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Anticancer Activity of Polyether Ionophore-Salinomycin

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Abstract: Since the discovery of unusual anti-tumor activity of natural polyether antibiotic – Salinomycin, this compound, along with its derivatives, has been intensively studied against different human cancer cells, both *in vivo* and *in vitro*. Salinomycin has shown strong inhibition activity against the proliferation process of many different cancer cells, including multi-drug resistance (MDR) cancer cells, as well as cancer stem cells (CSCs), *i.e.* leukemic stem cells, colon carcinoma stem cells, prostate cancer stem cells and many others. Additionally, the application of Salinomycin has been proved to enhance the anti-cancer effect of radio- and chemotherapy. Preliminary clinical studies have shown tumor regression and only transient acute side effects after application of Salinomycin. Up to now, major efforts have been devoted to elucidate the biological mechanisms of anti-tumor activity of Salinomycin and it is expected that the results may provide new therapeutic strategies based on biological modulation of Salinomycin activity. This review is focused on and describes the possible role of Salinomycin in cancer therapy and gives an overview of its properties.

Keywords: Anticancer activity, Apoptosis, Cell proliferation, CSC, Polyether ionophores, Sensitizing effect

INTRODUCTION

The compounds of natural origin are widely used in medicine, veterinary, pharmaceutical and cosmetic industries. They are applied in both natural as well as chemically modified forms [1]. Natural products with potential applications as anti-tumor agents can be produced by plants (Etoposide, Paclitaxel, Vinblastine, Vincristine), marine (Aplidine, Citarabine, Dolastatin 10) or microbes (Bleomycin, Dactinomycin, Doxorubicin) [2].

Detailed data show that 60% and 75% of the drugs used in the fight against cancer and infectious diseases, respectively, are of natural origin [3]. Other data indicate that due to technical improvements in screening programs as well as high advancement in isolation as well as separation techniques, the total number of natural compounds discovered to the present time is much more than 1 million, wherein more than half of them are produced by plants (e.g. alkaloids, carbohydrates, flavonoids, steroids or terpenoids) and simultaneously only 5% are isolated from microorganisms [4]. Among all natural substances discovered, only 20-25% show interesting biological activity, wherein the largest number of them is produced by actinomycetes, fungi, unicellular bacteria [5] as well as about 10% (over 22 500) by microbes [4].

It therefore seems that the easiest way to discover new effective anti-cancer drugs is through chemical modifications of naturally-occurring substances with proven high biological activity, such as polyether ionophores. Among them Salinomycin seems to be the most interesting and it will be described in detail in the following sections of this review.

Chemical and biological investigation for the search of novel bioactive natural products involves the extraction, isolation, purification and structure elucidation (classical natural product isolation methodologies), which can be challenging and time consuming. The chemical modification of active natural compounds is often employed to enhance their activity and lower adverse effects. Therefore, polyether ionophores, as examples of biologically active substances, are very good candidates for the preparation of clinically useful derivatives and the starting point for extensive structure-activity relationship (SAR) studies. Thus, the properties of Salinomycin derivatives and their *in vitro* activity against human cancer cell lines are also discussed herein.

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ANTI-CANCER ACTIVITY OF POLYETHER IONOPHORES

Polyether ionophores represent a large group of naturally occurring compounds produced by *Strpetomyces* spp. (see Table **1**) and up to now over 120 of these substances have been isolated and described [6]. Seven of them (Laidlomycin, Lasalocid acid, Maduramycin, Monensin, Narasin, Semduramycin and Salinomycin) have found commercial application in veterinary medicine as a ruminant non-hormonal growth promoters as well as coccidiostatic agents in livestock and poultry breeding [7-9]. Coccidiosis is a widespread and economically significant disease of livestock caused by *Eimeria* protozoan parasites. This worldwide disease costs the animal agricultural industry many millions of dollars every year [8]. Additionally, polyether antibiotics can also be used in the production of ion-selective electrodes because of their strong complexing properties [10, 11].

Table 1. Selected polyether ionophores and their isolation sources.

Polyether Ionophore	Isolation Source
Ionomycin	*Streptomyces conglobatus*
Kalcimycin	*Streptomyces chartreusensis*
Lasalocid acid	*Streptomyces lasaliensis*
Monensin	*Streptomyces cinnamonensis*
Nigericin	*Streptomyces violaceoniger*
Salinomycin	*Streptomyces albus*
Valinomycin	*Streptomyces fulvissimus*

One of the most important biological activities of polyether ionophores is their antibacterial activity, especially towards Gram-positive bacteria, including anaerobic bacteria both against drug-sensitive and multi-drug resistant (MDR) bacteria strains [12]. What is important, the activity against Gram-positive bacteria is much stronger compared to the activity against Gram-negative bacteria. It has been explained by much greater complexity of the structure of cell wall of Gram-negative bacteria [13]. The outer cellular membrane of these types of bacteria is impervious to

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hydrophobic substances and their complexes, such as polyether ionophores. They also exhibit antifungal [14-16], antimalarial [17], antiviral, including human immunodeficiency virus (HIV) [18], as well as coccidiostatic activity [8].

Recently, ionophores have attracted the attention of researchers because of their high and uncommon anti-tumor activity. The most interesting in this respect appear to be Inostamycin, Ionomycin, Lasalocid acid, Monensin and, of course, Salinomycin **(**Fig. **1)**.

Below we provide a very short description of the most interesting anti-cancer properties of common polyether ionophores for better understanding of the unique anti-cancer activity of Salinomycin.

Anti-Tumor Activity of Inostamycin

Inostamicin was isolated and structurally determined for the first time in 1990 [19]. Already in 1991 the first mention of anti-tumor *in vitro* activity of this antibiotic appeared, when it was shown that Inostamycin reversed MDR in KB cells. In tests, 0.5-2.0 μg/ml of Inostamycin increased the accumulation of vinblastine, an alkaloid with a cytostatic effect, in MDR KB-C4 cells. Simultaneously, Inostamycin did not enlarge accumulation of vinblastine in case of drug-sensitive KB-3-1 cells. Moreover, 1 μg/ml of Inostamycin inhibited efflux process of vinblastine from KB-C4 cells, but what is worth to empasize not from KB-3-1 cells [20]. The accumulation of vinblastine increased for the next 48 hours, when MDR KB-C4 cells were preincubated with Inostamycin for 30 minutes [21]. KB is a cell line derived from a human carcinoma of the nasopharynx and it has been used as an anti-cancer assay for screening tests to discover new chemotherapeutic agents. KB activity may lead to discovery of vinblastine, vincristine and podofilox [22]. It has been suggested that Inostamycin inhibits irreversibly the P-glycoprotein (P-gp) functions by binding cell membranes through phosphatidylethanolamine, which significantly enhances its anti-cancer properties [21]. P-gp, also known as a multi-drug resistance protein 1 (MDR1), ATP-binding cassette subfamily B member 1 (ABCB1) or cluster of differentiation 243 (CD243), is a ATP-dependent efflux pump of the cell membrane with broad substrate specificity, which pumps many foreign substances out of cells, including therapeutic drugs, peptides and lipid-like compounds [23]. Finding ways to influence these proteins is an important goal of effective anti-tumor therapy.

In 1992 it was demonstrated that Inostamycin is a highly effective inhibitory agent of cytidine 5'-diphosphate 1,2-diacyl-sn-glycerol (CDP-DG):inositol transferase, which leads *in vitro* to the reduction of epidermal growth factor (EGF)-induced invasion in the case of tongue carcinoma cells [24]. Besides, in 2000, it was proved that Inostamycin suppresses invasion ability as well as cell motility of tongue carcinoma HSC-4 cell line by reducing production of matrix metalloproteinase-2 (MMP-2) as well as metalloproteinase-9 (MMP-9) [25].

Additionally, Inostamycin has been shown to inhibit the proliferation process in normal rat kidney (NRK) cells through effective G1 phase blocking, which is a result of the inhibition of cyclins D1 and E [26]. Subsequently, Inostamycin at low concentrations has been found to force human small lung carcinoma Ms-1 cells to accumulate in the G1 phase, leading to their apoptosis. Inostamycin is also known to diminish cyclin D1 as well as increase cyclin-dependent kinase inhibitors, including p21 and p27, in Ms-1 cell line. In higher concentrations, Inostamycin induces DNA fragmentation and morphological apoptosis in tested cancer cells, without significantly affecting p53, Bcl-2 as well as Bax proteins expression. Apoptosis induced by Inostamycin is also related to the function of this compound as an inhibitor of caspase-3 [27]. According to the definition, "apoptosis is a physiological process of cell death, the function of which is to control the population of cells. Some of the downstream elements of the pathways, which regulate this process, are conserved from nematodes to mammals [28]".

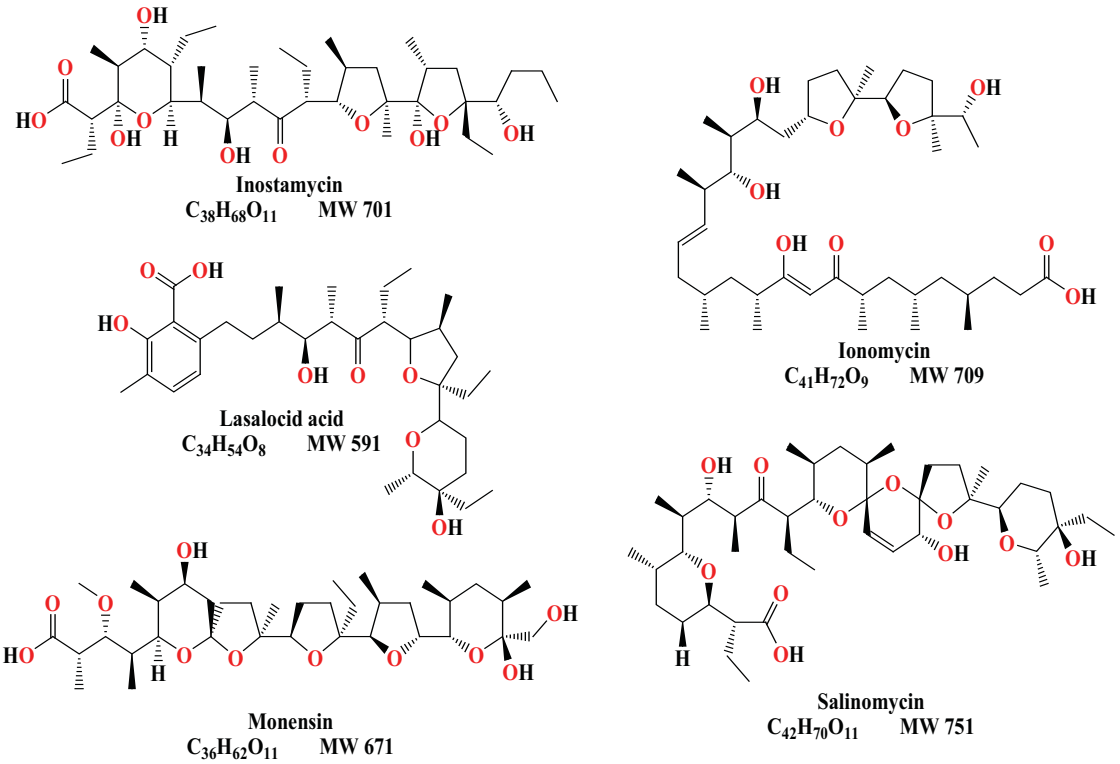

Fig. (1). Structures of polyether ionophores with proven anti-tumor activity.

