were effective to eradicate CSCs through decreasing the stemness and suppress the formation of CSC-enriched mammospheres. The efficacy of SAL/Ce6@kVE kNPs is associated with improved permeability into the inner mammosphere region. Following penetration into the inner mammosphere region, the therapy exerted its effect. Moreover, treatment of zebrafish embryos with SAL/Ce6@kVE kNPs showed that these particles inhibited Wnt pathway (Avancini et al., 2021).

Nanoparticles which are prepared with poly (lactic-co-glycolic acid) (PLGA) due to have some advantages have been applied recently in clinical studies. These polymeric nanoparticles contain improved permeability to tumor cells as well as retention effects. Therefore, they improve passive targeting and prolonged systemic circulation. Since,

PLGA can metabolize and produce lactic acid as well as glycolic acid, cannot be harmful. Lactic acid can be removed through the kidney easily.

Aptamers are oligunucleic acid or peptides that are able to bind to distinct target molecules. As aptamers have low molecular weights and lack of immunogenicity are ideal candidates for targeted therapy. Several aptamers have been synthesized targeting extracellular ligands and cell surface proteins which are involved in carcinogenesis. One of these aptamers which has been applied successfully to target CD133 positive cancer cells is A15. It is an RNA aptamer that can interact and bind to CD133. In one study, A15–CD133 interaction has been applied in order to deliver SAL to osteosarcoma CSCs over expressing CD133. The anti-tumor functions of SAL-loaded PLGA nanoparticles (SALNP) conjugated with CD133 aptamers (Ap-SAL-NP) were examined. Using Ap-SAL-NP enhanced the efficacy of SAL against Saos-2 CS133+ cells. As a result, it could decrease the proportion of Saos-2 CD133+ and the tumorsphere formation capability of Saos-2 cells (Ni et al., 2015).

Nanoparticles composed of SAL and curcumin were loaded onto PLGA-PEG through double emulsion method. Curcumin has been considered as an effective anti_cancer agent which is able to inhibit the formation and spread of tumors.

In order to target CD44+ cells, the nanoparticles were conjugated with hyaluronic acid (HA). Hydrophilic characteristic of PEG caused to improve penetration and release great amount of drug. The release of both SAL and curcumin was optimum at pH 5.0. Therefore, SAL and curcumin can be released under the same condition in tumor cells. In vitro study indicated that these nanoparticles were effective to inhibit cell proliferation and

induce G2 cell cycle arrest. Indeed, the co-delivery of SAL and curcumin promoted G1/S arrest and consequently, induced apoptosis. Moreover, these nanoparticles were able to inhibit migration through upregulation of E-cadherin and downregulation of vimentin (Zhao et al., 2021).

In another study, SAL- loaded polymer–lipid hybrid nanoparticles conjugated with antiHER2 antibodies were synthesized with the aim of improved delivery of SAL to breast cancer cells including, MDA-MB-361 and BT-474. Moreover, CSCs were isolated using ALDH as a breast CSCs marker. As over expression of HER2 in normal mammary epithelial cells or breast cancer cells is responsible for tumorigenesis, invasion as well as metastasis, it can be a promising target in order to treatment of breast cancer cells. Moreover, breast CSCs demonstrate more HER2 expression compared with breast cancer cells. It was observed that SAL-NP-HER2 was more efficacious to prevent tumor growth in mice bearing BT-474 breast cancer xenografts compared to SAL-NP and SAL. The improved efficacy was related to enhanced targeting (Li et al., 2017).

Vitamin E derivatives have been applied in drug delivery due to its physicochemical and biological properties, including, lipophilicity and solubilizing water-insoluble dugs, anticancer activity. Vitamin E derivatives such as, TOS and TPGS1000 have ability to induce apoptosis and sensitize chemotherapeutic response. The prepared conjugation of vitamin E with water insoluble drugs intensifies the lipophilicity and self-aggregation. The nanoparticles are composed of vitamin E contain enhanced stability, high drug loading capacity and decreased toxicity. Based on the mentioned advantages, vitamin E can be a good carrier for SAL. In one study, the nanoparticles of vitamin E conjugated

with SAL and hyaluronic acid was synthesized. Firstly, TOS-ss-SAL (TS) was fabricated by formation of cystamine linkages between SAL and carboxyl groups of TOSSES. TS NPs and hyaluronic acid (HA)-coated TS NPs (HTS NPs) were synthesized in order to deliver both paclitaxel and SAL. The intracellular uptake of lipophilic TS prodrug by CSCs was performed easily and then cleaved into SAL to demonstrate the anti-CSCs action. Following uptake by cancer cells, TS NPs were degraded by intracellular glutathione (GSH) and cleaved into free SAL. Treatment of cells caused to arrest G0/G1 cell phase and inhibit mammospheres formation (Liang et al., 2018).

The combination of silver nanoparticles (AgNPs) and SAL was synthesized targeting human ovarian cancer cells (A2780). Treatment of A2780 cells with the synthesized nanoparticles caused a decrease in cell viability. The combinational therapy affected morphology of cells through altering the shape of cells to be rounder and decreasing cell density. Based on the observation, it was concluded that the synthesized compound caused cell damage and death. It was noticed that combinational therapy led to the release of LDH, MDA and decreased the levels of SOD and GSH-PX. The increased levels of LDH and MDA were a result of activation of lipid oxidation and also the decrease in GSH, SOD as well as CAT levels demonstrated induced oxidative stress. Therefore, the therapy was able to stimulate cellular damage. Moreover, the combination of SAL and AgNPs resulted in a loss of MMP which is concomitant with the increased ROS production.

Since following treatment with AgNPs and SAL, the upregulation of Bax, Bak, p53 and p21 genes was observed, these could activate apoptosis. It was found that the amount

of procaspase-3 was increased confirming caspase-dependent apoptosis was activated upon treatment. Moreover, the genes which are correlated with autophagy and responsible for the autophagolysosomes formation, including, Atg3, Atg5, Atg6, Atg7, Atg12, and Atg17 were activated. The induced autophagy was a protective mechanism against cytotoxicity by the treatment indicating the cytotoxic effects by SAL. Indeed, the formation of autophagy can be considered as a protective mechanism against cytotoxicity by the treatment (Zhang & Gurunathan, 2016).

There is limited penetration of drugs into the brain due to tight junction between brain epithelial cells and the expression of efflux transporters, such as P-glycoprotein (P-gp) in these cells. Therefore, current chemotherapeutics cannot penetrate the blood–brain barrier (BBB) sufficiently and consequently, the efficacy is decreased. In order to improve the efficacy, the dose of the agents should be increased, however it cannot be effective due to the systemic side effects.

