

Salinomycin as a potential chemotherapeutic compound in cisplatin-resistant ovarian cancer: effects and mechanisms

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ABBREVIATIONS

ABC	ATP-Binding Cassette
AJCC	American Joint Committee on Cancer
ALDH	Aldehyde Dehydrogenase
ATR	Ataxia Telangiectasia and Rad-3-related kinase
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
Bcl-2/BCL2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
CA-125	Cancer Antigen 125 or Carbohydrate Antigen 125 (also known as Mucin 16)
Caspases	Cysteine-aspartic proteases or Cysteine-dependent aspartate-directed proteases
CHEK1	Checkpoint kinase 1
CHEK2	Checkpoint kinase 2
CI	Confidence interval
CRC	Human Colorectal Cancer
CSCs	Cancer Stem Cells
CTLA-4	Cytotoxic T Lymphocyte-Associated Antigen 4
CTR1	Copper Transporter 1
DAPI	4, 6-diamino-2-phenylindole
DCs	Dendritic Cells
DDR	DNA Damage Response
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DYRK1B	Dual-specificity Y-phosphorylation Regulated Kinase 1B (also known as MIRK)
EGFR/ErbB	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
EOC	Epithelial Ovarian Cancer
ERKs	Extracellular-signal-regulated kinases or classical MAP kinases
ETO	Etoposide
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FIGO	International Federation of Gynaecology and Obstetrics

ABBREVIATIONS

FITC	Fluorescein isothiocyanate
G	Grade
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GOG	Gynecologic Oncology Group
GSH	Glutathione
GST	Glutathione-S-Transferase
HE	Hematoxylin Eosin
HER2/neu	Human Epidermal Growth Factor Receptor 2
HMG	High-Mobility-Group proteins
HNPCC	Hereditary Nonpolyposis Colorectal Cancer (formerly called Lynch syndrome)
HR	Homologous Recombination
HSP	Heat-Shock Proteins
IAP	Inhibitor of Apoptosis Protein
IC50	Half maximal Inhibitory Concentration
IFN- γ	Interferon gamma
I κ B α	Nuclear factor of Kappa light polypeptide gene enhancer in B-cells Inhibitor, alpha
IL	Interleukins
JNKs	c-Jun N-terminal kinases
LRP6	Lipoprotein Receptor Related Protein 6
MAPK	Mitogen-Activated Protein Kinases
MDR	Multidrug Resistance
MET	Mesenchymal-Epithelial Transition
MMP	Mitochondrial Membrane Potential
MMR	Mismatch Repair
MOMP	Mitochondrial Outer Membrane Permeabilization
MRP2	Multidrug-Resistance Protein 2
MUC1	Mucin 1
NCCN	National Comprehensive Cancer Network
NCXs	Na ⁺ /Ca ²⁺ exchangers
NER	Nucleotide Excision Repair
NF- κ B	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
Oct	Octreotide
PARP	Poly-ADP Ribose Polymerase
PBS	Phosphate Buffered Saline

PDGF	Platelet-Derived Endothelial Cell Growth Factor
P-gp	P-glycoprotein
PI	Propidium Iodide
PI3Ks	Phosphatidylinositol 3-kinases (PI 3-kinases)
PKC	Protein Kinase C
PTEN	Phosphatase and tensin homolog
PTX, Taxol	Paclitaxel
RB	Retinoblastoma protein
ROS	Reactive Oxygen Species
Sal	Salinomycin
SSTR	Somatostatin Receptors
TAA	Tumor-Associated Antigens
TAG-72	Tumor-Associated Glycoprotein 72
TILs	Tumor-Infiltrating Lymphocytes
TNF- α	Tumor Necrosis Factor alpha
TRAIL	TNF-Related Apoptosis-Inducing Ligand
Tregs	Regulatory T Cells
VDAC	Voltage-Dependent Anion Channel
VEGF	Vascular Endothelial Growth Factor
Ver	Verapamil
WART	Whole-Abdominal Radiation Therapy
XIAP	X-linked Inhibitor of Apoptosis Protein

1. INTRODUCTION

1.1 Ovarian cancer

Among the gynecological malignancies, ovarian cancer is the leading cause of mortality in developed countries with 225,500 new cases and 140,200 estimated deaths each year worldwide, and its incidence is rising [1]. In Switzerland, statistical data from the Swiss Association of Cancer Registries (www.nicer.org) showed that ovarian cancer is the seventh most common cancer and the fifth cause of death from cancer in Swiss women [2, 3]. Ovarian cancer has the highest fatality-to-case ratio of all the gynecologic malignancies, primarily because of the difficulty in detecting the disease before widespread dissemination [4]. Ovarian cancer are usually asymptomatic until they have metastasized, so patients have advanced disease at diagnosis in more than two thirds of the cases with a resultant poor prognosis [5]. Even with the use of multimodality approach to treatment, including aggressive cytoreductive surgery and combination chemotherapy, the 5-year survival rate for carefully and properly staged patients with stage I disease is 76% to 93%, depending on the tumor grade. The 5-year survival for stage II is 60% to 74%. The 5-year survival rate for stage IIIa is 41%, for stage IIIb about 25%, for stage IIIc 23%, and for stage IV disease 11% [5]. Therefore, in clinic ovarian cancer represents a major surgical challenge, requires intensive and often complex therapies, and is extremely demanding of the patient's psychological and physical energy [5].

1.1.1 Risk factors

The incidence of ovarian cancer increases with age [6] and the peak incidence of invasive epithelial ovarian cancer is at 56 to 60 years of age [5]. About 30% of ovarian neoplasms in postmenopausal women are malignant, whereas only about 7% of ovarian epithelial tumors in premenopausal patients are frankly malignant. The average age of patients with borderline tumors is approximately 46 years [5]. The frequency of ovarian cancer varies among different geographic regions. The incidence of ovarian cancer in Western European countries and in the United States is higher, with a five to sevenfold greater incidence than age-matched populations in East Asia. Whites are 50% more likely to develop ovarian cancer than blacks living in the United States [4]. A woman's risk at birth of having

ovarian cancer sometime in her life is 1% to 1.5%, and that of dying from ovarian cancer almost 0.5% [5].

Epidemiologic studies have identified risk factors in the etiology of ovarian cancer. A 30% to 60% decreased risk of cancer is associated with younger age at pregnancy and first birth (25 years or younger), the use of oral contraceptives, and/or breast-feeding [7]. Conversely, nulliparity or older age at first birth (older than 35 years) confers an increased risk of cancer. Because parity is inversely related to the risk of ovarian cancer, having at least one child is protective of the disease, with a risk reduction of 0.3 to 0.4 [5]. Oral contraceptive use reduces the risk of epithelial ovarian cancer. Women who use oral contraceptives for 5 or more years reduce their relative risk to 0.5 (*i.e.*, there is a 50% reduction in the likelihood of development of ovarian cancer) [4, 5]. Additionally, recent data suggest that hormone therapy [8] and pelvic inflammatory disease [9] may increase the risk for ovarian cancer. The risk of borderline ovarian cancer may be increased after ovarian stimulation for *in vitro* fertilization [10].