In vitro tests carried out in 2001 showed that Inostamycin is anti-tumor active against five oral squamous carcinoma cells (SCC), when used at concentrations of 62.5-125 ng/ml. Additionally, flow cytometric analysis has demonstrated that this substance induces an extension in G1/G0 cells, which clearly shows that Inostamycin can be a very useful compound in the treatment of oral SCC [29].

The same research group has proven that Inostamycin is an effective compound in the fight against head and neck SCC by reducing their motility and forcing them to apoptosis. The same compound has been shown "to abrogate the stimulatory effect of vascular endothelial growth factor (VEGF) on growth and migration activities of endothelial cells by targeting extracellular signal-regulated kinase-cyclin D1 as well as p38 pathways, respectively [30]".

The synergistic effect of Inostamycin in combination with anti-cancer drugs has been confirmed. Firstly, Inostamycin has been shown to be a chemo-sensitizer of Paclitaxel and to enhance the ability of this drug to effectively induce apoptosis process in Ms-1 cell line. The use of Inostamycin significantly reduced the amount of Paclitaxel necessary to effectively induce Ms-1 cell death. What is interesting, this specific effect is observed only for Paclitaxel. It has been presented that Inostamycin did not enhance the cytotoxicity effect of other anti-tumor compounds in tests conducted, including Adriamycin, Camptothecin, cisplatin, Etoposide, Methotrexate and Vinblastine, against Ms-1 cell line [31]. Secondly, recent studies have indicated that Inostamycin can be a chemo-sensitizer of other anti-tumor drug, e.g. tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). "TRAIL has been considered as a possible therapeutic agent for cancer treatment, because of its selective cytotoxicity against various cancer cells, simultaneously without a detrimental effect on normal cells. Unfortunately, it has been reported that the potential application of TRAIL is limited, because many cancer cells have been found to be resistant to TRAIL action". However, the synergistic effect of use of Inostamycin in combination with TRAIL against HCT116 colon cancer cell line has been observed. In addition, "Inostamycin increased the expression of DR5 on the cell surface and therefore Inostamycin-increased cell surface expression of DR5 could have contributed to the enhancement of TRAIL-induced apoptosis [32]".

Anti-Tumor Activity of Ionomycin

Ionomycin is a compound having a very high affinity to calcium Ca^{2+} cations [33]. The first information about the anti-tumor activity of Ionomycin appeared in 1996, when this ionophore was found *in vitro* effective towards human promyelocytic leukaemia HL-60 cells. This activity is associated with increasing the amount of Ca^{2+} cation and thus intracellular pH inside the cancer cells [34].

In subsequent years Ionomycin has been presented as *in vitro* and *in vivo* active compound against human bladder HT1376 cancer cell line. The *in vitro* growth rate of HT1376 cells was suppressed by Ionomycin in a dose- and time-dependent manner. Additionally, characteristic apoptotic DNA degradation was observed in HT1376 cells. Ionomycin treatment induced a marked reduction in the ratios of Bcl-2 to Bax mRNA and protein in HT1376 cells. Intra-tumoral injection of Ionomycin into subcutaneous HT1376 tumors reduced the tumorigenicity in nude mice. Moreover, *in vivo* growth-inhibitory effects of Ionomycin were significantly enhanced by pre-treatment with commonly used cytostatic drug – cisplatin [35].

"Functional activation of β-catenin/Tcf signaling plays an important role in the early events in colorectal carcinogenesis. What is important, it has been proved that the association of β-catenin and Tcf-4 is disrupted as well as the amount of β-catenin product in the nucleus is decreased by Ionomycin in a concentration-dependent manner in colon cancer cell line. Furthermore, the inhibitory mechanism of Ionomycin activity has been related to the decreased nuclear β-catenin products and to the suppressed binding of Tcf complexes to consensus DNA [36]". Slightly earlier it has been also shown that Ionomycin-induced calpain activation facilitates the reduction in the level of Bcl-2 proteins, which in turn leads to apoptosis of cancer cells tested [37].

In several publications a synergistic effect of the use of Ionomycin with other biologically active substances has been demonstrated. In 2001 the synergistic anti-tumor effect of Ionomycin in combination with cisplatin against renal cell carcinoma (ACHN) was presented *in vitro* and *in vivo*. "The *in vitro* growth rate of ACHN cell line was more suppressed, while DNA ladder and fragmentation were more obvious, when the cells were incubated by Ionomycin and cisplatin in combination than by either of them alone, both in the *in vitro* and *in vivo* tests". Moreover, Ionomycin treatment was evidenced to increase the expression level of Bax protein [38]. In the same year it was documented that "adoptive transfer of tumor-sensitized lymphocytes activated *in vitro* with bryostatin-1 and Ionomycin, and expanded in culture, can induce regression of small established tumors. Adoptive transfer of bryostatin-1/Ionomycin-activated cells, with or without long-term expansion, induced regression of early and late stage of mouse breast 4T07 cancer cell line dependent on $CD8^+$ but not $CD4^+$T cells [39]".

Recently, it has been shown that Ionomycin strongly inhibits human acute T lymphocyte leukemia progress in combination with phorbol-12,13-dibutyrate (PDBu) through the inhibition of ERK1/2 signaling, activation of caspase-3 as well as the attenuation of TGF-β mediated by the [Ca^{2+}] and pH enhancement [40]. Furthermore, it has been proved that phorbol myristate acetate (PMA) in the presence of Ionomycin causes death of glioblastoma cells [41].

Anti-Tumor Activity of Lasalocid Acid

The anti-cancer activity of Lasalocid acid was proved in 2013. Its activity was tested against five different cancer cell lines, such as human breast adenocarcinoma MCF-7 cell line, human colon adenocarcinoma HT-29 cell line, human lung adenocarcinoma A-549 cell line, human lung microvascular endothelial HLMEC cell line and murine leukemia P-388 cell line. In some cases, the anti-tumor activity is higher and the cytotoxicity to normal murine embryonic fibroblast BALB/3T3 cell line is lower than those of the commonly used cytostatic drug – cisplatin [42-43].

Anti-Tumor Activity of Monensin

At the beginning of the 21st century Monensin was proved to be an effective substance towards human colon SNU-C1 cancer cell line, lymphoma and myeloma NCI-H929 cell lines as well as to induce G1 and/or G2-M phase arrest. Detailed analysis demonstrated that Monensin induces the lymphoma cells proliferation and forces these cells to apoptosis in two different ways: firstly, by inducing cell cycle arrest and, secondly, by losing of mitochondrial trans-membrane potentials [44-46].

Additionally, recent tests have also proved that Monensin is one of the most specific and potent inhibitory agent from among the well-known drugs as well as drug-like substances in screening program of prostate cancer. Monensin and 3 other substances from 4910 compounds tested effectively forces prostate cancer cells to death with high selectivity at low (nanomolar) concentrations. Detailed analysis clearly showed that anti-tumor activity of Monensin against prostate cancer cell lines is connected with reduction of mRNA androgen receptors as well as proteins and improved intracellular oxidative stress, which consequently leads to apoptosis process. Furthermore, the anti-proliferative activity of Monensin has been found to be potentiated by combinational treatment with antiandrogens [47].

In addition, the Monensin potential in *in vitro* cytotoxicity towards immunotoxins and its beneficial role in overcoming MDR of cancer cells has been demonstrated. It has been shown that the anti-tumor effects of immunotoxin SWA11 ricin A-chain is much higher (about 100-fold) in the combination of this substance with Monensin. The kinetic studies have disclosed that "Monensin 2-fold enhances the rate of protein synthesis inhibition and eliminates the lag phase". Furthermore, Monensin has been proved to be a potent proliferation inhibitor of KB parent as well as KB/MDR cells and to markedly reduce Doxorubicin efflux from the interior of KB/MDR cells [48-53]. In view of short half-life and relatively high lipophilicity of Monensin, it is necessary to create suitable systems for efficient use of this compound *in vivo*. For these reasons, very interesting delivery systems, including long-circulating liposomes as well as nanoparticles have been proposed. For example, long-circulating Monensin liposomes have been shown to overcome the human Doxorubicin-resistant breast adenocarcinoma MCF-7/DX cells. These cells were treated with various anti-tumor agents, including Doxorubicin, etoposide and Paclitaxel, alone as well as in combination with long-circulating Monensin liposomes. What is interesting, it has been documented that "long-circulating Monensin liposomes overcome the drug resistance in MCF-7/DX cells to Doxorubicin, etoposide and Paclitaxel by 16.5, 5.6 and 2.8-times, respectively". Moreover, separate use of Doxorubicin and long-circulating Monensin liposomes induce minimal apoptosis (<10%) in these cells, whereas Doxorubicin in the conjunction with long-circulating Monensin liposomes induce apoptosis in approximately 40% MCF-7/DX cells [54-57]. Additionally, it has been presented that Monensin incorporated into liposomes is effective *in vitro* against different human cancer cell lines (malignant mesothelioma H-MESO-1, colorectal carcinoma LS174T as well as human glioblastomas MG-1, U87 and U373) and *in vivo* (in mice), when it has been used with specific immunotoxins. The combination of immunotoxin with liposomal Monensin is 5-fold more effective against H-MESO-1 cells, 1000-fold more effective against U373 cells and 2200-fold more effective against U87 cells than immunotoxin and Monensin acid in buffer. Moreover, *in vivo* studies have demonstrated that "liposomal Monensin in combination with immunotoxin substantially prolong survival of about 21% of mice bearing H-MESO-1 cells for more than 160 days of the treatment [58]".