In order to solve the problem, nanoparticles such as iron oxide nanoparticles (IONPs) have been developed with the aim of delivering chemotherapeutics into the brain. IONPs contain site-specific targeting and are coated with biocompatible polymers that have good safety profile. Magnetic iron oxide. (IONPs have) been used for SAL delivery to glioblastoma cells. It was observed that the nanoparticles efficiently were up taken by both mouse brain-derived micro vessel endothelial (bEnd.3) and human U251 GBM cell lines. The release of SAL under acidic conditions showed the potential of drug release in acidic tumor environment. The nanoparticles are devised in order to accelerate the initial release of drug. The release was correlated with the absorption of the drug on the exterior parts of

the polymer coating on the IONPs and the electrostatic bonds between the drug and the coating polymer. The electrostatic bonds were formed between PEG and SAL due to their hydrophilic characteristics. Moreover, PEG helped to decrease the non-specific protein adsorption on the nanoparticles and prevent their clearance through RES. As a result, the drug access to the tumor cells was improved. The anticancer mechanism exerted by SAL-PEI-PEG-IONPs was related to inhibit cell migration and induce apoptosis. It was noticed that SAL-PEIPEG-IONPs through changing the actin cytoskeleton to a shrunken structure could prevent cell migration. The induced apoptosis by SAL-PEI-PEG-IONPs was associated with inhibition of topoisomerase II and Wnt1 expression.

Treatment of U251 cells with SAL-PEI-PEG-IONPs caused up regulation of RbI2 and consequently, cell cycle arrest. The RbI2 is a tumor suppressor preventing cell cycle progression by inhibiting E2F family of cell-cycle-promoting transcription Factors. Moreover, SAL-PEI-PEG-IONPs activated the expression of long non-coding RNA (lncRNA) growth-arrest-specific 5 (Gas5), which is tumor suppressor and is downregulated in many cancers, including glioma. Furthermore, treatment of U251 cells with SAL-PEI-PEG-IONPs inhibited MiR155 expression, which is suppressor of caspase-3 (Norouzi et al., 2020).

In one study, iTEP-SAL-ABA NP was fabricated with the aim of SAL delivery to the breast cancer cells. To synthesis the nanoparticle, SAL was modified though binding to ABA, 4-(aminomethyl) benzaldehyde, which is a pH-sensitive linker. In order to generate a cleavable hydrazine bond, SAL was conjugated with ABA then attached to MPBH, a bi- functional linker. iTEP, immune-tolerant elastin-like polypeptides were used

in the synthesis of nanoparticles. The advantage of iTEP was due to its containing immune-tolerant elastin-like polypeptides, they did not cause to elicit antibody responses. Furthermore, as iTEPs are biodegradable, they were not accumulated in the body for the long-time and so do not result in forming of toxicity and side effects.

The selective induced toxicity by SAL and the capability of NP to penetrate and deliver SAL to CSCs is an advantage of this nanoparticle and demonstrated a synergy between SAL and the iTEP-SAL-ABA NP. The selective toxicity induced by SAL led to inhibit metastasis of 4T1 orthotopic tumor by NP. In order to enhance the survival and the metastasis-free survival for mice bearing 4T1 tumors, iTEP-SAL-ABA NP was used for delivery of PTX. Due to heterogenecity of tumors consisting of two differentiated tumor cells and CSCs populations, tumors have drug susceptibility. As a result, the combination therapy helped to simultaneously inhibit tumor growth and metastasis. Also, the results of the combinational therapy were better than monotherapy as it could prevent metastasis as well as increase the overall survival of tumor-bearing animals (Zhao et al., 2016).

Nanoscale drug delivery system (NDDS) composed of SAL and clathrin was synthesized with the aim of decreased premature drug release (PDR) while burst intracellular drug ensuring (BIDR).-NDDSs are fabricated to increase the efficacy of small molecules by solving problems correlated with low solubility, short circulation time, several unwanted effects and lack of target effects. They can mediate the delivery of the small molecules to their targets which are problematic to reach by themselves.

Clathrin modified SLN (CMSLN) was synthesized by installation of clathrin polymerize on the surface of SLN. To clathrin polymerization on SLN surface, there is

need to lipid membrane enclosed particles. SLN-SAL acted to activate the polymerization of clathrin. Therefore, SLN-SAL was inserted into CMSLNNDDS. It was revealed that there was no difference between surface charge, hydrophilicity and solubility of CMSLN-SAL and SLN-SAL. By polymerization of clathrin, a cage-like lattice structure is formed on the surface of particles enclosing SLN. The formed lattice structure prevents deformation due to external and internal pressure and caused the capability of anti-deformation of CMSLN-SAL. The particle expansion resulted from internal pressure caused to resist external pressure. Utilizing clathrin shell helped to decrease PDR. The modified clathrin prepares the opportunity for SLN-SAL to pass through cell membrane as a whole particle. Following the internalization of CMSLN, the clathrin coat undergoes depolymerization via cytoplasmic enzyme, HSC70 leads to release the SLN particle. The formed lipids due to SLN release in cytoplasm fuse with the lipid membrane organelle and the drug is released in a burst way. The improved intracellular uptake of CMSLN-SAL compared to SLN-SAL can be explained by clathrin-mediated endocytosis mediating the CMSLN-SAL uptake. The CMSLN- SAL activated apoptosis in HepG2 cells and more importantly, the induced effect by CMSLNSAL was more than SLN-SAL indicating the role of modified clathrin in order to improve function (Li et al., 2019).

The EGFR aptamer-conjugated SAL-loaded polymer-lipid hybrid nanoparticles (EGFR-SNPs) was synthesized with the aim of targeting osteosarcoma cells as well as CSCs. As epidermal growth factor receptor (EGFR) is overexpressed in different cancers such as osteosarcoma, the fabricated nanoparticles target and bounded to EGFR. SAL was encapsulated in polymer-lipid hybrid nanoparticles composed of EGFR-SNPs. The

EGFR-SNPs were consisted of lecithin, PLGA, DSPE-PEG 2000), aptamers which are biodegradable and do not contain side effects.

U2OS and MG63 cells were tested expressing CD133+. The results of qPCR revealed that the expression of EFGR was increased in the CD133+ cells and there is a positive correlation between CD133 and the amount of EGFR expression in both U20S and MG63 cells. The uptake of nanoparticles in U20S and MG63 cells was correlated with the conjugated EGFR-aptamers. The released SAL caused to decrease tumorsphere formation and the population of CD133+ cells (Yu et al., 2018).

Li. et al. using molecular docking model observed that SAL could be able to inhibit angiogenesis through interfering VEGFR2 phosphorylation and the downstream STAT3 in HUVECs. The assay revealed that SAL could interact with hydrophobic amino acids in binding pocket and impede the interaction of ATP and VEGFR2. In addition, SAL through interfering STAT3 binding to DNA was able to prevent STAT3 activation. Following inhibition of STAT3 activation, the expression of genes, such as Bcl-2, Bcl-xL and VEGF was decreased, and apoptosis was stimulated (Li et al., 2016).