Most epithelial ovarian cancer is sporadic, with familial or hereditary patterns (involving first- or second-degree relatives with a history of epithelial ovarian cancer) accounting for 5% to 10% of all malignancies [4, 5]. Having a first-degree relative (*i.e.*, mother, sister, daughter) with an epithelial carcinoma gives a 5% lifetime risk for ovarian cancer, whereas having two first-degree relatives increases this risk to 20% to 30% [4]. Hereditary ovarian cancers in general occur in women approximately 10 years younger than those with nonhereditary tumors [4, 5]. Most hereditary ovarian cancer is associated with germline mutations in the *BRCA1* gene; a small proportion of inherited disease is associated with mutations in the gene *BRCA2*. The mutations are inherited in an autosomal dominant fashion, and therefore a full pedigree analysis (*i.e.*, both maternal and paternal sides of the family) must be carefully evaluated [5, 11-15]. Additionally, women affected with hereditary nonpolyposis colorectal cancer (HNPCC; formerly called Lynch syndrome) have approximately a 13-fold greater risk of developing ovarian cancer than the general population [4, 12, 16]. In high-risk women (with either *BRCA1* or *BRCA2* mutations), oophorectomy is associated with a reduced risk of ovarian and fallopian tube cancer, however, there is a residual risk for primary peritoneal cancer in these high-risk women

after prophylactic salpingo-oophorectomy [17-19]. The risk of surgery include injury to the bowel, bladder, ureter, and vessels [20].

1.1.2 Symptoms and signs

The majority of women with epithelial ovarian cancer have vague and nonspecific symptoms [5]. Symptoms of ovarian cancer are often confused with benign conditions or interpreted as part of the aging process, with the final diagnosis often delayed [4]. In early-stage disease, the patient may experience irregular menses if she is premenopausal. If a pelvic mass is compressing the bladder or rectum, she may report urinary frequency or constipation [21, 22]. Occasionally, she may perceive lower abdominal distention, pressure, or pain, such as dyspareunia. Acute symptoms, such as pain secondary to rupture or torsion, are unusual. In advanced-stage disease, patients most often have symptoms related to the presence of ascites, omental metastases, or bowel metastases. The symptoms include abdominal distention, bloating, constipation, nausea, anorexia, or early satiety [5, 23]. The most common symptoms in order from highest percentage to lowest are abdominal fullness or distension, abdominal or back pain, decreased energy or lethargy, and urinary frequency, especially if these symptoms are new and frequent (> 12 days/month) [4, 24].

The most important sign of epithelial ovarian cancer is the presence of a pelvic mass on physical examination [5]. Palpation of an asymptomatic adnexal mass during a routine pelvic examination is the usual presentation for ovarian cancer. The presence of a solid, irregular, fixed pelvic mass on pelvic examination is highly suggestive of an ovarian malignancy. The diagnosis of malignancy is almost certain if a fixed, irregular pelvic mass is associated with an upper abdominal mass or ascites [5, 23].

1.1.3 Diagnosis

Because of the location of the ovaries and the biology of most epithelial cancers, it has been difficult to diagnosis ovarian cancer at an earlier more curable stage [6, 21, 22, 25]. The early diagnosis of ovarian cancer is also made even more difficult by the lack of effective screening tests [4]. Given the false-positive results for both CA-125 and transvaginal ultrasonography, particularly in premenopausal women, these tests are not

cost-effective and should not be used routinely to screen for ovarian cancer [5]. Furthermore, randomized data do not yet support routine screening for ovarian cancer in the general population, and routine screening is not currently recommended by any professional society [20, 26, 27]. To date the efficacy of ovarian cancer screening methods as tumor markers and ultrasonography remains to be proven [6].

CA-125 should not be routinely used to screen for ovarian cancer, but, instead, should be used to follow response to therapy and evaluate for recurrent disease [4]. Serum CA-125 levels have also been shown to be useful in distinguishing malignant from benign pelvic masses [28]. For a postmenopausal patient with an adnexal mass and a very high serum CA-125 level (>200 U/mL), there is a 96% positive predictive value for malignancy. However, a normal CA-125 measurement alone does not rule out ovarian cancer, because up to 50% of early-stage cancers and 20% to 25% of advanced cancers are associated with normal values. For premenopausal patients with symptoms, a CA-125 measurement has not been shown to be useful in most circumstances and the specificity of the test is low, because the CA-125 level tends to be elevated in common benign conditions, including uterine leiomyomata, pelvic inflammatory disease, endometriosis, adenomyosis, pregnancy, and even menstruation [4, 5]. In the premenopausal patient, a period of observation is reasonable, provided the adnexal mass does not have characteristics that suggest malignancy (*i.e.*, it is mobile, mostly cystic, unilateral, and of regular contour).

The diagnosis of an ovarian cancer requires an exploratory laparotomy for definitive histologic staging, even though tumor markers (e.g., serum CA-125) and ultrasound examination may be helpful in predicting a higher likelihood of a malignant tumor than a benign tumor [5]. The threshold for surgical intervention is lower in postmenopausal women and those with cysts >3 cm should undergo exploratory surgery, laparotomy, or laparoscopy [23].

1.1.4 Patterns of spread

Ovarian epithelial cancers spread primarily by exfoliation of cells into the peritoneal cavity, by lymphatic dissemination, and by hematogenous spread (Fig.1). The most common and earliest mode of dissemination of ovarian epithelial cancer is by exfoliation of cells that

implant along the surfaces of the peritoneal cavity [5]. This process explains widespread peritoneal dissemination at the time of diagnosis, even with relatively small primary ovarian lesions [4]. Lymphatic dissemination to the pelvic and para-aortic lymph nodes is common, particularly in advanced-stage disease [29]. Hematogenous dissemination at the time of diagnosis is uncommon [5].