Recent studies have also indicated that "Monensin overcomes TRAIL resistance in glioma cells *via* endoplasmic reticulum stress, DR5 upregulation as well as c-FLIP downregulation. These results suggest that combined treatment of glioma cells with TRAIL and Monensin may offer an effective therapeutic strategy [59]".

DISCOVERY OF THE ANTI-CANCER ACTIVITY OF SALINOMYCIN

Salinomycin (Fig. **1**), isolated from *Streptomyces albus*, is an antibiotic belonging to the group of polyether ionophores. For the first time Salinomycin was isolated in 1974 [60]. Total synthesis of this compound was proposed in 1998 [61] and in 2012 a biosynthetic model for preparation of this compound was also proposed [62]. Basic data about Salinomycin are collected in Table **2**.

Salinomycin shows potent antibiotic activity especially against different Gram-positive bacteria, including *Staphylococcus aureus*, mycobacteria, fungi, *Plasmodium falciparum*, *Eimeria spp.*, parasites, and protozoa, which are responsible for coccidiosis – a disease of poultry. It has found commercial application in veterinary medicine as a coccidiostatic and non-hormonal growth promoting agent [63].

However, Salinomycin has become a subject of international interest since 2009, when Gupta *et al.* declared that this ionophore is about 100-fold more effective towards breast cancer stem cells (CSCs) than widely used drug in the fight against this type of cancer disease – *Taxol* [64]. One of the reasons why cancer diseases are so difficult to cure is the presence of CSCs. These aggressive cells are not numerous, but well hidden in the tumor, which significantly hinder their detection and destruction. What is worse, if not removed during therapy they often cause recurrence of the disease. Moreover, CSCs exhibit very strong radio- and chemoresistance [65].

Table 2. Characterisation and toxicity of Salinomycin.

Name	Salinomycin, Salinomycin Acid
CAS Number	53003-10-4
Molecular weight	751.00 g/mol
Molecular formula	$C_{42}H_{70}O_{11}$
Composition	C 67.17%; H 9.39%; O 23.43%
Melting point	112.5–113.5ºC
Appearance	At room temperature crystalline cream solid
Toxicity in mice:	
LD_{50} oral administration	50 mg/kg
LD_{50} intravenously	18 mg/kg

Gupta *et al.* have performed tests on about 16 000 biologically active substances, of which only 32 were destroying CSCs studied and the most effective proved to be Salinomycin. The use of Salinomycin in mice resulted in inhibiting mammary tumor growth *in vivo* as well as inducing enhanced epithelial differentiation of these tumor cells. Furthermore, "Salinomycin treatment resulted in the loss of expression of breast CSC genes previously identified by analyses of breast tissues isolated directly from patients [64]". Since then, very extensive research works have been undertaken to explain the extremely effective anti-tumor properties of this ionophore and up to now more than 100 publications describing the remarkable anti-tumor properties of Salinomycin have been published.

IN VITRO STUDIES OF ANTI-CANCER ACTIVITY OF SALINOMYCIN

Anti-Cancer *in vitro* Activity of Salinomycin Against Cancer Cells

In 2010 it has been proved that Salinomycin overcomes programmed cell death (apoptosis) of dangerous tumor cells, such as leukemia cells. The studies relied on collecting blood samples from patients with leukemia and isolating lymphocytes. The cells were treated with various doses of this substance. It was found that the application of the highest dose of this ionophore resulted in apoptosis of almost all leukemic cells, simultaneously with no damage to the normal cells of the body [63].

Moreover, Salinomycin causes concentration- and time-dependent reduction in viability of human lung A-549 and LNM35 cancer cell lines. It should be noted that metastasis caused by lung cancer is a major cause of death in this type of disease. In this connection, it has been demonstrated that Salinomycin induces a time- as well as concentration-dependent inhibition of cancer cells migration and their invasion [66].

Additionally, Salinomycin inhibits the growth and migration of chemoresistant prostate cancer cells, which is a result of accumulation of reactive oxygen species (ROS). This leads to the depolarization of the mitochondrial membrane and cell apoptosis. It has been documented that "Salinomycin decreases viability of the androgen-dependent LNCaP as well as androgen-independent PC-3 and DU-145 prostate cancer cell lines in a time- and dose-dependent manner [67]". "PC-3 cells have high metastatic potential

in comparison to DU-145 cells, which have a moderate metastatic potential, and to LNCaP cells, which have low metastatic potential. The non-malignant RWPE-1 epithelial prostate cell line is resistant to the drug-induced lethality at a lower dose of Salinomycin [68]". "Salinomycin is the most effective in inhibiting VcaP cells, whereas non-malignant prostate epithelial RWPE-1, EP156T as well as PrEC cells are non-responsive [69]". Moreover, Salinomycin efficiently inhibits proliferation and invasion of nasopharyngeal CNE-1, CNE-2 and CNE-2/DDP cancer cells [70].

In 2013 Salinomycin was proved to be able to destroy human breast MCF-7 as well as T47D cells and triple negative MDA-MB-231 human breast cancer cells. These tests have shown that "Salinomycin is able to induce growth inhibition, permanent cell cycle arrest, apoptosis and senescence of different breast cancer cells [71]". T47D cells are the most sensitive breast cancer cell line among all cell lines tested [72]. The influence of Salinomycin on growth as well as migration in pancreatic and human hepatocellular HepG2, SMMC-7721 and BEL-7402 carcinoma cell lines has been also studied [73]. Moreover, "Salinomycin suppresses late stage of hepatocellular carcinoma cell autophagy, leading to impaired recycling as well as accumulation of dysfunctional mitochondria with increased of ROS-production [74]".

It has been also proved that Salinomycin affects cell cycle progression in ovarian OVCAR-8 and OV2008 cancer cell lines as well as two MDR ovarian NCI/ADR-RES and DXR cancer cell lines, which are derived from parental cells. OVCAR-8 cells show sensitivity to several anti-cancer drugs, but NCI/ADR-RES and DXR exhibit resistance to the action of several drugs [75, 76]. Furthermore, Salinomycin induces both apoptosis and autophagy in U2OS and MG-63 osteoblastoma cells [77].

Finally, "Salinomycin is an effective agent against cholangiocarcinoma cells and might be a potential candidate for the treatment of these cells in future. Cell cycle analyses reveal G2-phase accumulation of human cholangiocarcinoma cells after treatment with Salinomycin. Apoptosis is induced in two of three cancer cell lines tested [78]". The third cell line reveals decreased proliferation as well as migration of cholangiocarcinoma cells [78].

Anti-Cancer *in vitro* Activity of Salinomycin Against Cancer Stem Cells (CSCs)

It has been shown that Salinomycin is capable of inducing apoptosis of human tumor cells exhibiting MDR, for example leukemic CSCs exhibiting resistance by expression of ATP-binding cassette transporters (ABC) [79-80]. ABC transporters belong to a family of transmembrane proteins, which are responsible for removing various anti-cancer drugs from the cytosol of cells. This process leads to the development of MDR in these cells, which is a major obstruction in the fight against different neoplastic diseases [81-82]. Besides, Salinomycin has been found to inhibit Wnt signaling pathway and induce tumor cell apoptosis in chronic lymphocytic leukemia [83].

Furthermore, the ability of Salinomycin to reduce colorectal CSCs and its appreciable activity against human colon cancer cells has been observed. What is interesting, these cancer cells are more sensitive to the effects of Salinomycin than to oxaliplatin, the anti-tumor drug, which is commonly used in anti-cancer chemotherapy of colorectal cancer [84].

In 2011 and 2012 six other publications on the activity of Salinomycin against CSCs appeared. The *in vitro* tests have shown that Salinomycin demonstrates potent anti-tumor activity against human lung adenocarcinoma [85], breast [86-87], gastric [88], head and neck squamous [89] as well as osteosarcoma [90] CSCs. Furthermore, in 2013 it was shown that Salinomycin induces apoptosis as well as inhibits Wnt signaling pathway, proliferation, migration, invasiveness and tumorigenicity of human endometrial CSCs [91].

Sensitizing effect of Salinomycin in Cancer Diseases

Salinomycin can substantially sensitize cancer cells treated with irradiation. This process can lead to DNA damage by two pathways and to p21 protein levels decrease. Finally, these damages, by inhibiting CSCs division, support anti-apoptic effect in tumor cells [92-94].

Additionally, sensitizing effect of Salinomycin in combination with different anti-tumor agents has been also demonstrated [95]. Salinomycin is a stronger sensitizer than the well-known P-gp inhibitor *Verapamil* [96]. The CSCs resistant to the treatment of *Taxol*, 5-fluorouracil or cisplatin are liable to the action of Salinomycin [88, 93, 95-96]. Furthermore, Salinomycin sensitizes cancer cell treated with colchicines, *Docetaxel* as well as vinblastine [88, 96]. Strong synergistic anti-tumor effect of Salinomycin in combination with gemcitabine against human pancreatic cancer has been demonstrated. In this case, Salinomycin inhibits the growth of CSCs while gemcitabine effectively dampens the viability of these cancer cells [97]. Also Salinomycin induces the process of apoptosis in cisplatin-resistant colorectal and cisplatin-resistant ovarian cancer cells. This happens by accumulation of ROS as well as inhibition of cell signaling molecules, such as Akt or NF-KB [98-99].

In addition, it has been proved that Salinomycin has the potential to control the proliferation of imatinib-resistant murine gastrointestinal stromal CSCs and increases their sensitivity to imatinib [100].

In 2014 a combination of Salinomycin and 5-fluorouracil was shown to result in a synergistic anti-tumor effect against liver cancer cells. "Salinomycin reverses the 5-fluorouracil-induced increase in $CD133^+$ $EPCAM^+$ cells, epithelial-mesenchymal transition as well as activation of the Wnt/ β-catenin signaling pathway [101]". In the same year, a combination of Salinomycin and TRAIL was indicated as successful to overcome TRAIL-resistance in glioma cells as well as suggested for treatment of these types of cancers [102].

IN VIVO STUDIES OF ANTI-CANCER ACTIVITY OF SALINOMYCIN

For over 30 years Salinomycin was commercially used only in veterinary medicine as a feed additive and up to 2009 it had not aroused particular interest among scientists and doctors. The reason was the substantial difference in the toxicity of this ionophore, observed in the *in vitro* and *in vivo* tests. What is interesting, this toxicity to a large extent depends on the species tested. Salinomycin was less toxic to livestock and poultry, but more harmful to alpaca, cats, dogs, horses and pigs [103-106]. For this reason, no attempt had been made to use Salinomycin in humans and, simultaneously, the exact effect of this ionophore on the human body has not been identified [103]. Literature only provided a description of the case of a 35-year-old man, who accidentally swallowed about 1mg/kg of Salinomycin, which resulted in the appearance of acute nausea, photophobia, weakness in the limbs, high blood pressure, elevated of creatine kinase level as well as muscle pain [107]. The breakthrough in the perception of Salinomycin as a potential anti-cancer drug as well as detailed studies on the impact of this ionophore on the human body occurred after the previously mentioned discovery of high anti-cancer activity of Salinomycin, documented by Gupta *et al.* [64].