In one study, the docking assay showed that SAL has high affinity toward Protein Kinase A (PKA) and Casein Kinase 1 gamma (CK1γ). Following Wnt binding Fzd, PKA phosphorylates LRP5/6 causes dephosphorylation of beta catenin and its stabilization. SAL through binding to PKA and CK1γ and inhibiting their phosphorylating activity caused to lower the levels of β-catenin and LRP-6 and consequently, inhibited Wnt pathway (Ragunathan & Ravi, 2015).

The immune tolerance pathway in which the metabolic conversion of tryptophan to kynurenine is achieved and regulated by Indoleamine 2,3 dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO). The immunosuppressive mechanism induced by IDO results in inhibiting T-cell activity and is upregulated in several tumors including breast cancer and is related to tumor progression as well as invasiveness. Different signaling pathways are involved in regulating of IDO expression such as, the (NF)-κB pathway and the (JAK-STAT) pathway. Interferon (IFN)-γ is produced by activation of Tcell is a main stimulator of IDO1 expression in different types of cancer. Considering the role of IDO in immunosuppression, its inhibition or suppressing downstream targets effects has been attended in cancer therapy. Three enzymes, including IDO1, IDO2, and TDO have a role in the catalysis and conversion of tryptophan into kynurenine. A molecular docking experiment was utilized to detect the interaction of SAL with IDO1. The results showed that SAL through forming of hydrogen bonding and hydrophobic interaction can align in the active site of IDO1. SAL through competitive inhibition caused to prevent IDO1 activity. The formation of hydrogen and hydrophobic bonds between SAL and amino acids like Arg, Ala and Gln stabilized the intermediate. It has been shown that SAL inhibited the activity of IDO2 without any effect on TDO2 function in MDA-MB-231 and MCF-7 and 4 T1 cells. The obtained results indicated that SAL demonstrated immune modulatory effect and IDO1 can be a potential target in breast cancer immunotherapy (Ebokaiwe et al., 2020).

Chapter 4

Salinomycin Modification

Studies showed that chemical modification of SAL can alter the ability and selectivity of binding to metal cations and prepare novel compounds with less toxicity for humans. Selective chemical modification can develop compounds with improved selectivity against CSCs as well as help to better structural understanding of the mechanism of action of SAL. The modifications include the oxygen functions such as carboxylic acid, hydroxyl and ketone groups which participate in the ion binding to form cation complex. The most obtained derivatives of SAL are related to modification of C1 carboxyl group to produce SAL amides and esters. The performed tests confirmed that all of SAL C1 esters and amides showed anti-cancer activity against cancer cells.

4.1. Modification of The Carboxyl Group

4.1.1. Amides

Considering that SAL is very sensitive to acidic conditions and heating, Mild reaction conditions for performing the amidation of the molecule should be selected. The first practical approach to the synthesis amide was first described by Huczynski and coworkers in 2012, applying DCC (N, N-dicyclohexylcarbodiimide) as a coupling agent and HOBt (1-hydroxybenzotriazole) as an activator. The reaction was processed at room temperature on SAL carboxylic acid. The advantages of the procedure included easy work-up, purification of the products through dry column vacuum chromatography and crystallization.

The desired product was produced by applying aliphatic and aromatic primary amines (a), the aliphatic secondary amines (b), the mono-substituted benzyl amines with fluorine, chlorine and bromine atoms as well as nitro group in ortho, meta and para positions (c) and the methyl esters of selected naturally occurring amino acids (d) effectively at the -ortho position. The performed analysis demonstrated that the derivatives which were modified contained the most efficacy against cancer cells and the least effect was related to the derivatives substituted at the -para position. In addition, all SAL N-benzyl amides were less toxic toward normal cells compared to the current cytostatic agents, such as cisplatin and doxorubicin. The efficacy of modified compounds could be explained based on the Warburg effect and/or with different mechanisms of ion transport by SAL. The Warburg effect is responsible for providing energy through high rate of glycolysis. Moreover, anti-cancer function of SAL is associated with ability of cation transport. Due to being highly acidic, common electroneutral transport cannot be performed efficiently in cancer cells and the COOH moiety cannot be deprotonated (Huczynski et al., 2012).

4.1.2. Esters

In the beginning work, the methyl and the p-bromophenacyl esters of SAL were obtained through reacting SAL with an excess of ethereal diazomethane and with pbromophenacyl bromide in ethanol, respectively. In 2014, Antoszczak et al. introduced two beneficial methods for preparing SAL esters by the reaction between SAL and the alcohol in the existence of DCC, PPy (4-pyrrolidinopyridine) and p-TSA (ptoluenesulfonic acid) (method A), and the alkylation of carboxylate ion with the proper alkyl halide

(chlorides or bromides) using 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU) (method B). It was shown that these methods are solvent dependence: The esterification methods also show a remarkable solvent dependence: dichloromethane was the most desirable solvents for method A and toluene for method B (Antoszczak et al., 2014).

The reversible masking of the carboxylic acid of SAL by esterification makes it possible for obtaining selective derivatization of the C9, C20, and C28 hydroxyl groups. The introduction of such protective groups improves the stability as well as purification of intermediates in the selective O-acylation of SAL. The appropriate protective groups for the carboxylate include TMSEt (2-trimethylsilylethyl-) and allyl bromide, in the presence of Cs2CO3 (Borgstrom et al., 2013).

4.2. Modification of The Hydroxyl Groups

4.2.1. Conjugates (O-Acylates at C9, C20, And C28)

Recently, selective methods for modification of the hydroxyl groups of SAL at C9, C20, and C28 have been reported. First, aliphatic acyl anhydrides were used for esterification of the C20 hydroxyl group, however, this method had restricted applicability to unhindered aliphatic anhydrides (formyl, acetyl, propionyl, n-butyril, n-valeryl) and was not very successful based on the obtained results. Studies have shown that protected SAL-TMSEt-ester unlike SAL or its sodium salt can react at the C20 hydroxyl with various reagents such as acid chlorides (RCOCl), isocyanates (RNCO), and chloroformates (ROCOCl) to obtain C20-esters, carbamates, and carbonates. The SALTMSEt-ester could

be efficiently cleaved with TBAF yielding SAL derivatives which are selectively acylated at the 20-position. To facilitate selective derivatization of the C28 alcohol, silyl groups reacted with C20 hydroxyl of SAL with TESCl (triethylsilyl chloride) and imidazole in DCM following the carboxylic acid protecting, however, for modifying the least reactive C9 hydroxyl, bis-silylation of both C20 and C28 alcohols with TESCl in DMF was required. Treatment of SAL which is protected at C1 (TMSEt or Allyl) and at C20 (TES), using isocyanates (PhNCO and EtNCO), with CuCl and DMF, yielded carbamoylated C28-position. Removal of the protective groups with TBAF produced C28-carbamates. Acylation of the C9 alcohol was achieved by reacting SAL, protection of C1 (Allyl), C20 (TES) and C28 (TES), using triphosgene after the addition of methanol to allow the methyl carbonates. Following the deprotection of the carboxylic group under Pd (0) catalyst, removal of the TES protective groups with TBAF, made the proper C9-carbonate (Borgstrom et al., 2013).