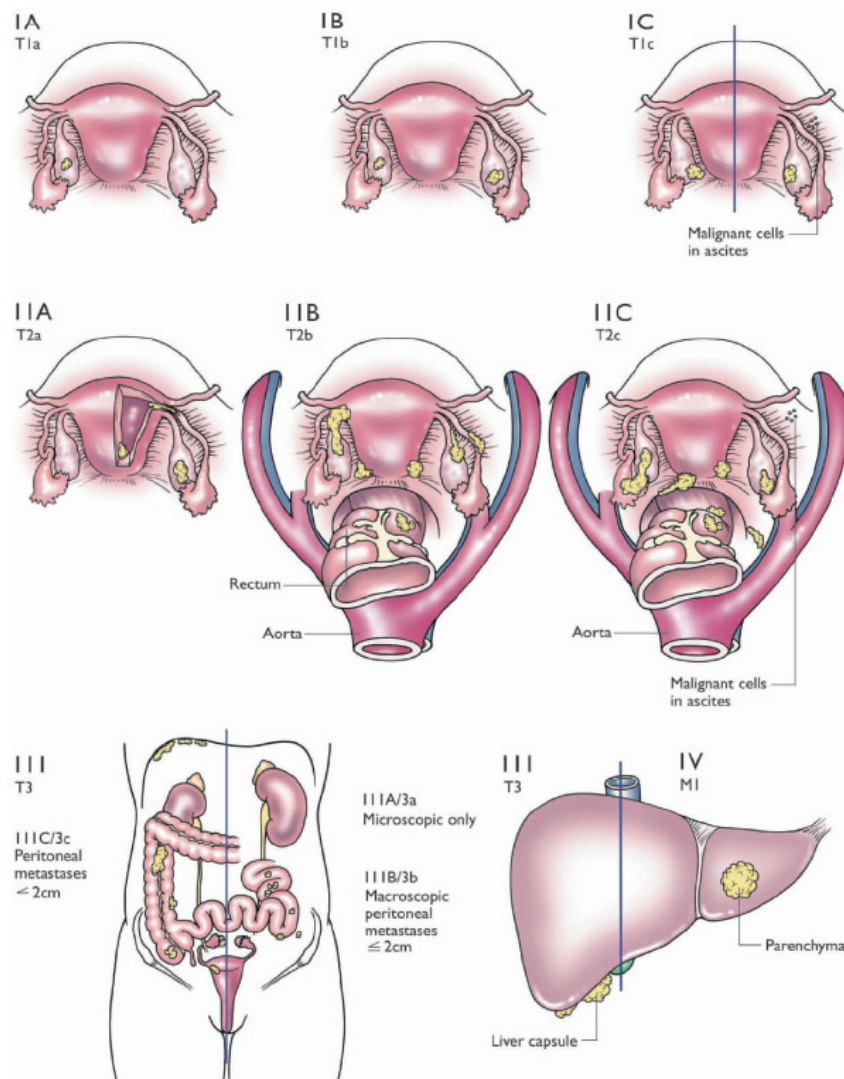


Fig. 1 Staging ovarian cancer: primary tumor and metastases (FIGO and TNM). [Adapted from Heintz, A.P., et al.: *Int J Gynaecol Obstet*, 2006.]

1.1.5 Histologic classification

The classification of ovarian tumors by cell line of origin is presented in Fig.2 [30]. Both benign and malignant tumors can arise from each of the three ovarian cell types [30]:

- **Germ cell tumors** include the most common ovarian neoplasm in reproductive-age women [4]. In the first two decades of life, almost 70% of ovarian tumors are of germ cell origin, and one third of these are malignant [5], which account for 3-5% of ovarian cancers [31]. In contrast to the relatively slow-growing epithelial ovarian tumors, germ cell malignancies grow rapidly. The most common types of malignant germ cell tumors are dysgerminomas, immature teratomas, and endodermal sinus tumors. Preservation of fertility should be standard in most patients. The most effective chemotherapy is *bleomycin, etoposide, and cisplatin* (BEP) combination [5].

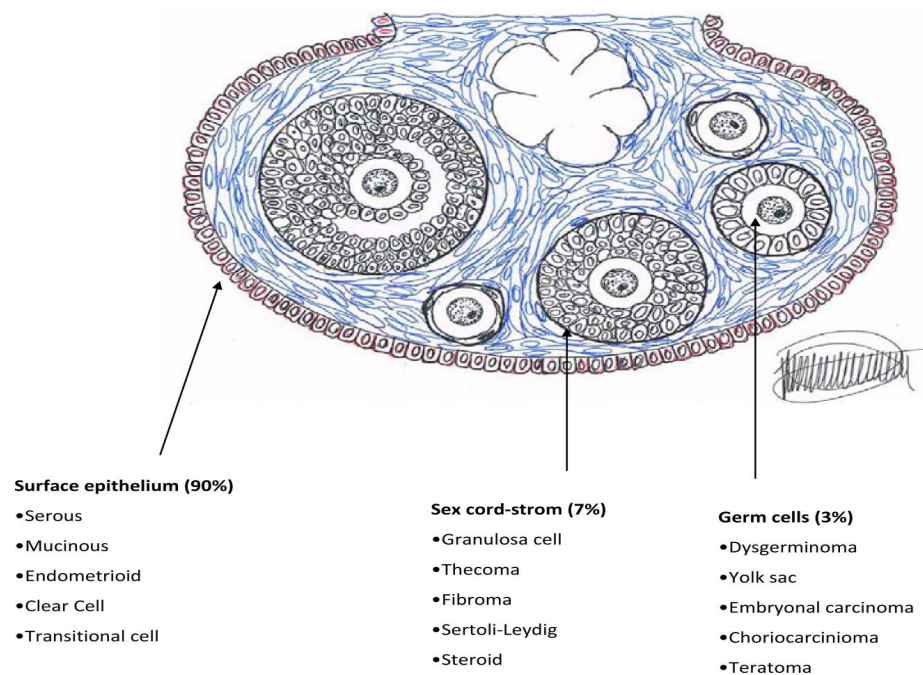


Fig. 2 Different ovarian tumors originate from different cell subtypes. Prevalence of malignant components in parentheses. [Adapted from Chen, V.W., *et al.*:*Cancer*, 2003.]

- **Sex-cord-stromal tumors** arise from the ovarian connective tissue, often secrete hormones, and can occur in women of all ages, comprising approximately 7% of all ovarian malignancies [30]. Stromal tumors include granulosa cell tumors, which are

low-grade malignancies. In premenopausal women, they can be treated conservatively. Adjuvant chemotherapy is of unproven value [5].

- ***Epithelial cell tumors*** is the largest class of ovarian neoplasm [4]. Approximately 90% of ovarian cancers are derived from tissues that come from coelomic epithelium or mesothelium [23] and more than 80% of epithelial ovarian cancers are found in postmenopausal women [5]. The types of epithelial tumors classified are as follows: serous, mucinous, endometrioid, clear cell (mesonephroid), Brenner, mixed epithelial, undifferentiated and unclassified [5]. Seventy-five percent of epithelial cancers are of the serous histologic type. Less common types are mucinous (20%), endometrioid (2%), clear cell, Brenner, and undifferentiated carcinomas, and each of the last three types represents less than 1% of epithelial lesions [5].

In addition to benign and malignant epithelial lesions, borderline tumors of low-malignant potential contain morphologically and molecularly partially transformed epithelial cells that do not invade underlying stroma [30]. These tumors generally remain confined to the ovary, are more common in premenopausal women (30 to 50 years of age), and have good prognoses [32]. About 20% of such tumors show spread beyond the ovary. They require carefully individualized therapy following the initial surgical resection of the primary tumor. If frozen section pathology demonstrates borderline histology, unilateral oophorectomy with a staging procedure and follow up is appropriate, assuming the woman wishes to retain ovarian function and/or fertility and understands the risks of such conservative management [4]. Approximately 10% of borderline tumors can recur after resection and prove lethal [30].