Just after three years, in 2012, Salinomycin was approved for testing on humans. The tests were made on a small group of patients with invasive carcinoma of the head, neck, breast and ovary in the screening studies. "Patients were administered 200-250

μg/kg of Salinomycin intravenously every second day for three weeks. Two cases are described in literature in detail. In both cases the administration of Salinomycin resulted in inhibition of disease progress over an extended period of time. Acute side effects were rare and the serious long-term adverse side effects were not observed [108]".

Because of the promising results of these studies, in 2013 phase I and II clinical trials with VS-507 (registered by Verastem Inc., Cambridge, MA, USA the name of Salinomycin) were performed in patients with triple-negative breast cancer disease [108].

Moreover, *in vivo* activity of Salinomycin in several tests carried out in mice, has been also presented. For example, it has been demonstrated that Salinomycin inhibits proliferation as well as induces apoptosis process of human hepatocellular carcinoma cells *in vivo* and the potential mechanism of this action is inhibition of Wnt/*β*-catenin signaling pathway *via* increased intracellular Ca^{2+} levels [73]. Moreover, in nude mice the anti-tumor effect of Salinomycin against nasopharyngeal carcinoma cells has been associated with the downregulation of *β*-catenin expression. These studies have proved that Salinomycin effectively inhibits tumor growth *in vivo*, probably via the inhibition of Wnt/*β*-catenin signaling pathway, suggesting that Salinomycin can be potential candidate for the chemotherapy of nasopharyngeal carcinoma cells [70]. Furthermore, growth inhibitory effects of Salinomycin in the ovarian A2780, A2780-cp, C13, OV2008, OVCAR3 and SKOV3 cancer cell lines, which are associated with the p38 MAPK activation, have been observed in the *in vivo* tests [109]. Finally, *in vivo* activity of Salinomycin against breast [64], endometrial [91] and osteosarcoma [90] cancer cells by targeting its tumor stem cells has been proven.

All these results indicate that in the near future Salinomycin can become a widely used agent not only in veterinary medicine, but also in medicine as an anti-tumor agent in the fight against different cancer diseases. Anti-tumor activities of Salinomycin against different cancer cells are summary collected in Table **3**.

MECHANISMS OF ANTI-TUMOR ACTIVITY OF SALINOMYCIN

The reason why Salinomycin exhibits so numerous pharmacological as well as biological effects is the ability of this ionophore to disrupt an intracellular ion balance, which consequently leads to apoptosis of the cell. Intracellular concentration of potassium cations is higher than sodium cations and extracellular concentrations are reversed. The cation concentration gradients are essential for normal cell functioning [110]. Salinomycin can readily penetrate biological membranes thanks to their lipophilic properties and, simultaneously, effectively interfere with the intracellular ionic balance, leading to the destruction of cancer cells.

The exact mechanism of anti-tumor action of Salinomycin is not yet understood, but it might be related with the ability of this ionophore to lower intracellular pH, which results in subsequent inhibition of DNA synthesis [9]. Additionally, it has been shown that Salinomycin, demonstrating a strong affinity especially to potassium cations, promotes the outflow of these ions from the mitochondria as well as cytoplasm. What is important, it has been proved that a decrease in potassium concentration is necessary for apoptosis of *i.e.* human lymphoma and human lung cancer cells [79]. This suggests that Salinomycin is involved in exhausting the cytoplasmic as well as mitochondrial potassium concentration and/or interferes with the potassium membrane potential. Excessive potassium channels expressivity, which is noted in many human cancer cells, plays a very important role in the cell cycle progression, proliferation and apoptosis of tumor cells [64]. Moreover, it has been noted that "Salinomycin induces cell death with autophagy through activation of endoplasmic reticulum stress in human cancer cells [111]".

Induction of apoptosis by the commonly used anti-cancer drugs depends on the p53 protein expression. What is interesting, Salinomycin causes programmed cell death irrespective of the level of these proteins. It has been demonstrated that Salinomycin is involved in the activation of a separate apoptotic pathway, which is not assisted by a change in the cell cycle and which is independent of the cancerous p53 protein suppressor, proteasomes as well as caspase activity. Furthermore, Salinomycin has been found capable of inducing apoptosis of different tumor cells characterized by high resistance to other anti-cancer drugs by increased expression of the Bcl-2 protein, P-gp as well as the 26S proteasome [79].

Salinomycin has been documented to induce human lung cancer cell apoptosis through the caspase 3/7-associated cell death pathway. The same studies clearly showed that "Salinomycin induces a marked increase in the expression of the pro-apoptotic protein NAG-1, which leads to the inhibition of lung cancer cell invasion, but not cell survival [66]". Moreover, Salinomycin induces apoptosis in prostate cancer cells by increasing the intracellular level of ROS, which is associated with a reduction in mitochondrial membrane potential, Bax protein translocation to mitochondria, release of cytochrome c into the cytoplasm, activation of caspase-3 as well as cleavage of the key enzyme in many nuclear physiological processes – PARP-1 [67].

Additionally, high anti-tumor activity of Salinomycin against nasopharyngeal carcinoma cells is accompanied by activation of caspase-3 and caspase-9 as well as decreases mitochondrial membrane potential. The Wnt/*β*-catenin signaling pathway has been established to be involved in Salinomycin-induced apoptosis of nasopharyngeal carcinoma cells [70]. Furthermore, Salinomycin activity is found to be "correlated with induction of H3 and H4 histones hyperacetylation as well as upregulation of the level of p21 protein [71]". Besides, Salinomycin inhibits proliferation and decreases proliferation of cell nuclear antygen (PCNA) levels as well as the proportion of subpopulation of $CD133^+$ cells in hepatocellular carcinoma cells [73]. What is interesting, tests performed on different ovarian cancer cell lines clearly proved that "Salinomycin causes cell growth inhibition as well as apoptosis in all three cell lines tested *via* downregulation or inactivation of cell cycle-associated oncogenes, such as Stat3, cyclin D1 and Skp2 [75]". Salinomycin induces both apoptosis as well as autophagy in osteoblastoma cells by activating of AMPK-dependent (AMP-activated protein kinase) autophagy, which is a negative regulator against apoptosis in osteoblastoma cells [77]. It has been also found to be able to overcome drug-resistance and anti-apoptotic ABC transporters ability of human leukaemic CSCs, as a result of the expression of these systems [79-80]. Moreover, the role of Salinomycin as P-gp inhibitor, which supports the flow of anti-cancer drugs from cells, has been documented [112]. Salinomycin has been proved to be effective in strong inhibition of proximal Wnt/*β*-catenin signaling as well as phosphorylation process blocking of the Wnt-LRP6 coreceptor, which leads to its degradation. It is very important, because "Wnt/*β*-catenin signaling pathway plays a significant role in stem cell renewal and, simultaneously, is involved in the pathogenesis of different types of cancer cells. However, abnormal activation of this signaling pathway in normal stem cells might result in their transformation into CSCs [113]". For this reason, compounds like Salinomycin, which affect Wnt/*β*-catenin signaling pathway, are potential agents in the fight against CSCs.

"Salinomycin reduces the proportion of $CD133^+$ glycoproteins in cancer cell lines tested. It also decreases colony-forming ability as well as cell motility and, simultaneously, downregulates the expression of vimentin as well as induces the E-cadherin in these

Table 3. Anti-tumor activity of Salinomycin against different cancer cells.