4.2.2. Modification of Ketone Group and Ring C of Salinomycin

Strand, et al. modified SAL through diasteroselective reduction of C11 ketone group using the Luche reduction (NaBH4) and obtained a highly syn isomer with respect to the C10 methyl group. Treatment of breast cancer cells, JIMT-1 and MCF-7, with these synthesized analogues, did not induce significant toxicity compared to SAL. Since, C11 ketone group participates directly in the ionophore characteristic of SAL, its oxidation resulted in decreasing the potency of the modified molecule. Moreover, the hydrogenation of the double bond between C18 and C19 utilizing an Adams catalyst and then the

chemoselective oxidation of the product using Dess-Martin periodinane yielded diketone. The cytotoxicity of the compound was lower than SAL (Borgstrom et al., 2017).

4.2.3. Reduction and Oxidation

For investigating the SAR of SAL and in order to assess the function of the carboxylic acid and the β-hydroxy ketone groups in formation of cation-complex, LiAlH4 was used for the concurrent reduction of the carboxylic acid and the β-hydroxy ketone groups, resulting 11-hydroxy-salinomycinol which was inactive completely. Moreover, the selective reduction of the ketone group with NaBH4 produced the 11hydroxysalinomycin which did not contain any anti_microbial and ion-transport activities. The 18,19-dihydro-SAL exhibited a moderate lowered cation affinity. However, the selective oxidation of the hydroxyl group at C20 with active manganese dioxide led to the C20-keto-SAL, an α, β-unsaturated ketone maintained one-half of antibacterial activity compared with SAL (Miyazaki et al., 1976).

4.3. Salinomycin Hybrid Compounds

One of the present approaches for development of novel anti_cancer drugs is formation of hybrid compounds (molecular hybridization/bioconjugation) using the present drugs. The achievement is correlated with improved affinity and efficacy compared to those of the parent drugs. The covalent combination of SAL with floxuridine, Cinchona alkaloids and silybin (SIL), has been examined for superior efficacy compared to the single target drugs, to decrease the undesirable side effects and also permit a synergic action. The SAL-SIL hybrid was prepared through selective esterification of C23-OH alcoholic group of SIL with the carboxylic moiety of SAL. This reaction was done under mild conditions

in the presence of DCC, PPy and p-TSA. Regarding the structure of SIL which exists in two forms of C10 diastereoisomers, (Silybin A and Silybin B), this procedure produced SAL-SIL biconjugate in 43% yield as a mixture of two diastereomers (Piperno et al., 2015).

Huczynski et al. reported the biological characteristics of SAL and modified nucleosides for the first time. They introduced two strategies for this purpose: the formation of ester linkage starting from SAL and floxuridine (FdU) under mild condition and the copper(I) conjugation catalyzed by click Huisgen cycloaddition reaction carried out between 3'-azido-2',3'-dideoxy-5-fluorouridine (AddFU) and SAL-propargyl amide (Huczynski et al., 2012).

In other study, it has been made effort to improve the efficacy of acylated SAL. Shi et al. showed that the inversion of C20 configuration could solve the problem due to the steric hindrance and consequently, improved ion chelation and efficacy. In this experiment, conversion of the C20 hydroxyl group with an azido group was selected because it could provide different SAL analogs and conjugates with use of the Cu-catalyzed azide-alkyne cycloaddition (CuAAC) reaction under mild conditions. Indeed, a library of analogs was obtained by the application of CuAAC reaction. First, SAL was esterified with TMS(CH2) 2OH using a reversible strategy for carboxylic group masking. Then, C20 reacted with an azido group through the Mitsunobu reaction with diphenylphosphoryl azide (DPPA) as a nucleophile. This led to the generation of azide with a 73% yield. Using tetrabutylammonium fluoride (TBAF) to unmask the carboxylic group caused an intermediate with 68% yield. A various library of SAL analogs was created using the CuAAC reaction, a panel of alkynes were chosen, including phenyl-containing, nitrogen-

containing, hydroxyl-containing, and ethercontaining alkynes. Each alkyne was coupled with azide, in the existence of CuSO4 and sodium ascorbate under mild conditions to produce triazols with good yields. To describe the structures of obtained triazols, single-crystal X-ray structure of one of these triazols with Na+ and K+ was synthesized. It was observed that the compound was able to acquire a suitable conformation to chelate ions, Na+ and K+, by its oxygen atoms. Indeed, the Mitsunobu reaction which was performed at C20 led to inverse configuration. A triazol side arm was made which stretched out of the ion chelation pocket. So, both the problems related to the steric hindrance and chelation perturbation of ion chelation were solved. The cytotoxicity of these triazols together with SAL was tested against glioma (U87), cervical (Hela), breast (MCF-7) and colorectal (Caco2) cancer cells as well as normal human liver cells.

It was observed that among the ether-containing triazols, perfluoro-tert-butyl ether was the most efficient one with an EC50 of 1.52 μM, which is 2- fold more efficacious than SAL. Moreover, C20 showed less toxicity to the normal cells than SAL. It was found that bulky substituents on the C20 trizol are suitable for the desired potency, however, analogues with the least bulky substituent on C20 hydroxyl acyl demonstrated most efficacy. Indeed, the inversion of C20 configuration enhances the effectiveness. In order to investigate the mechanism of activity of SAL derivatives against CSCs, F NMR/MRI was used. It was noticed that perfluoro-tert-butyl, due to being bulky group, is proper for F magnetic resonance imaging (F MRI). Based on the finding, SAL analog containing triazols, perfluoro-tert-butyl can be ideal for imaging-guided cancer therapy regarding its high cytotoxicity, selectivity, and F MRI sensitivity. Moreover, the synthesis of fluorinated

SAL analogues can be served as a promising approach for cancer treatment (Shi et al., 2016).

In one study, the hybrids of SAL and 3'-azido-3'-deoxythymidine (AZT) were synthesized. These hybrids were synthesized by formation of ester bond between SAL and AZT under mild reaction condition. The advantage of such types of derivatives was their easy penetration of the hydrophobic cell membrane and subsequent hydrolysis inside the cancer cells. It was observed that at low micromolar concentrations, such types of derivatives could be effective to overcome drug-resistance of the cancer cells. Moreover, these hybrids contained high selectivity indexes (low toxicity) against normal cells (Antoszczak et al., 2017).