1.1.6 Staging

The staging of ovarian carcinoma is based on extent of spread of tumor and histologic evaluation of the tumor [4]. Ovarian malignancies are surgically staged according to the 2002 revised American Joint Committee on Cancer (AJCC) and International Federation of Gynaecology and Obstetrics (FIGO) joint staging system, which is presented in Table 1 and Table 2 [6, 33]. Clinical and radiological evaluation may affect the final staging. Histopathological typing is to be considered at staging [5, 23].

Table 1 Carcinoma of the ovary: FIGO nomenclature (Rio de Janeiro 1988) [Adapted from Heintz, A.P., *et al.*: *Int J Gynaecol Obstet*, 2006.]

Stage I	Growth limited to the ovaries
Ia	Growth limited to one ovary; no ascites present containing malignant cells. No tumor on the external surface; capsule intact
Ib	Growth limited to both ovaries; no ascites present containing malignant cells. No tumor on the external surfaces; capsules intact
Ic ^a	Tumor either Stage Ia or Ib, but with tumor on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings
Stage II	Growth involving one or both ovaries with pelvic extension
IIa	Extension and/or metastases to the uterus and/or tubes
IIb	Extension to other pelvic tissues
IIc ^a	Tumor either Stage IIa or IIb, but with tumor on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings
Stage III	Tumor involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastases equals Stage III. Tumor is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum
IIIa	Tumor grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologic proven extension to small bowel or mesentery
IIIb	Tumor of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative
IIIc	Peritoneal metastasis beyond the pelvis >2 cm in diameter and/or positive retroperitoneal or inguinal nodes
Stage IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV

^a In order to evaluate the impact on prognosis of the different criteria for allotting cases to Stage Ic or IIc, it would be of value to know if rupture of the capsule was spontaneous, or caused by the surgeon; and if the source of malignant cells detected was peritoneal washings, or ascites.

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FIGO Annual Report, Vol. 26

Histopathologic grade (G)

- GX: Grade cannot be assessed
- G1: Well differentiated
- G2: Moderately differentiated
- G3: Poorly or undifferentiated

Table 2 Carcinoma of the ovary: Stage grouping for ovarian cancer [Adapted from Heintz, A.P., *et al.*: *Int J Gynaecol Obstet*, 2006.]

FIGO	UICC		
	T	N	M
Ia	T1a	N0	M0
Ib	T1b	N0	M0
Ic	T1c	N0	M0
IIa	T2a	N0	M0
IIb	T2b	N0	M0
IIc	T2c	N0	M0
IIIa	T3a	N0	M0
IIIb	T3b	N0	M0
IIIc	T3c	N0	M0
	any T	N1	M0
IV	any T	any N	M1

1.2 Ovarian cancer and treatments

Primary treatment for presumed ovarian cancer consists of appropriate surgical staging and cytoreduction, followed in most (but not all) patients by systemic chemotherapy [6]. Over the last three decades, 5-year survival for ovarian cancer patients has increased from 37 to 45%, related to more consistent use of cytoreductive surgery and combination chemotherapy with platinum compounds and taxanes [34]. However, ovarian cancer still ranks as the most deadly gynecologic cancer and less than 40% of all stages can be cured [30, 35]. Currently, emerging treatment strategies have focused on targets which are integral to tumor growth and metastasis, and some innovative agents are being developed and under investigation in clinical trials [35].

1.2.1 Surgical management

Surgery is necessary for diagnosis, accurate staging and optimal cytoreduction, and is crucial for the successful treatment of ovarian cancer [23]. The importance of thorough surgical staging cannot be overemphasized, because subsequent treatment will be determined by the stage of disease [5].

Primary surgical therapy is indicated in most of the ovarian malignancies, using the principle of cytoreductive surgery, or “tumor debulking”. The rationale for cytoreductive surgery is that adjunctive radiation therapy and chemotherapy are more effective when all tumor masses are reduced to less than 1 cm in size [4]. Because direct peritoneal seeding is the primary method of intraperitoneal spread, multiple adjacent structures commonly contain tumor, resulting in cytoreductive procedures that are often extensive [4]. Patients with advanced-stage disease should undergo “debulking” or cytoreductive surgery to remove as much of the tumor and its metastases as possible, if the patient is medically stable. A thorough abdominal exploration, total abdominal hysterectomy, bilateral salpingo-oophorectomy, lymphadenectomy, omentectomy, and removal of all gross cancer are standard therapy for malignant ovarian tumor. The performance of a debulking operation as early as possible in the course of the patient's treatment should be considered the standard of care [5, 36]. Surgery can be performed after neoadjuvant chemotherapy

[37], when optimal cytoreduction is not considered feasible at initial diagnosis. Survival increases with the expertise of the surgeon [38], and optimal cytoreductive surgery is an independent prognostic factor [39].

In several retrospective series, cytoreductive surgery for recurrent disease has been associated with improved survival when all macroscopic cancer can be removed [40, 41]. Two ongoing prospective trials in Europe and the United States are evaluating criteria and outcomes for secondary cytoreduction [30].

1.2.2 Adjuvant therapy

1.2.2.1 Chemotherapy

Because most ovarian cancer presents at an advanced stage, adjunctive treatment using systemic chemotherapy is usually necessary. However, patients with stage IA or IB disease (who have been completely surgically staged) and who have borderline, well- or moderately differentiated tumors do not benefit from additional chemotherapy because their prognosis is excellent with surgery alone [6, 23, 42].

Chemotherapy improves survival and is an effective means of palliation of ovarian cancer. In patients who are at increased risk of recurrence (stage I G3 and all IC-IV), chemotherapy is recommended. Sequential clinical trials of chemotherapy agents demonstrate that cisplatin (or carboplatin) given in combination with paclitaxel is the most active combination identified [23]. Carboplatin is an alkylating agent that binds covalently to DNA, creating adducts that form intrachain and interchain cross-links. Paclitaxel binds noncovalently to microtubules and increases their stability, interfering with mitotic spindle formation. Both agents induce apoptosis. Chemotherapy has generally been administered intravenously, but three randomized phase III trials have shown a 20-25% relative risk reduction in mortality after intraperitoneal therapy for patients who have been optimally cytoreduced [43-45]. For advanced stage epithelial ovarian cancer, the choice of intravenous versus intraperitoneal platinum and taxane chemotherapy should be individualized [5].