Cancer Type	Cell Line	Cancer Cells IC_{50} and/or % of Apoptosis/Viability of Cancer Cells	References
Breast cancer	MCF-7 MDA-MB-231 T47D	The IC_{50} at 24 h is approximately 40 μM for the MCF-7 and T47D, while the same concentration gives only about 35% inhibition of proliferation in the MDA-MB-231 cells. Following 48 h treatment, IC_{50} is 15 μM for the MCF-7 and T47D, and about 35 μM for the MDA-MB-231 cells. On the other hand, T47D is the most sensitive cell line among the breast cancer cell lines tested	[71] [72] [95]
Chronic lymphocytic leukemia	HEK293	Incubation of the malignant lymphocytes with Salinomycin induces apoptosis within 48 h, with a mean IC_{50} of 230 nM. Under the same conditions, Salinomycin fails to induce apoptosis in peripheral blood mononuclear cells at 100-fold higher concentrations	[83]
Cholangiocarcinoma	EGI-1 Mz-ChA-1 TFK-1	After 24 h treatment of Mz-ChA-1, TFK-1 and EGI-1 cancer cells with 5 μM of Salinomycin, about 65%, 86% and only 10% of these cells undergo apoptosis process, respectively. After increasing the dose of Salinomycin to 10 μM, significant changes are not observed in Mz-ChA-1 and TFK-1 cells, but number of EGI-1 cells that compliant an apoptosis process increases to about 18%	[78]
Colorectal cancer	RKO SW480 SW620	At the 10 μM concentration of Salinomycin, the cell viability decreases by 95% in comparison to the solvent control in all colorectal cancer cell lines. The decrease in viability is highly significant, post-hoc testing reveals that the difference between solvent control and Salinomycin treatment is significant for all concentrations >1 μM after 72 h	[72]
Glioblastoma	T98G U251 U87MG	Dose-response experiments carried out on cells exposed to increasing concentrations of Salinomycin (from 0.6 to 10.0 μM) show a decrease of viable cells, marked in U87MG cells as well as moderate in T98G and U251 cells. Maximal effects are observed at a Salinomycin concentration corresponding to 5-10 μM. On the other hand, the addition of TRAIL, even at low concentrations, *i.e.* at 10 ng/ml, to 1.2 μM of Salinomycin causes a marked reduction of viable cells	[102]
Hepatocellular cancer	BEL-7402 HepG2 Huh7 LM3 SMMC-7721	The IC_{50} value is about 14.7 μM, 18.6 μM and 17.1 μM for HepG2, SMMC-7721 as well as BEL-7402 cell lines after 24 h treatment of Salinomycin, respectively. These values decrease to about 10.2 μM, 15.3 μM and 13.8 μM after 48 h treatment of Salinomycin for these cells. On the other hand, treatment of hepatocellular cells in the combiantion of Salinomycin with 5-fluorouracil for 48 h results in a decrease in cell viability, which is greater than either Salinomycin and 5-fluorouracil alone	[73] [74] [95] [101]
Lung cancer	A-549 LNM35	The IC_{50} concentrations at 24 h are in the range of 5 to 10 μM of Salinomycin for both cell lines. After 48 h treatment, the IC_{50} concentrations decreased to the range of 1.5 to 2.5 μM of Salinomycin with 90% inhibition of cell viability in both cells at the concentration of 50 μM	[66]
Nasopharyngeal cancer	CNE-1 CNE-2 CNE-2/DDP	Salinomycin at a concentration of 8 μM inhibits about 53.0%, 48.0% and 42.3% of the survival of CNE-1, CNE-2 and CNE-2/DDP cells after 48 h treatment, respectively. When CNE-1 cells are exposed for 72 h to 16 μM of Salinomycin, the inhibition ratio is up to 70%	[70]
Osteoblastoma	HEK-293T MG-63 U2OS	No significantly cell viability loss is observed with treatment of Salinomycin at concentration lower than 1 μM. On the other hand, 48 h treatment with 10 μM of Salinomycin reduces viability of U2OS, MG-63 and HEK-293T cells to about 50%, 53% and 52%, respectively	[77]
Ovarian cancer	OVCAR-8 OV2008	The viability of OVCAR-8 cell line is reduced to about 53% and 45% after 72 h treatment of 4 μM as well as 8 μM of Salinomycin, respectively. On the other hand, IC_{50} (95% confidence interval) of Salinomycin on OV2008 cell line for 24, 48 and 72 h treatment is 7.44, 4.78 as well as 3.20 μM, respectively	[75] [76]
Ovarian epithelial cancer	A2780	Salinomycin induces a moderate pro-apoptotic effect on A2780 cells, particularly evident at days 2-3 of culture and at Salinomycin dosages of 1-5 μM	[112]
Prostate cancer	DU-145 LNCaP PC-3 VCaP	At 1.33 μM concentration of Salinomycin LNCaP cells manifest a stronger inhibition – viability reduced to about 55%, 38%, 35% and 22% (after 12, 24, 36 and 48 h, respectively), whereas >50% of PC-3 and DU-145 cells remain viable after 36 h treatment of Salinomycin and even at 48 h >30% of PC-3 cells as well as >50% of DU-145 cells remain viable. On the other hand, Salinomycin is the most effective in inhibiting VcaP cells (EC_{50} = 380 nM), whereas non-malignant prostate epithelial RWPE-1, EP156T as well as PrEC cells are non-responsive (EC_{50} > 10 μM)	[67] [69]
Cancer Type	**Cell Line**	**Radio- and/or Drug-Resistant Cancer Cells** **IC_{50} and/or % of Apoptosis/Viability of Cancer Cells**	**References**
Burkitt's lymphoma	Namalwa cells	Salinomycin induces apoptosis in about 73% Namalwa cells after adding 5 μM of this ionophore for 24 h. Similar results are obtained using 10 μM of Salinomycin	[79]
Colon adenocarcinoma	LoVo LoVo/DX	The IC_{50} of LoVo cell line for Salinomycin is about 1.5 μM, while the IC_{50} of Doxorubicin-resistant LoVo/DX subline is about 1.0 μM, respectively	[118]
Colorectal cancer	SW620	After treatment with Salinomycin for 48 h, the cell apoptotic rate in cisplatin-resistant SW620 cells (about 38%) is significantly higher than that of SW620 cells (about 17%)	[98]
Ovarian cancer	A2780 DXR NCI/ADR-RES	The viability of DXR and NCI/ADR-RES cell lines is reduced to about 50% after 72 h treatment of 4 μM of Salinomycin. The treatment of 8 μM of Salinomycin causes reduction of viability of DXR and NCI/ADR-RES cell lines to about 43% and 37%, respectively. On the other hand, the cisplatin-resistant A2780 cell growth is decreased by about 55% in 5 μM of Salinomycin	[75] [99]

Table 3. contd….

Cancer Type	Cell Line	Radio- and/or Drug-Resistant Cancer Cells IC_{50} and/or % of Apoptosis/Viability of Cancer Cells	References
Promyelocytic leukemia	HL-60 HL-60/vinc	The IC_{50} of HL-60 cell line for Salinomycin is about 1.2 μM, while the IC_{50} of vinristine-resistant HL-60/vinc subline is about 4.7 μM, respectively	[118]
T-cell leukemia	Molt-4 Jurkat cells $CD4^+$ cells	Salinomycin induces apoptosis in about 50% and 71% Molt-4 cancer cells after adding 5 μM and 10 μM of this ionophore for 24 h, respectively. The same concentrations of Salinomycin induce apoptosis in about 88% and 90% Jurkat cells. Moreover, Salinomycin induces apoptosis in about 82%, 84% and 88% $CD4^+$ cancer cells after adding 0.5 μM, 1.0 μM and 2.0 μM of this ionophore, respectively	[79]
Uterus sarcoma	MES-SA MES-SA/Dx5	5 μM and 10 μM of Salinomycin induces apoptosis process in about 71% and 80% MES-SA/Dx5 cancer cells after 24 h treatment, respectively	[79] [95]
Cancer Type	**Cell Line**	**Cancer Stem Cells or Cancer Stem-Like Cells** **IC_{50} and/or % of Apoptosis/Viability of Cancer Cells**	**References**
Breast cancer	4T1 BT-474 MCF-7-SP MCF-7-NSP MDA-MB-231	No synergistic effect of the combination of 0.5 μM of Salinomycin with different concentrations of trastuzumab is observed against triple negative MDA-MB-231 breast cancer cell line. Treatment of MCF-7 as well as BT-474 mammospheres with a combination of Salinomycin and trastuzumab demonstrates more cell death in comparison with single treatments. On the other hand, for the 48 h test, when the concentration of Salinomycin is increased to 0.5 μM, the inhibitory rate of MCF-7-SP cells is significantly higher than that of MCF-7-NSP cells. Moreover, Salinomycin treatment leads to an about 2-fold reduction for 4T1 cells relative to control DMSO treatment	[64] [86] [87]
Colorectal cancer	HT29 SW480	Salinomycin exhibits significant activity against HT29 cells with IC_{50} value about 8 μM. HT29 cells are more sensitive to Salinomycin than oxaliplatin, a commonly used anti-colorectal cancer chemotherapeutic agent (>10-fold increase in IC_{50}). Comparable results are observed with cells of the SW480 human colorectal cancer line (IC_{50} about 10 μM)	[84]
Endometrial cancer	Hec1-SP Hec1-NSP	The proliferation and viability of Hec1-NSP cells is inhibited at all concentrations tested (0.1, 1.0 and 5.0 μM) for 72 h. The viability of Hec1-SP cells is suppressed by treatment with concentrations of Salinomycin more than 1.0 μM. Chromatin fragmentation is observed in Hec1-SP cells treated with 1.0 μM of Salinomycin	[91]
Gastric cancer	BGC-823 NCI-N87 SGC-7901 SNU-1	The IC_{50} of NCI-N87 and SNU-1 for Salinomycin is about 3.35 μM and 3.21 μM, while, it is about 13.83 μM and 10.63 μM in SGC-7901 and BGC-823 cells, respectively. It means that sensitivity of Salinomycin for $ALDH^{high}$ cell lines NCI-N87 and SNU-1 is about 4.13 or 4.31-fold and 3.17 or 3.31-fold higher than that for $ALDH^{low}$ cell lines SGC-7901 and BGC-823, respectively	[88]
Head and neck squamous cancer	JLO-1 UMSCC-10B	JLO-1 cells experience significant toxicity towards Salinomycin in a dose dependent manner, with an IC_{50} close to 2 μM, while UMSCC-10B cells exhibit less sensitivity to Salinomycin treatment, with an IC_{50} beyond 8 μM. At 2 μM, there is a substantial increase in the proportion of CSCs undergoing apoptosis compared to the control	[89]
Murine gastrointestinal stromal cancer	D2211B	4 μM of Salinomycin inhibits the growth of D2211B cells without causing cell death. Submaximal doses of Salinomycin (0.25 or 1.00 μM) combined with imatinib caused a significantly greater inhibition of cell proliferation than Salinomycin alone	[100]
Myeloblastic and promyeloblastic leukemia	KG-1 KG-1a	After 24 h treatment, Salinomycin induces apoptosis in about 65% and 91% KG-1 as well as 52% and 77% KG-1a cells during use of 5 μM and 10 μM of this ionophore, respectively	[80]
Osteosarcoma	MG63 SAOS2 U2OS	The IC_{50} of MG63 and U2OS ostesarcoma CSCs for Salinomycin is about 8 μM and 5 μM, respectively, while for MG63 as well as U2OS parental cell lines is about 6 μM and 4 μM, respectively	[90]
Pancreatic cancer	AsPC-1 SW1990	Combined treatment of 5 μM of Salinomycin and 5 μg/ml of gemcitabine eradicate both $CD133^+$ and $CD133^-$ cells more efficiently than either these compounds alone in cancer cell lines tested	[97]
Cancer Type	**Tested on**	***In vivo* Activity Against Cancer Cells** **Applied dose/effects of the use**	**References**
Breast cancer	Mice	Animals were administered either ethanol (vehicle), Salinomycin (5 mg/kg) or Paclitaxel (5 mg/kg) daily by intraperitoneal injection for 5 weeks. While palpable tumors developed in vehicle-treated mice within about 1.5 weeks, Paclitaxel and Salinomycin treatment both delayed palpable tumor formation by about 2 weeks. Subsequent tumor size in Salinomycin-treated mice was reduced relative to tumors in vehicle-treated mice. Four weeks after cancer cell injection, tumors were analyzed for the presence of surviving CSCs with *in vitro* tumorsphere formation assays. Tumors from the Paclitaxel-treated cohort had a 2-fold increase in tumorsphere-forming cells relative to either Salinomycin- or vehicle-treated cohorts. Tumors from Salinomycin-treated mice had increased necrosis and apoptosis compared to comparably sized tumors from vehicle-treated mice. Viable cancer cells in tumors from Salinomycin-treated mice were mostly restricted to the periphery of the tumor mass	[64]
Endometrial cancer	Mice	Animal experiments were performed on 5-week-old nude mice and the tests were conducted for 6 months. The size of the tumor was measured every week and the weight of each mouse tested was recorded once a week. After palpable tumors (1 cm^3) had developed, 0.26 μM of Salinomycin or DMSO was injected into the tumors. Tumors in DMSO-treated mice continued to grow, but tumors in Salinomycin-treated mice stopped growing. Tumor size in Salinomycin-treated mice was reduced compared with tumors in DMSO-treated mice	[91]

Table 3. contd….