Regarding hydroxamic anti-cancer properties and ability of ion chelating, it has been made effort to develop hybrids of SAL and hydroxamic acid. Huang et al. presented such compounds varying in N- and O-alkylation. Since hydroxamic acid is hydrolytic and carboxylate group of SAL is low accessible, the synthesis was challenging. However, they could achieve these by one-step protocol which was significant. One of such hybrids was synthesized by reaction of SAL with free base hydroxylamine in DMF. In the reaction HATU was used as the coupling agent. Another protocol was presented which was not valuable due to lower result. In this method, an O-tert-butyldimethylsilyl (TBS) was formed. Intermediate was protected and silyl group was removed.

Then, single crystals a neutral form of this compound form by its binding a sodium atom with noncoordinating PF6-. The scXRD structure of newly formed compound

confirmed that cation could coordinate through 1,3- binding of the cation by the hydroxamic acid group which is similar to crystal structure of SAL.NA. The comparison of SA·Na and novel compounds showed that similarly to the crystal structure of SA.Na, hydroxamic acid hybrids coordinate to the metal ions by the oxygen atoms of the D- and E-rings as well as from the C11 carbonyl group. The head-to-tail conformation in SA.Na structure is stabilized via formation of hydrogen bonds from the C9 and C8 hydroxyl groups to the carboxylate, however, a new form of hydrogen bond network which is formed between C20 hydroxyl group serves as hydrogen bond donor and the hydroxamate OH giving proton to the C28 oxygen atom. Since the efficacy of SAL is related to its capability to bind and exchange ions, the ability of these compounds to bind and exchange metal ions was examined. It was noticed that washing an EtOAc solution of compound a with K_2CO_3 or Na_2CO_3 gained metal cation complexes. H NMR spectrum confirmed that it lost acidic proton ($\delta = 10.3$). Unlike SAL, however, washing an EtOAc solution of formed complexes did not lead to dissociation of the metal ion. The result indicated that these complexes did not contain acidic proton. Instead, they formed inclusion complexes and expanded signals in the 1H NMR spectra showed chemical exchange.

Moreover, it was observed that in biological systems, such compound is able to exchange alkali metal ions regardless of its high affinity toward such species. Also, it was shown that none of the other synthesized compounds was deprotonated or formed inclusion complexes under the same condition. Both the salinomycin structure and hydroxamic acid motifs of these compounds did not show any noticeable decomposition under these acidic or basic conditions. The cytotoxicity of obtained compounds was tested against two breast

cancer cell lines, JIMT-1 and MCF-7, with use of MTT based assay. The result confirmed that the hydroxamic acid derivatives are less active than SA itself, although showed IC50 in a range of micro molar. Although, in contrast to SAL, these hybrids showed no selective reduction of aldehyde dehydrogenase (ALDH). It was concluded that only SAL analogues containing free carboxylic acid show phenotype selectivity. Therefore, these hybrids were the first SAL C1 modified analogues with the ability to form complexes with metal alkali ions (Huang et al., 2016).

In one study, a synthetic derivative of SAL, which was named ironomycin (AM5) was investigated. It was observed that the derivative was accumulated and sequestered iron in lysosomes. Analogous of SAL were synthesized using chemoselective oxidation followed by a stereoselective reductive amination at C20. These modifications gained derivative AM5 with its methylated counterpart AM9. These analogous were tested against HMLER CD44high/CD24low cells (HMLER CD24low). As AM5 was zwitterionic, demonstrated higher toxicity against HMLER CD24low cells compared to SAL. Moreover, derivative AM9 to contain methyl was less effective compared to AM5 demonstrating that free carboxylate at C1 is essential for functioning. AM5 had more toxicity against (ALDH+) subpopulation of one model of CSCs, namely iCSCL-10A2 cells20, compared to SAL and showed little toxicity against primary breast cells.

This derivative inhibited tumorsphere formation in CSCs at doses as low as 30 nM. Inversely, AM9 had no efficacy indicating the role of free carboxylate for CSCs targeting. In addition, AM5 suppressed tumor growth without generic toxicity as the body weight was constant and the peripheral tissues were unchanged.

Using chemical and biochemical markers of lysosomes, including a lysotracker, the Ras-related protein Rab7 and the lysosomal-associated membrane protein 1 (Lamp1), revealed that the tested compounds were accumulated in lysosome whereas the lysosomal pH was unaltered according to acridine orange staining.

The results confirmed that AM5 did not aggregate with the early endosome antigen 1 maker EEA1 showing an endocytosis-independent entry mechanism and fusion across lipophilic membranes. Following treatment of HMLER CD24low and iCSCL-10A2 cells with both SAL and AM5, a response-characteristic of cytoplasmic depletion of iron27, such as enhanced levels of iron responsive element-binding protein 2 (IRP2) and transferrin receptor (TfR) together with decreased levels of ferritin was observed. The decreased levels of ferritin were correlated with its re-localization to the lysosomes. Since similar results were obtained following treatment of the cells with the iron chelating agent deferoxamine (DFO), it was concluded that these agents inhibit iron form release from the lysosomes.

Therefore, as AM5 is a lipophilic compound, it accumulates in the lysosome, interacts with iron (II) and prevents the iron translocation into the cytosol. Lysosomal iron accumulation results in suppression of cathepsins. Moreover, depletion iron triggered ER stress and degradation of ferritin in lysosomes. As a result of ferritin degradation, further levels of iron were accumulated in the lysosomes. Iron caused to stimulate production of ROS led to lysosomal membrane permeabilization, promoting a cell death pathway consistent with ferroptosis. Moreover, the induced ferroptosis by SAL or AM5 was inhibited by the ferroptosis inhibitor ferrostatin-1, however, the apoptosis and necrosis inhibitors such as Z-VAD-FMK and necrostatin-1, were not able to alter the cell death

profiles. As Wnt signaling pathway needs iron to inhibit E-cadherin expression, it was observed that the Wnt1 protein expressed more in HMLER CD24low cells compared to the control cells. Therefore, iron is important for proliferation of cells containing CSC characteristics. Moreover, it was noticed that cytokine oncostatin M (OSM) elevated the expression of ferritin in MCF-7 cells and knocking down of ferritin caused to decrease the stemness characteristics including, the expression of fibronectin, vimentin, zeb1 and the ration of CD44high/CD24low and ALDH+ cells which were induced by OSM. Based on this observation, it seemed that molecules targeting the pathways which are dependent on iron homeostasis can be served as potential therapeutic agents in CSCs treatment (Mai et al., 2017).

In line with this result, among a library of SAL, which was obtained by C20-amination, C1-esterification, C9-oxidation, and C28-dehydration, the aminated compound was the most effective against HMLER CD24low/CD44 high cells and exhibited the least IC50. This derivative was due to contain a cyclopropylamine at position C20 was zwitterionic at physiological pH. The presence of the positive charge of the amine affected the binding mode and specificity for different metal ions (Versini et al., 2020).