Six cycles of carboplatin and paclitaxel chemotherapy are considered standard adjuvant treatment for newly diagnosed ovarian cancer after cytoreductive surgery [30]. Recommendations for the number of cycles of treatment also can vary with the stage of the disease. For patients with advanced-stage disease (stages II-IV), 6-8 cycles of chemotherapy are recommended, whereas 3 to 6 cycles are recommended for earlier-stage disease [46]. Chemotherapy is generally administered every 3 weeks, but weekly dose-dense administration of paclitaxel has produced improved survival in one trial from Japan [47], and a confirmatory trial has not yet been completed.

More than 70% of patients with advanced ovarian cancer will experience disease recurrence and become candidates for second-line chemotherapy, within 12 and 18 months. Retreatment with carboplatin and paclitaxel is associated with a 20-50% response when platinum-sensitive disease recurs more than 6 months after primary chemotherapy [30]. Although recurrent disease is not curable, combinations of drugs can prolong survival. Disease that recurs in less than 6 months is considered platinum resistant. In this setting, several drugs produce response rates ranging from 10-30% and increase progression-free survival such as liposomal doxorubicin, weekly paclitaxel, and topotecan. Other drugs have demonstrated activity in phase II clinical studies, including gemcitabine, bevacizumab, docetaxel, and etoposide [6, 30].

1.2.2.2 Radiation therapy

Radiation therapy has only a limited role in the management of ovarian cancer [4]. Whole-abdominal radiation therapy (WART) given as a salvage treatment has been shown to be associated with a relatively high morbidity [5], so WART in patients with low-bulk stage III disease is no longer included as an option for initial treatment or consolidation treatment in ovarian cancer [6]. Palliative localized radiation therapy is an option for symptom control in patients with recurrent disease. Patients who receive radiation are prone to vaginal stenosis, which can impair sexual function. Women can use vaginal dilators to prevent or treat vaginal stenosis [48, 49].

1.2.3 Novel therapeutic strategies

The next horizon for ovarian cancer treatment is molecularly targeted agents, immunotherapy, and gene therapy [4].

1.2.3.1 Molecularly targeted agents

Emerging treatment strategies have focused on targets which are integral to tumor growth and metastasis. Targeted molecular strategies have been employed in the treatment of ovarian cancer. These strategies attempt to manipulate processes critical to ovarian carcinogenesis, including cellular growth and proliferation, cellular adhesion, intracellular signaling pathways, angiogenesis, and DNA repair pathways [35, 50].

Several kind of agents are currently available that target specific molecules or proteins in ovarian cancer cells. For example, angiogenesis is critical to tumor growth/metastasis and several proangiogenic factors, including vascular endothelial growth factor (VEGF), IL-8, platelet-derived endothelial cell growth factor (PDGF), angiogenin, and fibroblast growth factor (FGF), have been implicated in tumorigenesis [51]. Inhibitors of proangiogenic proteins such as VEGF (bevacizumab and aflibercept), angiopoietins (AMG386), PDGF (imatinib and pazopanib), or their receptors VEGF receptor (pazopanib, sorafenib, sunitinib, and BIBF1120) are being tested for possible treatment of EOC in the clinic [30]. Of these targeted molecules, VEGF has been the most commonly studied, given that it is abundantly present in the serum of patients with EOC [52, 53], and that elevated VEGF levels have been associated with poor survival [54]. Bevacizumab is a monoclonal antibody designed to target the VEGF protein and inhibit angiogenesis in tumors. Two recent trials have added a VEGF-binding antiangiogenic antibody, bevacizumab, to standard treatment during and for up to 15 months after chemotherapy. Improved progression-free but not overall survival was reported [55, 56]. According to the data from GOG 0218 and ICON7, recently the National Comprehensive Cancer Network (NCCN) Ovarian Cancer panel does not recommend the routine addition of bevacizumab to upfront therapy with carboplatin/paclitaxel or as maintenance therapy at this time. The NCCN panel encourages participation in ongoing clinical trials that are further investigating the role of anti-angiogenesis agents in the treatment of ovarian cancer, both in

the upfront and recurrence settings [6]. Additionally, trastuzumab is a DNA-derived monoclonal antibody to the HER2, a member of the epidermal growth factor receptor (EGFR/ErbB) family. Treatment with trastuzumab is currently indicated in patients with metastatic breast cancer whose tumors overexpress HER2 [57]. Some ovarian cancers express the HER2/neu receptor [58]; therefore, investigation is currently ongoing regarding the usefulness of this agent in ovarian cancer. Preliminary results from a phase II GOG trial suggested that the overall potential therapeutic benefit for trastuzumab in EOC might be limited [59]. Furthermore, one of the best examples of synthetic lethality to reach the clinic to date is provided by the activity of poly-ADP ribose polymerase (PARP) inhibitors in ovarian cancers that display BRCAness, *i.e.* a deficiency of BRCA1/2 function [60, 61] is associated with a better overall prognosis [62] and response to platinum compounds [63]. BRCA1 and BRCA2 mediate homologous recombination, which is one mechanism of DNA repair [64]. Cancers with BRCAness are deficient in homologous repair and cannot repair DNA double strand breaks induced by platinum compounds [65]. Inhibition of a second DNA repair pathway, base excision repair, by PARP inhibitors causes synthetic lethality in cancers with BRCAness [66]. Olaparib is the most studied PARP inhibitor in women with EOC. The results from clinical trials showed that olaparib might be an efficacious and safe treatment option in BRCA-mutated advanced ovarian cancer [67, 68]. In addition to targeted agents above-mentioned, other innovative agents targeting cellular adhesion molecules (such as Catumaxomab) [69] and folate metabolism (such as anti-human folate receptor-alpha monoclonal antibodies) [70, 71] currently are also under investigation in clinical trials.

1.2.3.2 Immunotherapy

Effective host anti-tumor immune responses have the potential to influence prognosis in patients with EOC. By manipulating the host immune system, it may be possible to enhance host antitumor immune responses and improve patient outcomes. Current immunotherapeutic approaches employ vaccines based on tumor-associated antigens (TAA), DC-based immunotherapy, adoptive immunity, antitumor cytokines or antibodies targeting co-stimulatory and immunosuppressive molecules [72].