Cancer Type	Tested on	*In vivo* Activity Against Cancer Cells Applied dose/effects of the use	References
Hepatocellular cancer	Mice	Animal experiments were performed on 6-week-old male nude mice. Saline, 5-fluorouracil (8 mg/kg), Salinomycin (4 mg/kg) as well as combination of 5-fluorouracil (8 mg/kg) and Salinomycin (4 mg/kg) were used in these tests. Observations of the anti-tumor effects of the substances tested were carried out for 4 weeks. The subcutaneous tumor volume was reduced in the combination therapy group compared to other three groups. The anti-tumor effect was observed by measuring tumor diameter in the animals tested twice per week. The relative tumor proliferation rate was slower and the tumor-growth inhibition rate was greater in the combiantion therapy than in the other three groups	[101]
Nasopharyngeal cancer	Mice	Animal experiments were performed on 4-week-old male nude mice. After tumors grew to about 4 mm, mice were randomly divided into a control group and a Salinomycin group (10 mg/kg). Drugs were given by intraperitoneal injection daily for 2 weeks. The animal weight and tumor volumes were monitored every other day. Assessment of tumor volume showed that the Salinomycin-treated group showed delayed tumor growth compared to the control group. The tumors were smaller in the experimental group than in the control group	[70]
Osteosarcoma	Mice	Animal experiments were performed on 6-8-week-old mice. Tests were conducted for 33 days and tumor volumes were monitored at day 15 and then every 2 days. The mice were treated with 5 mg/kg of Salinomycin or normal saline solution intraperitoneally every day. Salinomycin reduced tumor growth and acted synergistically with chemo-drug adriamycin. Salinomycin treatment had a minimal effect on the body weight of nude mice in comparison with treatment using normal saline solution	[90]
Ovarian cancer	Mice	Animal experiments were performed on 6-week-old female mice. The two experimental groups were administered Salinomycin (5 mg/kg) and 5% ethanol (vehicle), respectively, through intraperitoneal injection every other day for three weeks. The size of the tumor was measured every two days. Compared with the vehicle-treated controls, a significant reduction in the tumor volume was observed in the Salinomycin-treated mice. At the end of the test, the tumor volume of Salinomycin-treated and the control groups, in the C13 tumor model, was 84.2 ± 30.8 as well as 252.5 ± 63.4 mm^3, respectively	[109]
Invasive breast, head, neck and ovary carcinoma	Humans	Patients were administered 200-250 μg/kg of Salinomycin intravenously every second day for three weeks. Two cases are described in literature in detail. The first was a 40-year-old female patient with metastatic (bone and subcutaneous) invasive ductal breast cancer and the second was a 82-year-old female patient with advanced and metastatic (pelvic lymphatic metastasis) squamous cell carcinoma of the vulva. In both cases the administration of Salinomycin resulted in inhibition of cancer disease progress over an extended period of time. Acute side effects were rare and the seriuos long-term adverse side effects were not observed	[108]

cancer cells [84]". Finally, growth inhibitory effects of Salinomycin in the ovarian cancer cell lines, which are connected with the activation of p38 MAPK, have been observed [109]. Different routes of anti-tumor activity of Salinomycin, which are described in this chapter, are presented in Fig. **2**.

ANTI-CANCER ACTIVITY OF SALINOMYCIN DERIVATIVES

A very interesting direction of research is the chemical modification of Salinomycin, which can lead to obtaining various derivatives with significantly lower toxicity and better biological activity than those of the parental ionophore. Until now in the scientific literature the synthesis, structure and biological activity, including anti-tumor activity, of 18 amides, 13 esters (Fig. **3**), 22 *O*-acylated derivatives (Fig. **4**) as well as 2 C-ring-modified Salinomycin sodium salt analogues (Fig. **5**) have been described [114-120]. Anti-cancer activity of Salinomycin amides and esters have been determined in the *in vitro* tests against human leukemia vincistine-sensitive HL-60 and vincristine-resistant HL-60/vinc cells, human colon Doxorubicin-sensitive LoVo and Doxorubicin-resistant LoVo/DX cells as well as normal murine embryonic fibroblasts BALB/3T3. In these tests, Salinomycin derivatives activities have been checked in concentration ranges from 0.1 to 100 mM and as reference Doxorubicin and cisplatin have been chosen [114-118]. The structures of the most anti-tumor active Salinomycin amides and esters are presented in Fig. **3** as well as the anti-cancer activity of the selected derivatives are summarized and collected in Table **4**.

All Salinomycin amides and esters are more or less anti-cancer active in the specified concentration range. Their activities depend on the tested cancer cell line. Salinomycin amides and esters break the MDR of used cancer cells and this process strongly depends on the chemical nature of these derivatives. It has been shown that 4-fluorophenethyl (compound **6**) and dopamine (compound **7**) amides exhibit the highest anti-tumor activity (Fig. **3**) [114-118].

It has been noted that among all ester derivatives of Salinomycin, trifluoroethyl ester (compound **9**) evinces the highest ability to inhibit proliferation process of cancer cell lines tested. Simultaneously, this activity is higher than that of the chemically unmodified Salinomycin as well as Doxorubicin and cisplatin, commonly used anti-tumor agents in cancer chemotherapy. Besides, it has been documented that a given ester moiety to some extent determines the anti-cancer activity of these compounds. Generally, it has been proved that the most active ester derivatives are those, which contain trifluoroethyl ester group (compound **9**) and/or are accompanied by short aliphatic chain (compound **10**) or α - naphtylmethyl (compound **11**) ester substituents (Fig. **3**) [114, 118]. Furthermore, the tests performed on normal murine fibroblasts clearly show that some of Salinomycin amides and esters are characterized by a weaker toxicity against BALB/3T3 cell line, with IC_{50} value > 20 μM. Salinomycin as well as most of its amide and ester derivatives are less toxic than reference compounds used in tests, *i.e.* Doxorubicin and cisplatin [114-118].

The antiproliferative activity tests carried out on *O*-acylated derivatives of Salinomycin sodium salt against two human breast JIMT-1 and MCF-7 cancer cell lines showed that C20-acylated analogues of Salinomycin sodium salt (compound **13**, Fig. **4**)) display IC_{50} values to one fifth lower or similar to that of Salinomycin sodium salt against both cancer cells. Among them,

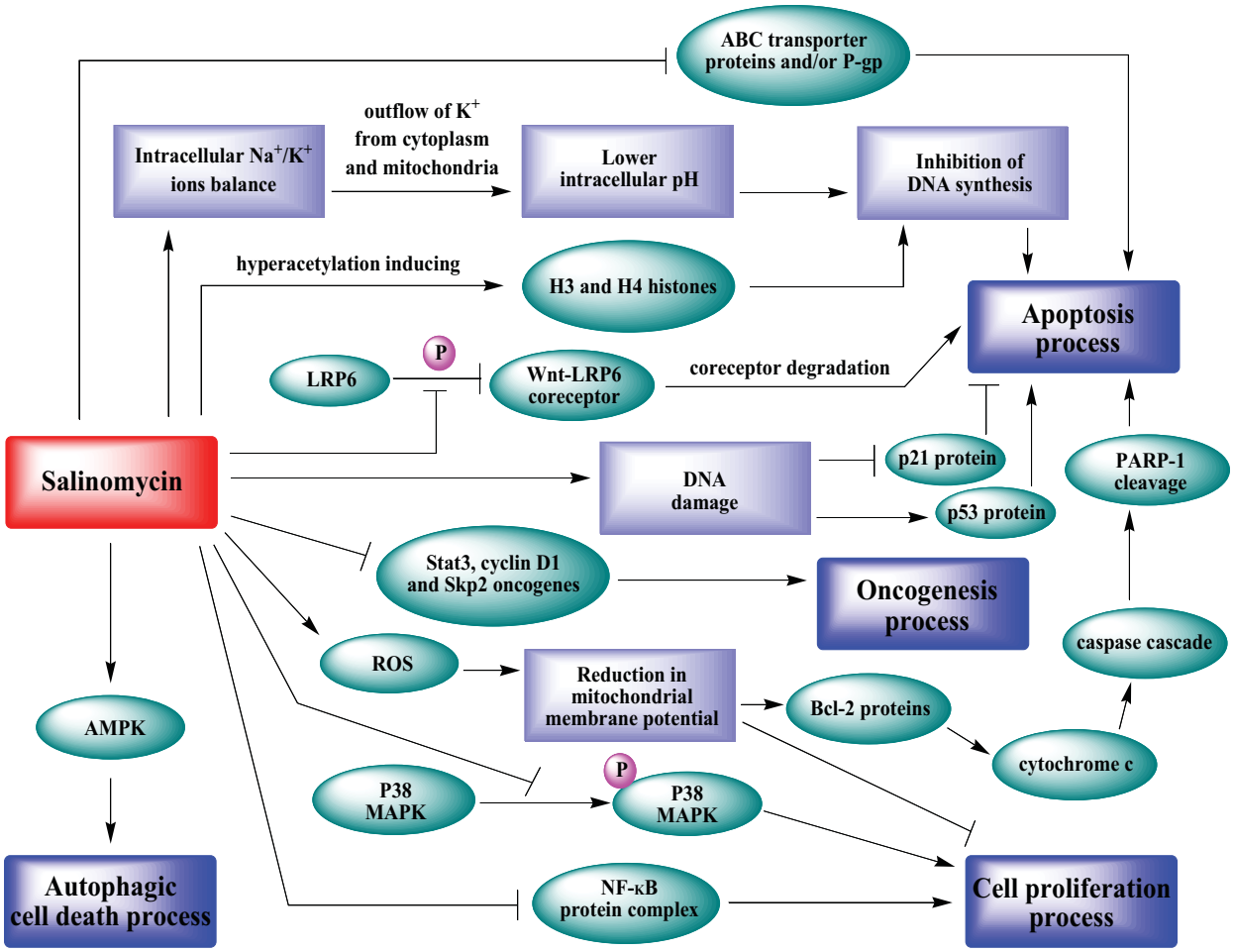

Fig. (2). Mechanisms of anti-tumor activity of Salinomycin. Salinomycin can easily penetrate biological membranes thanks to their lipophilic properties and exhausts cytoplasmic and mitochondrial potassium concentration and/or interferes with the potassium membrane potential, which leads to apoptosis of many cancer cells. Additionally, Salinomycin is capable of inducing apoptosis of different tumor cells by increased expression of P-gp as well as ABC transporter proteins. Furthermore, Salinomycin induces apoptosis of tumor cells by increasing the intracellular level of ROS, which is accompanied by a reduction in mitochondrial membrane potential, Bcl-2 proteins translocation to mitochondria, release of cytochrome c into the cytoplasm, activation of caspase cascade as well as cleavage of PARP-1. Salinomycin induces also apoptosis by activating of AMPK-dependent autophagy as well as by downregulation or inactivation of cell cycle-associated oncogenes. On the other hand, Salinomycin strongly inhibits proximal Wnt/*β*-catenin signaling as well as blocks phosphorylation process of the Wnt-LRP6 coreceptor, which leads to its degradation and, finally, to apoptosis of cancer cells [64, 67, 75, 77, 79, 80, 112, 113].

the most active C20-derivatives are those with the least bulky substituents in each series – ethyl carbonate (compound **14**), ethyl carbamate (compound **16**) and acetate (compound **19**). C9- as well as C28-acylated analogues of Salinomycin sodium salt are much less active against both breast cancer cell lines tested [119]. The structures of the C20-derivatives of Salinomycin sodium salt synthesized are presented in Fig. **4**.