Chapter 5

Methods

SAL analogs, including Sal-Nano, SV-KV- 034, SV-KV-032 and SV-KV-030 were obtained by SAL modification. Effects of SAL analogs on inhibition of viability, angiogenesis, metastasis and Wnt pathway in Triple negative breast cancer cells, including, MDA-MB-231, MDA-MB-468, MDA-MB-431, Hs578T, ZR75 and MCF-7 cells was evaluated. The cells were treated with (0.5, 1, 2.5, 5 µM) doses of Sal-Nano, SV-KV- 034, SV-KV-032 and SV-KV-030. The effect of SAL analogs on the cell viability was investigated by thiazolyl blue tetrazolium bromide (MTT) assay. Inhibition of Wnt signaling pathway was tested by Western blot assay.

The inhibitory effect of the modified analogs on angiogenesis was evaluated on HUVEC cells. The cells were treated with 0.5 µM and 2.5 µM doses of SAL 30, SAL 32 and SAL 34 and in vitro formation assay was performed. Moreover, inhibitory effect of the tested analogs on VEGF expression in MDA-MB-231 cells was assessed by western blot.

Also, inhibitory effect of the tested analogs on metastasis was evaluated by western blot.

Chapter 6

Results

MDA-MB-231, MDA-MB-468, MDA-MB-431, Hs578T, ZR75 and MCF-7 cells were treated with different doses (0.5, 1, 2.5, 5 µM) of Sal-Nano, SV-KV- 034, SV-KV-032 and SV-KV-030.

Applying MTT assay, comparison of IC50 showed that Sal-Nano was the most efficient one for inhibiting the growth of MDA-MB-231 cells, SV-KV-034 was the most potent for decreasing the viability of MDA-MB-431 cells and both SV-KV-032 and SV-KV-030 were the most efficacious compounds for reducing the viability of MCF-7 cells (Table 1).

Applying western bot, the effects of MDA-MB-231 cells treatment with SV-KV-030, SV-KV-032 and SV-KV-034 on phosphorylation and degradation of β catenin was examined. Following treatment for 48 h, the obtained results of western blot indicated that SV-KV-032 caused to inhibit phosphorylation of β-catenin at (T41/S45) residues and SV-KV-034 lowered the phosphorylation of β-catenin at (Ser 675) (Figure 1).

HUVEC cells were treated with 0.5 µM and 2.5 µM doses of SAL 30, SAL 32 and SAL 34 and in vitro formation assay was performed. It was observed that SAL 30 (2.5 µM) was the most potent analog for inhibit angiogenesis and SAL 32 and SAL 34 compared to SAL 30 demonstrated lower efficacy (Figure 3).

Moreover, inhibitory effect of the compounds on angiogenesis and metastasis was tested through using western blot. Following treatment for 48h, the obtained results showed

that SV-KV-030 was the most potent compound for suppressing angiogenesis through down regulation of VEGF (Figure 2).

Also, SV- KV-030 demonstrated the most efficacy for inhibiting metastasis by reducing the expression of MMP-9 and SV-KV-030 was the most potent compound for down regulation of C-Myc Proteins (Figure 2).

Table 1

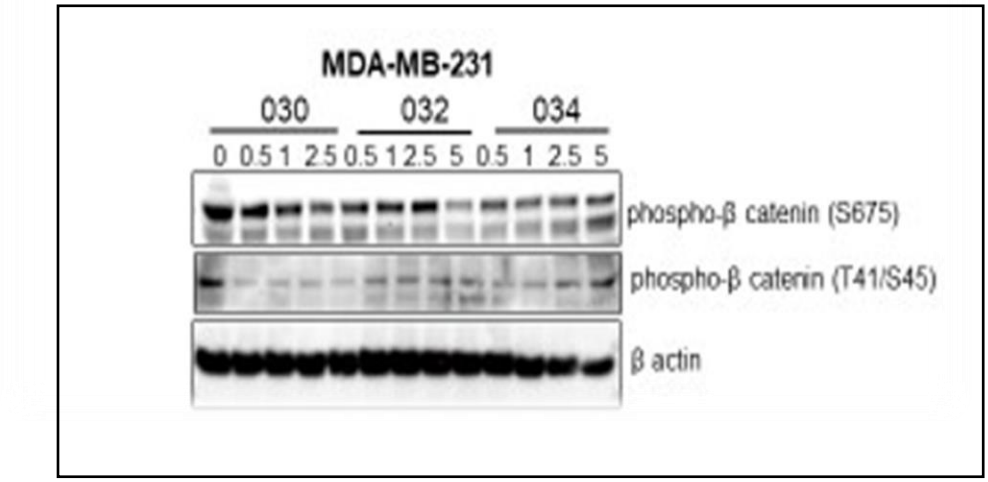
IC_{50} Value and Selective Index of Salinomycin Analogs Treatment on MDA-MB-231, MDA-MB-468, MDA-MB-431, Hs578T, ZR75 and MCF-7 Cells

Analogs	IC_{50} of Salinomycin analogs in Breast cancer cells (μM)					
	MDA-MB-231	MDA-MB-468	MDA-MB-431	Hs578T	ZR75	MCF-7
Sal-Nano	0.5 ± 0.02	1.0 ± 0.12	0.5 ± 0.15	5 ± 0.25	5 ± 0.75	2.5 ± 0.5
SV-KV-034	5 ± 0.3	10 ± 1.5	0.5 ± 0.02	>10	5 ± 1.15	2.5 ± 0.5
SV-KV-032	1 ± 0.15	5 ± 0.45	1 ± 0.15	2.5 ± 1.0	5 ± 0.05	0.5 ± 0.10
SV-KV-030	>10	1 ± 0.14	2.5 ± 0.47	2.5 ± 0.25	1.0 ± 0.25	0.5 ± 0.20

Note. Values are expressed as mean ± S.D. The results of MTT assay showed that Sal-Nano was the most efficient for reducing the viability of MDA-MB-231 cells, SVKV-030 was the most efficacious for inhibiting the growth of MCF-7 cells, SV-KV-032 was effective to arrest the growth of MCF-7 cells and SV-KV-034 was the most potent for decreasing the viability of MDA-MB-431 cells.