Tumor vaccines are currently being investigated for the treatment of ovarian cancer. Several proteins that are abnormally expressed in cancer cells, due to mutations, overexpression, or post-translational modifications, have been identified and are currently studied as targets for immunotherapy. In a recent analysis from the National Cancer Institute Pilot Project for the acceleration of translational research, 75 tumor antigens were priority ranked for cancer vaccine development [73]. Some of these antigens, including MUC1, CA-125, human epidermal growth factor receptor 2 (HER2)/neu, membrane folate receptor, TAG-72, mesothelin, and NY-ESO-1, are targets of therapeutic tumor vaccines in ovarian cancer [72]. Additionally, vaccine-approaches in EOC have also utilized whole tumor cell lysates and dendritic cells (DCs) in an attempt to boost host anti-tumor immune responses. The former affords the opportunity for broad tumor antigen exposure, while use of dendritic cells enhances anti-tumor immunity via specific tumor-antigen presentation and activation of effector T cells [50]. Adoptive immunity is a process by which immune cells, including T lymphocytes, B lymphocytes, natural killer cells, and macrophages, are removed from an individual, modified extracorporeally and then placed back into the same individual [74]. The adoptive transfer of autologous tumor-infiltrating lymphocytes (TILs) has proven to be high response rates in a sample of women with advanced or recurrent EOC [75], and subsequent studies in ovarian cancer have examined the utility of adoptive transfer with modified T cells to enhance antitumor activity [76]. Proinflammatory cytokines, including interleukins (IL) 2, 4, 7, 12 and 18, interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), and granulocyte-macrophage colony-stimulating factor (GM-CSF), have been utilized in preclinical models to induce anti-tumor immune responses [35]. In addition, investigations have begun focusing on molecules (e.g., cytotoxic T lymphocyte-associated antigen 4, CTLA-4) [77] and cell populations (regulatory T cells, Tregs) [78] which suppress host immune responses. These trials will hopefully reinforce the utility of these novel immunotherapeutic techniques in the treatment of recurrent EOC. Currently the response to this type of therapy has been modest, but studies are ongoing.

1.2.3.3 Gene therapy

Because some ovarian cancers result from loss of genetic function through DNA mutations, investigational therapies have also focused on genetic manipulation of the tumors, or gene therapy. For instance, because half of ovarian cancers exhibit deleterious mutations in the p53 gene, research has focused on delivering a normal p53 gene product to the tumor using a variety of viral vectors. The hope is that the wild-type gene product would then be expressed by the tumor and the growth would then be inhibited. So far, response has been minimal, but investigation continues [4].

The potential benefits of these novel therapeutic concepts are manifold, whether considered as primary or adjunct therapy. Work in this area is in the experimental stage or under investigation in clinical trials, but the goal of eliminating cancer cells with minimal toxicity remains the goal of cancer therapeutics [4].

1.3 Cisplatin-resistance in ovarian cancer

Cisplatin is one of the most potent antitumor agents known, displaying clinical activity against a wide variety of solid tumors, including ovarian cancer [79, 80]. Cisplatin often leads to an initial therapeutic success associated with partial responses or disease stabilization in ovarian cancer. Unfortunately, the initial response rate of up to 70% is not durable, and results in a 5-year patient survival rate of only 30% in patients with advanced ovarian cancer, primarily as originally sensitive tumors eventually develop chemoresistance, leading to therapeutic failure [81-84]. The onset of resistance creates a further therapeutic complication in that tumors failing to respond to cisplatin are cross-resistant to diverse unrelated drugs; therefore, the benefits of second-line chemotherapy diminish substantially, and eventually patients succumb to their disease [79]. In addition, the cytotoxicity of cisplatin (which is given intravenously as short-term infusion in physiological saline) also affects kidneys (nephrotoxicity), peripheral nerves (neurotoxicity) and the inner ear (ototoxicity) [85, 86]. Still, the main limitation to the clinical usefulness of cisplatin as an anticancer drug is the high incidence of chemoresistance [87].

1.3.1 Cisplatin and mode of action

The therapeutic activity of cisplatin is mediated by an active species, formed by aqueous hydrolysis as the drug enters the cell. This active species interacts with DNA, RNA and protein, but the cytotoxic effect seems to be primarily mediated via the formation of DNA interstrand and intrastrand crosslinks [79]. These platinum-DNA adducts are recognized by a number of proteins, including those involved in nucleotide excision repair (NER), mismatch repair (MMR), and high-mobility-group proteins (such as HMG1 and HMG2) [88, 89]. Platinum-induced DNA damage is normally repaired by the NER pathway [90-93]. However, proteins belonging to MMR system also participate in the recognition and resolution of cisplatin lesions [94]. When the extent of damage is limited, cisplatin adducts induce an arrest in the S and G2 phases of the cell cycle, a phenomenon that exerts cytoprotective effects by (1) allowing repair mechanisms to re-establish DNA integrity and (2) preventing potentially abortive or abnormal mitoses [95]. Conversely, if DNA damage is beyond repair, cells become committed to (most often apoptotic) death.

Cisplatin exerts anticancer effects via multiple mechanisms, yet its most prominent (and best understood) mode of action involves the generation of DNA lesions followed by the activation of the DNA damage response (DDR) and the induction of mitochondrial apoptosis [87]. The pathways involved in cisplatin-induced cytotoxicity are summarized in Fig.3.

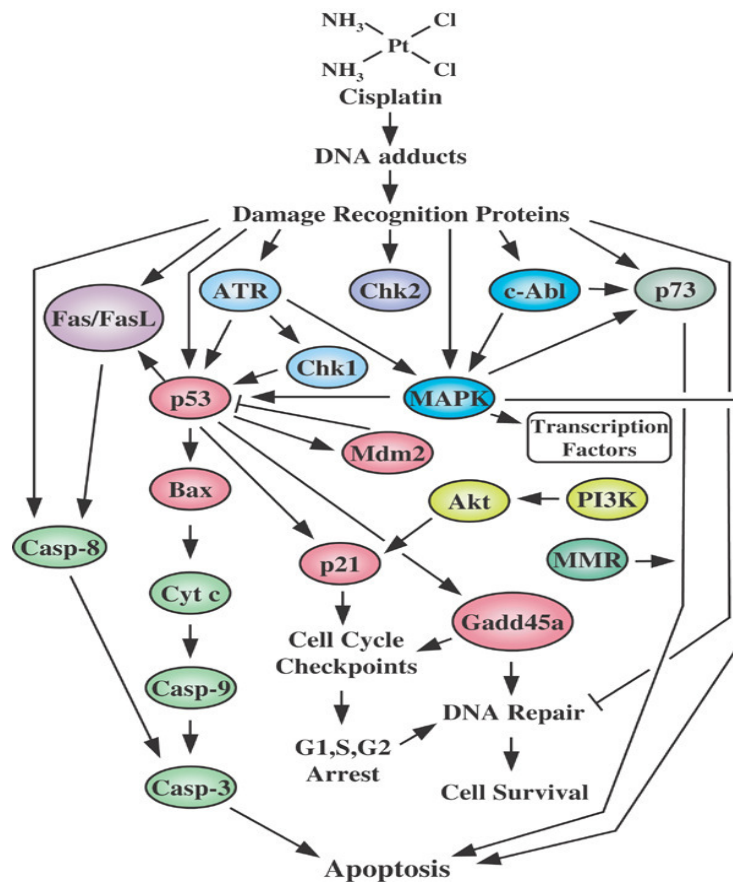


Fig.3 An overview of pathways involved in mediating cisplatin-induced cellular effects. [Adapted from Siddik, Z.H., *et al.*:*Oncogene*, 2003.]