In addition, the synthesis of two C-ring-modified Salinomycin sodium salt analogues, *i.e.* 20-deoxy Salinomycin (compound **31**) and 18,19-dihydro-20-deoxySalinomycin (compound **32**), have been documented. Both these compounds exhibit very similar activities against human breast JIMT-1 and HCC1937 cancer cell lines, but are less active than the parental ionophore. Salinomycin sodium salt is found more efficient than these two analogues "in reducing the proportion of $CD44^{+}/CD24^{-}$ cells, but all compounds target this phenotype with substantially complete selectivity in JIMT-1 cell line at concentrations below the respective IC_{25}. Similar biological profiles of Salinomycin sodium salt and these two analogues suggest that they act through a common mechanism [120]". The structures of C-ring-modified Salinomycin sodium salt derivatives are presented in Fig. **5** as well as the anti-cancer activity of C20-acylated and C-ring-modified analogues of Salinomycin sodium salt against breast cancer cell lines are characterised in Table **5**.

This high anti-tumor activity of Salinomycin derivatives may be connected with the Warburg effect and/or with different mechanisms of ion transport by polyether antibiotics, including Salinomycin**.** "Three different ion transport mechanisms through the biological membranes realized by polyether antibiotics are described in literature: (a) electroneutral transport, when the transmembrane potential is maintained, (b) electrogenic transport, when the transmembrane potential is changed and (c) biomimetic transport implemented by polyether antibiotics with the chemically modified carboxyl group [121]".

"The Warburg effect is observed in the most cancer cells, which predominantly produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria. It has been postulated that this change in metabolism is the fundamental cause of cancer diseases [122]".

Simultaneously, "high anti-cancer activity of Salinomycin derivatives is probably connected with the mechanism of cations transport realized by such compounds. In cancer cells that are

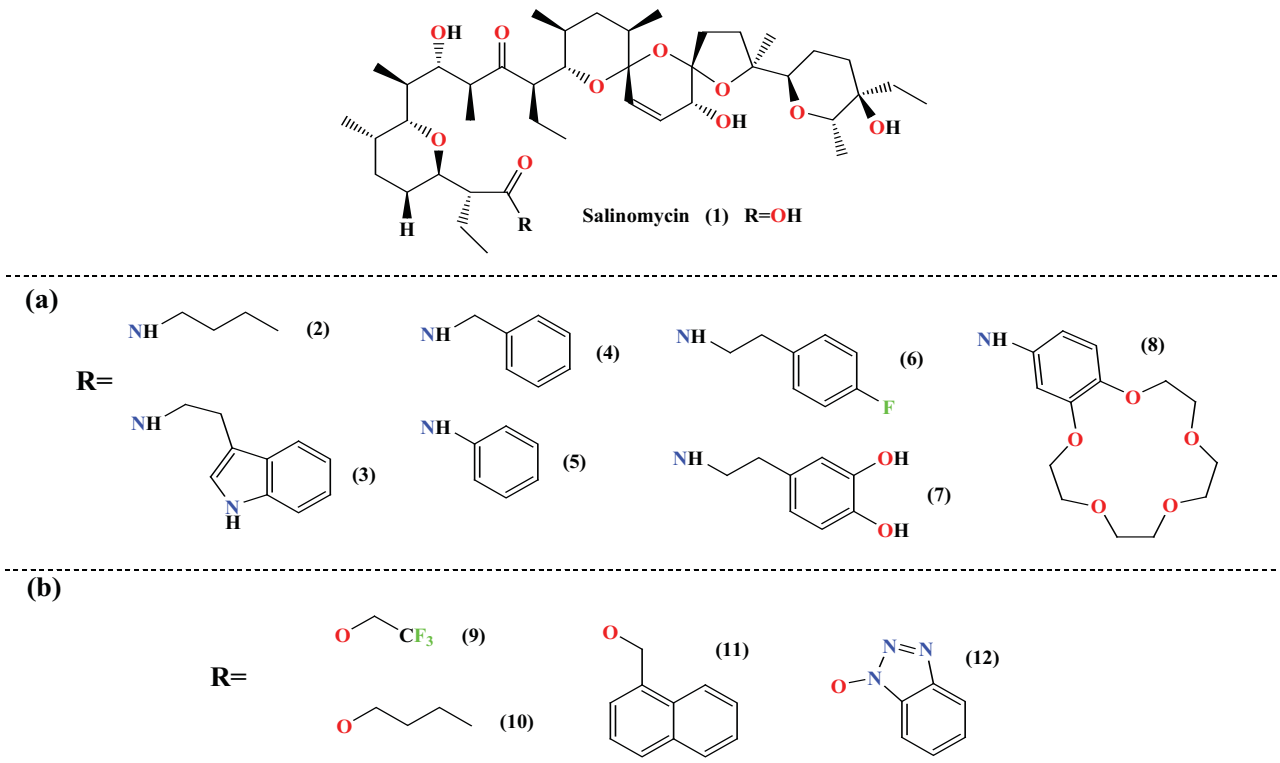

Fig. (3). Structures of the most anti-tumor active (**a**) amides and (**b**) esters of Salinomycin [114-118].

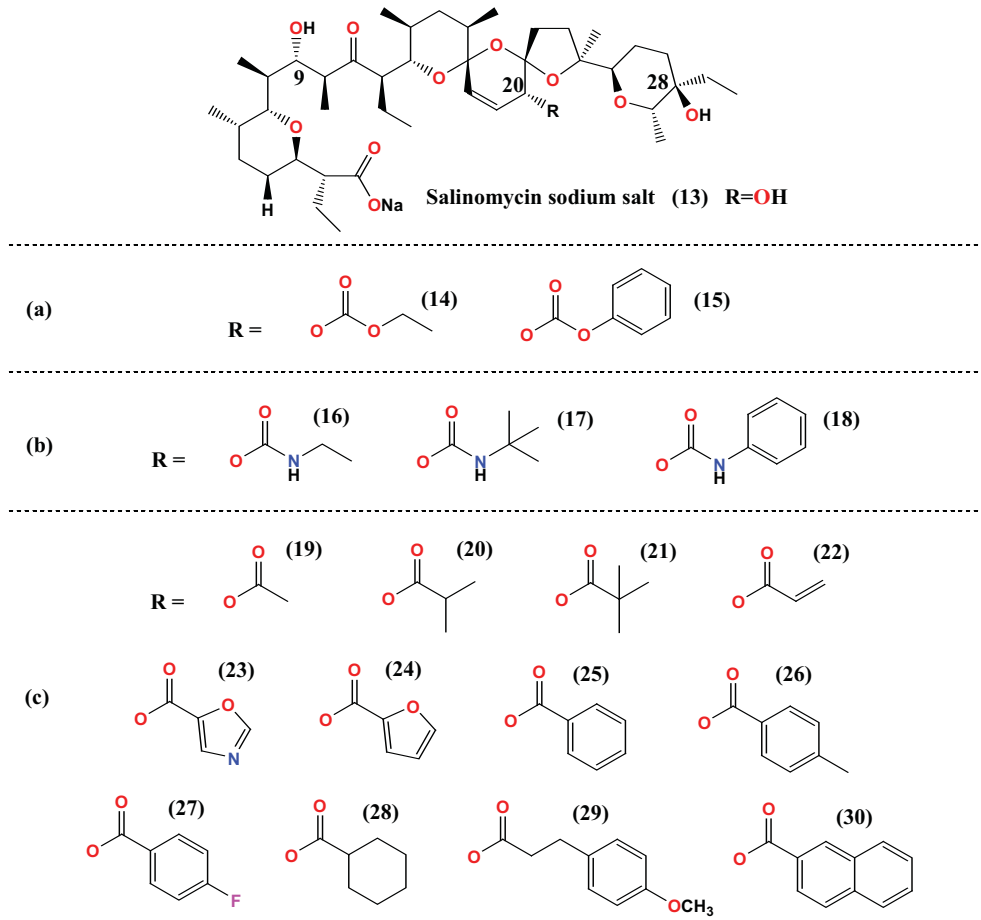

Fig. (4). Structures of (**a**) C20-carbonates, (**b**) C20-carbamates and (**c**) C20-esters of Salinomycin sodium salt [119].

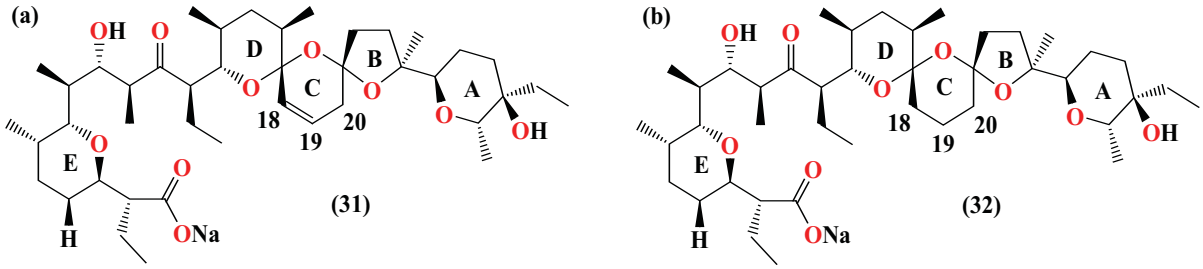

Fig. (5). Structures of C-ring-modified analogues of Salinomycin sodium salt: (**a**) 20-deoxy Salinomycin and (**b**) 18,19-dihydro-20-deoxy Salinomycin [120].