Figure 1

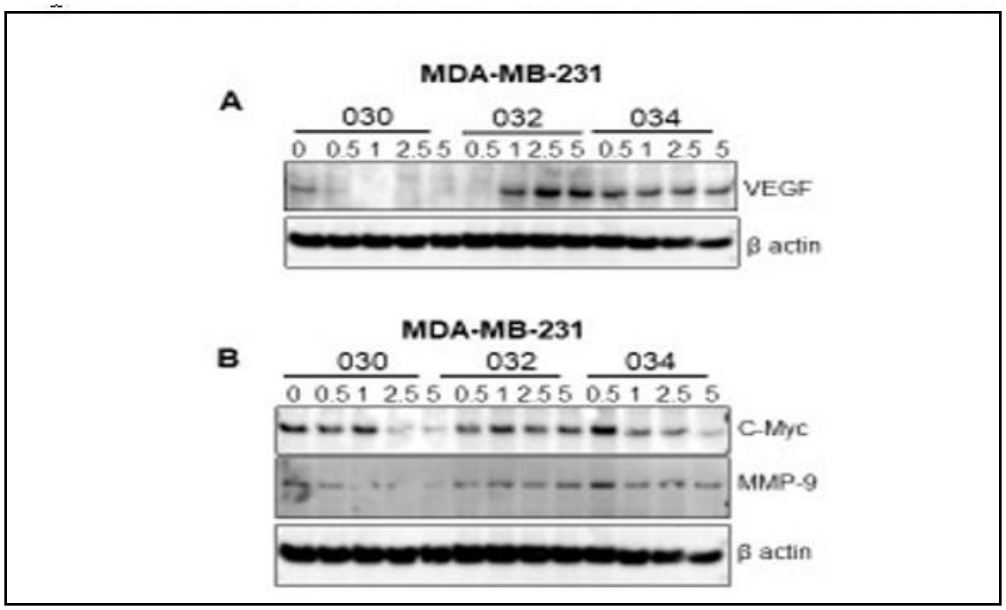
Effect of Salinomycin Analogs Treatment on β-Catenin Expression



Note. MDA-MB-231 cells were treated with 0.5 µM, 1 µM, 2.5 µM, 5 µM doses of SV-KV- 030, SV-KV-032 and SV-KV-034. After treatment for 48h, western blot test was performed, and levels of protein expression were analyzed. SV-KV-032 inhibited phosphorylation of β-catenin at (T41/S45) residues and SV-KV-034 lowered the phosphorylation of β-catenin at (Ser 675).

Figure 2

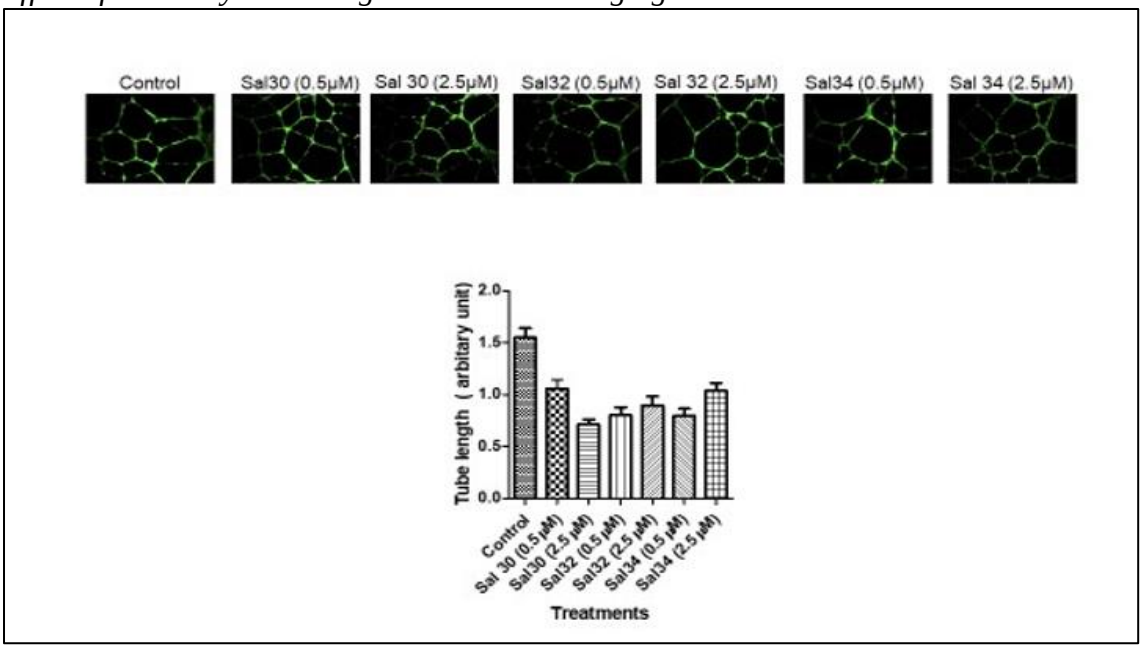
Effect of Salinomycin Analogs Treatment on Angiogenesis and Metastasis



Note. (A) MDA-MB-231 cells were treated with 0.5 µM, 1 µM, 2.5 µM, 5 µM doses of SV-KV- 030, SV-KV-032 and SV-KV-034. After treatment for 48h, western blot test was performed, and levels of protein expression were analyzed. It was noticed that SV-KV-030 was the most effective compound for inhibiting angiogenesis through down regulation of VEGF. (B) SV-KV-030 demonstrated the most efficacy for inhibiting metastasis by reducing the expression of MMP-9 and SV-KV-030 was the most efficient one for down regulation of C-Myc protein expression.

Figure 3

Effect of Salinomycin Analogs Treatment on Angiogenesis Inhibition



Note. HUVEC cells were treated with 0.5 µM and 2.5 µM doses of SAL 30, SAL 32 and SAL 34 and in vitro formation assay was performed. It was observed that SAL 30 (2.5 µM) was the most potent analog for inhibit angiogenesis and SAL 32 and SAL 34 compared to SAL 30 demonstrated lower efficacy.

Chapter 7

Discussion

Cancer is a threatening disease globally. New cases of cancer and death have been estimated in 2020 (1.8 million new cancer cases have been diagnosed). Currently several types of anticancer treatment, including chemotherapy, radiotherapy as well as surgical resection have been used (World Health Organization).

Breast cancer is one of the most common cancers among women. TNBC as a subtype of basal-like breast cancer is very metastatic. There are some obstacles toward cancer treatment especially TNBC, including, phenotypic heterogeneity within tumors, genetic mutations, metastasis and development of resistance to the current chemotherapeutic drugs. Therefore, several anti-cancer agents have failed to cancer treatment.

Moreover, molecular targeted therapies, such as effective apoptotic factors and monoclonal antibodies against certain enzymes, show high toxicity and result in serious systemic damage to the non-cancer cells. Therefore, there is need to develop novel therapeutic agents to selective targeting and overcome the resistance to the current chemotherapy with minimal side effects (Li et al., 2020).

Recently, SAL has been considered as an efficacious agent for eradication of CSCs as well as cancer cells. The molecule contains properties such as being ionophore as well as facilitation of cation transport through cell membranes selectively which potentiates its anti-cancer activity.

The applied mechanism by SAL is attributed to the interactions with multiple molecular targets. It has been reported that several cells signaling pathways and targets

including, Wnt pathway, apoptosis through the ROS-mediated mitochondrial pathway, autophagy, MAPK, ER stress, PI3K/AKT/mTOR, AMPK, EMT, iron homeostasis, angiogenesis, Hedgehog, NF-kB and Notch pathway, are affected by SAL. In addition, SAL as a P-gp inhibitor overcomes the developed resistance to the anti-cancer agents (Shuanf et al., 2013).