Aquated cisplatin can indeed bind a plethora of nucleophilic species, including cysteine and methionine residues on proteins and DNA bases. In the nucleus, this leads to the generation of inter- and intra-strand adducts that are recognized by the DNA damage-sensing machinery. If the extent of damage is beyond repair, cisplatin adducts trigger the activation of a DNA damage response that frequently involves the ATR kinase, CHEK1,

CHEK2, p73, MAPK and the tumor suppressor protein TP53. In turn, TP53 transactivates several genes whose products facilitate mitochondrial outer membrane permeabilization (MOMP), thereby triggering intrinsic apoptosis, as well as genes that encode for components of the extrinsic apoptotic pathway. MOMP sets off the caspase cascade as well as multiple caspase-independent mechanisms that eventually seal the cell fate. In the cytoplasm, the interaction between cisplatin and glutathione (GSH), metallothioneins or mitochondrial proteins like the voltage-dependent anion channel (VDAC) results in the depletion of reducing equivalents and/or directly sustains the generation of reactive oxygen species (ROS). ROS can directly trigger MOMP or exacerbate cisplatin-induced DNA damage, thereby playing a dual role in cisplatin cytotoxicity [87].

The cisplatin-resistant phenotype of cancer cells can derive from alterations in any of these molecular circuitries as well as from changes that affect the intracellular uptake of cisplatin or the execution of the apoptotic program.

1.3.2 Mechanism of cisplatin resistance

Drug resistance is thought to cause treatment failure and death in more than 90% of patients with metastatic disease. Reasons for the clinical failure of chemotherapy and ‘apparent drug resistance’ can be classified into three broad categories: pharmacokinetic, tumour micro-environmental and cancer-cell specific [81]. Pharmacokinetic resistance is caused by inadequate tumour-cell drug exposure due to interpatient differences in pharmacokinetic variables [96]. The tumour microenvironment can also modulate tumour-cell drug sensitivity. For example, hypoxia has long been known to induce radioresistance, and is now also implicated in chemoresistance [97]. Historically, the main research emphasis has been on tumour-cell-specific mechanisms of drug resistance and, in particular, on those that influence drug-target interactions and subsequent cell damage. Still, in ovarian cancer it seems likely that two parallel phenomena underlie clinical drug resistance [98, 99], and this is illustrated in Fig.4.

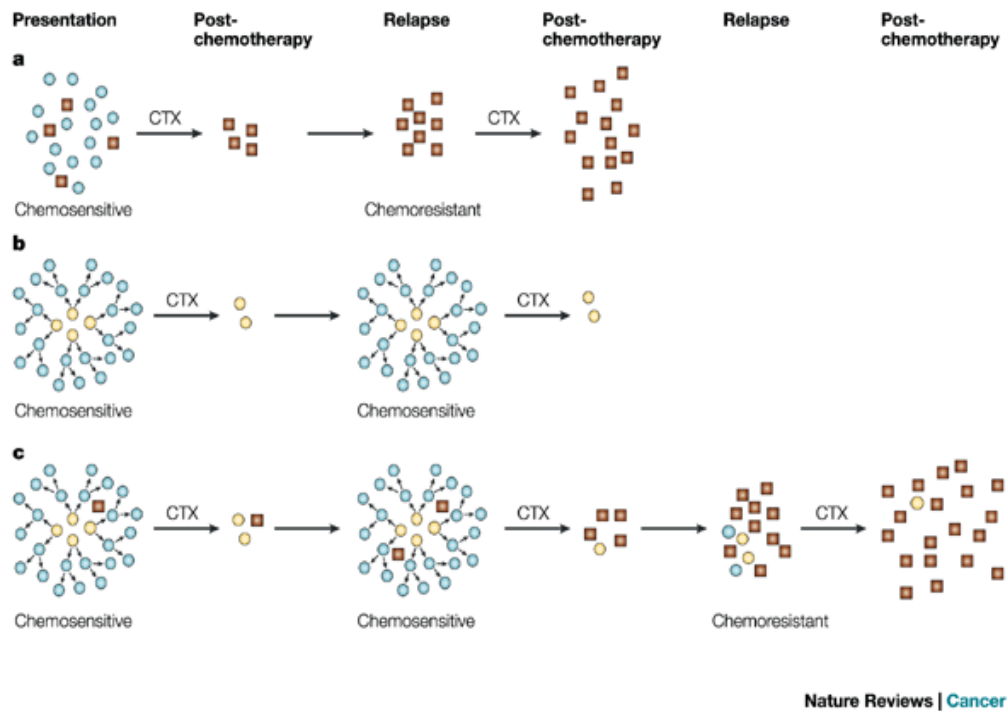


Fig.4 Models of treatment failure in ovarian cancer. CTX, chemotherapy. Chemosensitive cells, blue; stem cells that are drug insensitive due to relative quiescence, yellow; resistant cells due to somatic mutation, brown. [Adapted from Agarwal, R., *et al.*:*Nature Reviews Cancer*, 2003.]

a) The somatic mutation model of relapse proposes growth of resistant tumour-cell clones that remain at the end of chemotherapy, and provides the rationale for combination and/or sequential chemotherapy. However, it is important to consider that cytotoxic agents are primarily effective against proliferating cells and that, even in rapidly proliferating tumours, a significant proportion of cancer cells are in a quiescent state. These quiescent cells therefore show a degree of drug resistance relative to cycling cells [99].

b) Recent studies have shown that a number of proteins that are involved in cell-cycle regulation (such as MYC, RB, INK4A, cyclin-A-CDK2, E1A and E2F1) also interact with apoptotic pathways and cell death in response to chemotherapeutic agents and provide the molecular link between the degree of cell proliferation and intrinsic chemosensitivity [100]. Subsequent relapse is then due to the re-growth of persistent stem cells that were predominantly in G₀. This model goes some way to explaining the observation that

patients who relapse after initial chemotherapy can often be retreated with the same agents and achieve complete clinical responses.

c) In practice, chemoresistance develops despite initial chemosensitive relapses, and it therefore seems likely that both models presented in a and b underlie clinical drug resistance in ovarian cancer. These two processes presumably occur concurrently, but the relative proportions vary from individual to individual, and determine the clinical pattern of relapse and drug sensitivity.

The three reasons above-mentioned influence the response to chemotherapy by principally affecting intracellular active drug concentrations, drug-target interactions, target-mediated cell damage, damage-induced apoptotic signalling or the apoptotic effector machinery [81]. During the past 30 years an intense research has been conducted and several mechanisms that account for the cisplatin-resistant phenotype of tumor cells have been described. These mechanisms can be systematically classified in alterations (1) that involve steps preceding the binding of cisplatin to DNA (pre-target resistance), (2) that directly relate to DNA-cisplatin adducts (on-target resistance), (3) concerning the lethal signaling pathway(s) elicited by cisplatin-mediated DNA damage (post-target resistance) and (4) affecting molecular circuitries that do not present obvious links with cisplatin-elicited signals (off-target resistance) [87].