Table 4. Anti-cancer activity of Salinomycin (compound 1) and its the most active amides (compounds 2-8) and esters (compounds 9-12) against various cancer cell lines [114-118].

	IC_{50} ± SD [μM]				
	HL-60[a]	HL-60/vinc[b]	LoVo[c]	LoVo/DX[d]	BALB/3T3 [e]
1	0.44 ± 0.16	3.44 ± 0.32	1.11 ± 0.15	6.23 ± 1.72	28.08 ± 4.63
2	3.88 ± 0.04	5.31 ± 0.68	4.04 ± 0.17	3.26 ± 0.61	8.21 ± 1.14
3	3.52 ± 0.13	4.31 ± 0.52	3.45 ± 0.26	2.78 ± 0.47	9.98 ± 4.71
4	3.63 ± 0.25	6.02 ± 0.72	4.02 ± 0.17	3.31 ± 0.76	7.26 ± 1.02
5	3.79 ± 0.07	4.31 ± 0.53	4.11 ± 0.15	3.21 ± 0.49	7.08 ± 1.40
6	2.26 ± 0.24	6.74 ± 1.15	4.09 ± 0.14	2.34 ± 0.49	45.80 ± 20.94
7	2.77 ± 1.11	6.88 ± 1.08	3.88 ± 0.24	4.26 ± 0.74	5.69 ± 2.17
8	3.08 ± 0.25	6.87 ± 0.27	6.24 ± 1.08	5.65 ± 1.12	25.47 ± 4.24
9	0.47 ± 0.22	3.05 ± 0.38	0.78 ± 0.24	0.80 ± 0.07	23.82 ± 6.49
10	3.58 ± 0.45	4.15 ± 1.50	4.04 ± 0.09	3.99 ± 0.06	24.32 ± 7.27
11	3.73 ± 0.21	9.33 ± 1.47	7.34 ± 0.35	4.70 ± 0.28	35.80 ± 1.66
12	1.84 ± 0.37	5.25 ± 0.55	4.11 ± 0.20	6.61 ± 1.12	31.90 ± 5.33
Doxorubicin	0.04 ± 0.02	1.78 ± 0.33	0.17 ± 0.06	6.45 ± 1.82	0.48 ± 0.33
cispaltin	3.95 ± 3.22	10.53 ± 2.12	5.18 ± 1.06	8.84 ± 0.76	9.47 ± 0.29

[a-b]human leukemia cell line sensitive and resistant to vincristine (HL-60 and HL-60/vinc); [c-d]human colon cancer cell line sensitive and resistant to Doxorubicin (LoVo and LoVo/DX); [e]normal murine embryonic fibroblasts (BALB/3T3).

highly acidic, the most common electroneutral transport cannot be effectively carried out, because the carboxyl group does not undergo deprotonation. Biomimetic transport is then preferred. Moreover, in bacterial cells the electroneutral or electrogenic transport of ions is preferred and hence much lower anti-microbial than anti-tumor activity of the Salinomycin derivatives tested is observed [114-120]".

Table 5. Anti-cancer activity of Salinomycin sodium salt (compound 13), its C20-acylated derivatives (compounds 14-30) and C-ring-modified analogues (compounds 31-32) against breast cancer cell lines [119, 120].

	IC_{50} ± SD [μM]	
	JIMT-1[a]	MCF-7[b]
13	0.52 ± 0.09	0.59 ± 0.08
14	0.09 ± 0.02	0.13 ± 0.01
15	0.20 ± 0.02	0.24 ± 0.02
16	0.26 ± 0.04	0.16 ± 0.02
17	0.38 ± 0.01	0.42 ± 0.06
18	0.41 ± 0.06	0.81 ± 0.14
19	0.11 ± 0.01	0.11 ± 0.02
20	0.15 ± 0.04	0.19 ± 0.02
21	0.23 ± 0.02	0.17 ± 0.04
22	0.39 ± 0.07	0.61 ± 0.08
23	0.24 ± 0.08	0.23 ± 0.04
24	0.16 ± 0.05	0.13 ± 0.03
25	0.36 ± 0.12	0.20 ± 0.02
26	0.34 ± 0.10	0.40 ± 0.07
27	0.47 ± 0.13	0.50 ± 0.05
28	0.30 ± 0.03	0.24 ± 0.04
29	0.45 ± 0.09	0.38 ± 0.03
30	Not soluble in DMSO and thus not tested	
	JIMT-1[a]	HCC1937[c]
13	0.38 ± 0.03	4.36 ± 0.89
31	1.73 ± 0.28	> 10
32	2.08 ± 0.37	> 10

[a-c]human breast cancer cell lines (JIMT-1, MCF-7 and HCC1937).

CONCLUSIONS

According to the latest World Health Organization (WHO) data, "cancer diseases are a leading cause of death worldwide,

accounting for 8.2 million deaths in 2012". Among them, the most dangerous is the cancer of the lungs, liver, stomach, colorectal, breast and oesophagus. What is worse, it is expected that "annual cancer cases will rise from 14 million in 2012 to 22 million within the next two decades [123]".

For this reason it is extremely important to search for new substances with potential use in the fight against cancer diseases and to such substances without a doubt, Salinomycin should be ranked. Salinomycin shows strong anti-tumor and antiproliferative activity against various cancer cells, including multi-drug resistance (MDR), radio-resistance as well as cancer stem cells (CSCs), *i.e.* colorectal, gastric, leukemic, lung cancer stem cells and many others. Additionally, Salinomycin forces dangerous cancer cells to induced apoptosis process in many different ways.

A very interesting direction of research is the chemical modification of Salinomycin. Some of Salinomycin derivatives obtained are less toxic and, simultaneously, show stronger anti-tumor activity than unmodified ionophores and commonly used cytostatic agents like cisplatin or Doxorubicin.

All the above-mentioned facts about Salinomycin and its derivatives, such as their strong activity against MDR cancer cells and against CSC as well as a very promising result of preliminary clinical tests, which have shown the inhibition of human neoplastic diseases, together with observed marginal side effects, justify formulation of a thesis that both Salinomycin and its derivatives should be studied in clinical test in the near future.

Extremely promising therapeutic effects are expected from the use of combination of Salinomycin with other anti-cancer drugs, because synergistic effects for Salinomycin have been observed *in vivo* and *in vitro*. "Synergistic action of drugs may overcome side effects that result from high doses of single-target drugs, increase drug selectivity, and offer an opportunity for more precise control of cancer therapy. Drug combinations that simultaneously impact multiple targets are more effective to overcome MDR and lower side-effects in cancer cell inhibition".

The chemical modification of Salinomycin is one of the best ways, which can offer new compounds with strong anti-cancer effect, good selectivity and low toxicity. Therefore, chemists can contribute to the discovery of new anti-cancer drugs based on the derivatives of Salinomycin.

These all facts confirm the significance of the study on Salinomycin and its derivatives, which are much promising for effective as well as selective fight against cancer diseases.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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LIST OF ABBREVIATIONS

4T07	=	Mouse breast cancer cell line
A2780	=	Ovarian cancer cell line
A2780-cp	=	Ovarian cancer cell line
A-549	=	Lung adenocarcinoma cell line
ABC transporters	=	ATP-binding cassette transporters
ACHN	=	Renal carcinoma cell line
BALB/3T3	=	Normal murine embryonic fibroblast cell line
BEL-7402	=	Hepatocellular carcinoma cell line
C13	=	Ovarian cancer cell line
CNE-1	=	Nasopharyngeal cancer cell line
CNE-2	=	Nasopharyngeal cancer cell line
CNE-2/DDP	=	Nasopharyngeal cancer cell line
CSCs	=	Cancer stem cells
DNA	=	Deoxyribonucleic acid
DU-145	=	Androgen-independent prostate cancer cell line
DXR	=	Multi-drug resistant ovarian cancer cell line
EP156T	=	Epithelial prostate cancer cell line
EPCAM	=	Epithelial cell adhesion molecule
H-MESO-1	=	Malignant mesothelioma cell line
HCC1937	=	Breast cancer cell line
HCT116	=	Colon cancer cell line
HepG2	=	Hepatocellular carcinoma cell line
HIV	=	Human immunodeficiency virus
HLMEC	=	Lung microvascular endothelial cell line
HL-60	=	Vincristine-sensitive leukemia cell line
HL-60/vinc	=	Vincristine-resistant leukemia cell line
HSC-4	=	Tongue carcinoma cell line
HT1376	=	Bladder cancer cell line
HT-29	=	Colon adenocarcinoma cell line
IC_{50}	=	Half maximal inhibitory concentration
JIMT-1	=	Breast cancer cell line
KB	=	Nasopharynx carcinoma cell line
LNCaP	=	Androgen-dependent prostate cancer cell line
LNM35	=	Lung cancer cell line
LoVo	=	Doxorubicin-sensitive colon cancer cell line
LoVo/DX	=	Doxorubicin-resistant colon cancer cell line
LRP6	=	Low-density lipoprotein receptor-related protein 6
LS174T	=	Colorectal carcinoma cell line
MAPK	=	Mitogen-activated protein kinases
MCF-7	=	Breast adenocarcinoma cell line
MCF-7/DX	=	Doxorubicin-resistant breast adeno-carcinoma cell line
MDA-MB-231	=	Triple negative breast cancer cell line
MG-1	=	Glioblastoma cell line
MDR	=	Multi-drug resistance

Ms-1	=	Small lung carcinoma cell line
NAG-1	=	Non-steroidal anti-inflammatory drug activated gene
NCI/ADR-RES	=	Multi-drug resistant ovarian cancer cell line
NCI-H929	=	Lymphoma and myeloma cell line
OV2008	=	Ovarian cancer cell line
OVCAR-3	=	Ovarian cancer cell line
OVCAR-8	=	Ovarian cancer cell line
PARP-1	=	Poly[ADP-ribose]polymerase 1
PC-3	=	Androgen-independent prostate cancer cell line
PrEC	=	Epithelial prostate cancer cell line
P-388	=	Murine leukemia cell line
P-gp	=	P-glycoprotein
ROS	=	Reactive oxygen species
RWPE-1	=	Epithelial prostate cancer cell line
SCC	=	Squamous carcinoma cells
SKOV3	=	Ovarian cancer cell line
SMMC-7721	=	Hepatocellular carcinoma cell line
SNU-C1	=	Colon cancer cell line
T47D	=	Breast adenocarcinoma cell line
TRAIL	=	Tumor necrosis factor-related apoptosis-inducing ligand
U87	=	Glioblastoma cell line
U373	=	Glioblastoma cell line
VCaP	=	Epithelial prostate cancer cell line
WHO	=	World Health Organization

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