The major problem regarding the application of SAL is related to poor solubility. Some techniques, including salt formation, solubilization, chemical modification have been served for modifying SAL solubility (Kuran et al., 2021).

Considering that TNBC contains molecular phenotype, conventional chemotherapy and molecular targeted therapy cannot be effective. Currently, novel treatments have been developed to enhance efficacy of chemotherapy or target mutations and key signaling pathway underlying malignancy or resistance to chemotherapy. SAL recently has been studied to investigate whether it can be efficient for treatment or potentiate the current therapy for TNBC.

The present study examined the effect of Sal-Nano and the modified analogous of SAL, including, SV-KV-030, SV-KV-032 and SV-KV-034 on the viability, metastasis, Wnt pathway as well as angiogenesis of TNBC cell lines.

Wnt signaling pathway has a critical role in different cellular processes, such as cell proliferation, tissue homeostasis, cell polarity as well as fate determination throughout embryonic development. β-catenin is one of the determining factors in Wnt pathway. Wnt signaling pathway can act through either β-catenin dependent pathway or β-catenin-independent pathway. Phosphorylation of β-catenin by several kinases can be a key factor for its involvement in canonical or non-canonical pathways. Indeed, phosphorylation of β-

catenin at different sites can determine its location. Based on the site of phosphorylation, β-catenin can be presented in the cell membrane, cytoplasm or nucleus. In the absence of Wnt signaling pathway, β-catenin is phosphorylated by destruction complex and is targeted for degradation. In the destruction complex, β-catenin is phosphorylated on Ser 45 by CK1a and Ser 45 phosphorylation leads to phosphorylate Thr 41, Ser 37, and Ser 33 by GSK3b. Protein kinase C (PKC) isoforms have a critical role in the regulation of Wnt signaling pathway. One of the PKC isoforms serving as a tumor suppressor is PKC ζ which stimulates Ser 45 phosphorylation and GSK3b-mediated phosphorylation. The induction of phosphorylation by PKC ζ is independent of induced phosphorylation by CK1a.

The cyclic AMP (cAMP)-dependent protein kinase, protein kinase A (PKA), has dual role regarding β-catenin phosphorylation. PKA through β-catenin phosphorylation at different serine residues causing its degradation or stabilization. PKA by phosphorylation of Ser 45 plays a role in β-catenin degradation and through phosphorylation of Ser 552 and Ser 675 involves in β-catenin stabilization (Shah & Kazi, 2022).

Considering that up regulation of Wnt signaling pathway is one of the characteristics of TNBC, we examined the effects of the tested compounds on Wnt pathway.

For the first time, it has been observed that SAL (SV-KV-032) caused to inhibit of phosphorylation of β-catenin at (T41/S45) and SAL (SV-KV-034) lowered the expression of phospho-β-catenin (Ser 675). Therefore, SV-KV-034 may be effective to induce β-catenin degradation through down regulation of Ser 675. PKS by phosphorylation of S675 may enhance β-catenin transcriptional activity by promoting β-catenin stability. It seems

that SV-KV-034 through down regulation of phospho-β-catenin (Ser 675) may be effective for decreasing β-catenin stability.

Unregulated angiogenesis is a determining factor in tumor progression and tumor metastasis. Angiogenesis by providing blood supply system promotes invasion, growth and survival of tumor cells. It has been shown that MMPs and VEGF are correlated with ECM remodeling and microvascular permeability during angiogenesis. It seems that MMPs together with VEGF are involved in invasion and metastasis. It has been observed that inhibition of MMP-9 and MMP-2 expression caused lowered VEGF expression in retinoblastoma cell lines (Zhu et al., 2019).

Myc proto-oncogenes which code for transcription factors are controlling cell cycle, apoptosis, cell proliferation and ribosome biogenesis. The Myc family contains three human genes including, c-Myc (MYC), l-MyYCL), and n-Myc (MYCN).

Following translocation of β-catenin to the nucleus, it interacts with TCF/LEF and activates Wnt target genes, including, c-MYC, CCND1, EGFR and LRG5. C-Myc is a transcription factor that acts as the C-Myc/ Max complex. For being active, C-Myc must heterodimerize with Max and the formed C-Myc/ Max complex thorough binding to a specific DNA sequence (Enhancer-box or E-box) activate gene transcription. The MYC de regulation is related to tumor progression and metastasis. Specifically, C-Myc overexpression is related to progression of breast tumors.

Tumor microenvironment (TME) contains several types of cellular and non-cellular factors, including, cancer-associated fibroblasts, tumor-associated macrophages, vascular endothelial cells, myeloid-derived suppressor cells and immune cells. It was found that C-

Myc controls the biological processes that happened in TME, such as invasion, angiogenesis, migration, and immune evasion (Gao et al., 2023).

C-Myc regulates angiogenesis through controlling VEGF. C-Myc is able to affect angiogenesis by enhancing VEGF expression, blood vessel permeability and induce Wnt pathway. Also, C-Myc activation enhances matrix metallopeptidase 9 (MMP-9) expression. EMT through degradation of extracellular matrix (ECM) and cleavage basement membrane components are participated in EMT and cancer metastasis. Moreover, MMPs are involved in tumor progression by neovascularizatrion (Magid et al., 2003).

It has been observed that MMP-9 is expressed following MYC activation in TAM. MYC induces expression of MMP-2, MMP-3 and MMP-9 by macrophages which are involved in tumor invasion (Pello et al., 2012).

It has been observed that MMPs including, MMP-1, MMP-2 and MMP-9 together with VEGF are involved in invasion and metastasis of retinoblastoma cells (Wang et al., 2021).

In agreement with the previous studies, VEGF and MMP-9 expression decreased following MDA-MB-231 cells treatment with SV-KV-030. Moreover, SV-KV-030 down regulated Myc expression. Therefore, we indicated that VEGF and MMP-9 are correlated with C-Myc expression and decreased expression of C-Myc expression inhibiting angiogenesis and metastasis. As C-Myc has a critical role in progression of breast cancer through enhance VEFG expression and induce angiogenesis, it seems that the compound may have potential for inhibition of TNBC.

In summary, we demonstrated that the tested compounds may have the potential to inhibit angiogenesis, metastasis and alter Wnt signaling pathway. However, more studies are needed to clarify their effects on CSCs, in vivo and moreover, the effects on both tumor and normal cells.

To date, few clinical studies have reported SAL application. One of these studies is related to SAL administration to a female patient with triple negative metastatic breast cancer. The findings indicated that SAL was able to decrease tumor metastasis. Moreover, it was observed that SAL regressed tumor metastasis in other three patients with invasive breast cancer, one patient with metastatic ovarian cancer, and one patient with metastatic head and neck squamous cell carcinoma (Soni et al., 2023).

These studies indicated that SAL may be effective for clinical use. However, further clinical studies need to be conducted to conclude whether SAL can be used as a potential treatment with less toxicity compared to the conventional treatment.

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