1.3.2.1 Mechanisms of pre-target resistance

There are at least two mechanisms by which cancer cells elude the cytotoxic potential of cisplatin before it binds to cytoplasmic targets and DNA: (1) a reduced intracellular accumulation of cisplatin, including reduced uptake of cisplatin, such as downregulated copper transporter 1 (CTR1) in cisplatin-resistant cancer cells [101, 102], and increased efflux of cisplatin, such as upregulated ATP7A/ATP7B [103, 104] or multidrug-resistance protein 2 (MRP2) in cisplatin-resistant cancer cells [105-107]. (2) An increased sequestration of cisplatin by GSH, metallothioneins and other cytoplasmic 'scavengers' with nucleophilic properties [108]. This process is catalysed intracellularly by a family of glutathione-S-transferase (GST) enzymes, but studies correlating response and prognosis

following platinum-based chemotherapy and GST expression in ovarian cancer have yielded contradictory results [109, 110].

1.3.2.2 Mechanisms of on-target resistance

The recognition of inter- and intra-strand DNA adducts and the consequent generation of an apoptotic signal is often impaired in cisplatin-resistant cancer cells because of a variety of defects. Alternatively, cisplatin-resistant cells acquire the ability to repair adducts at an increased pace, or become able to tolerate unrepaired DNA lesions [87]. As discussed previously, cisplatin induces apoptosis by forming DNA-platinum adducts. DNA adducts can be removed and the DNA repaired via the NER pathway [90]. In this setting, damaged nucleotides are excised from DNA upon incision on both sides of the lesion, followed by DNA synthesis to reconstitute genetic integrity [111]. At least 20 proteins participate in NER, including excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC1). ERCC1 expression has been negatively correlated with survival and/or responsiveness to cisplatin-based regimens in several human neoplasms including ovarian cancer [112]. An alternative mechanism of DNA repair is via MMR, which normally handles erroneous insertions, deletions and mis-incorporations of bases that can arise during DNA replication and recombination [94, 113]. MMR-related proteins that participate in the recognition of GpG interstrand adducts include MSH2 and MLH1 [113, 114]. According to accepted viewpoints, MMR proteins would attempt to repair cisplatin adducts, fail, and hence transmit a proapoptotic signal [113]. MLH1 is silenced by methylation in a significant proportion of ovarian tumours, and this correlates with cisplatin resistance in some patients [115]. The methylation-dependent silencing of MLH1 has also been shown to predict poor survival in ovarian cancer patients [116]. In addition, cisplatin-induced inter-strand adducts can lead to the so-called double-strand breaks, DNA lesions that are normally repaired in the S phase of the cell cycle (or shortly after) by the machinery for homologous recombination (HR) [117]. Two critical components of the HR system are encoded by BRCA1 and BRCA2, two genes that are frequently mutated in familial breast and ovarian cancers [118, 119]. Notably, HR-deficient cancers have a different phenotype and are often more sensitive to crosslinking agents including cisplatin than their HR-proficient counterparts [120-122]. These

observations suggest that the HR status, at least in specific clinical settings, has an important prognostic and predictive value.

1.3.2.3 Mechanisms of post-target resistance

Post-target resistance to cisplatin can result from a plethora of alterations including defects in the signal transduction pathways that normally elicit apoptosis in response to DNA damage as well as problems with the cell death executioner machinery itself [87]. Because most tumours develop a broad cross-resistance to the different chemotherapeutic agents and radiotherapy that they encounter during treatment, recently research emphasis shifts away from drug-specific mechanisms of resistance to defects in the common apoptotic signalling and effector pathways downstream of drug-target interactions, as the probable causes of resistance in clinical practice [81]. A number of proteins involved in these pathways are oncogenes (such as RAS and AKT) and tumour-suppressor genes (such as TP53 and PTEN), whereas others are components of the apoptotic machinery (such as survivin, XIAP and the BCL2 family). One model of cisplatin-mediated cytotoxicity is based on the recognition of DNA damage by MMR proteins and activation of p53. The activation of p53, in turn, leads to transcriptional upregulation of pro-apoptotic proteins such as BAX, BAK, CD95 and TRAIL (TNF-related apoptosis-inducing ligand), and downregulation of anti-apoptotic proteins such as BCL2, BCL-XL and IAPs, resulting in cell death via apoptosis [123]. Inactivation of p53 could therefore result in drug resistance. This hypothesis is supported by *in vitro* data in some cell lines [124]. The role of p53 has been extensively evaluated in clinical studies that correlate p53 status with response to chemotherapy and overall survival [125]. In addition, the threshold for apoptosis in response to chemotherapy-induced cellular damage is modulated by signalling through the PI3K, MAPK and protein kinase C (PKC) pathways [126]. In cell lines, activation of the PI3K pathway has been shown to correlate with resistance to cisplatin [127]. Amplifications of PI3K and activation of AKT have been found in 30-40% of ovarian tumours in some studies, and represent potential mechanisms of drug resistance in clinical practice [128]. Preclinical studies suggest that other proapoptotic signal transducers such as MAPK family members might also contribute to the cisplatin-resistant phenotype in ovarian cancer [129, 130]. In particular, it has been proposed that cisplatin-resistant cells

would fail to activate MAPK1 (also known as p38 MAPK) and c-JUN N-terminal kinase in a sustained fashion in response to cisplatin [129, 131]. Contrarily to the case of TP53, so far no correlation has been found between the levels of MAPKs or MAPK-related proteins and cisplatin sensitivity in patients [87]. Furthermore, Alterations in any of the factors that regulate and execute apoptosis, be it triggered by DNA damage or oxidative stress via the mitochondrial pathway or be it mediated by the extrinsic route, have the potential to influence cisplatin sensitivity [87]. In ovarian cancer, the upregulation of anti-apoptotic proteins such as BCL2, IAPs and the heat-shock proteins (HSP70 and HSP90), and downregulation of pro-apoptotic proteins such as BAX, have been shown to affect cisplatin sensitivity *in vitro*. The role of BCL2 and the IAP survivin in mediating cisplatin resistance is also supported by clinical studies [132, 133]. The inhibitors of survivin or BCL2 are currently being evaluated as single agents or in combination with cisplatin for the treatment of ovarian cancer [134, 135].

1.3.2.4 Mechanisms of off-target resistance

Accumulating evidence suggests that the cisplatin resistant phenotype can also be sustained (if not entirely generated) by alterations in signaling pathways that are not directly engaged by cisplatin, yet compensate for (and hence interrupt) cisplatin-induced lethal signals. The ERBB2 protooncogene (also known as HER2 or NEU), which codes for a member of the epidermal growth factor receptor family of tyrosine kinases, is overexpressed in ovarian cancers [136] and can signal via both the MAPK and PI3K pathways [137]. Activation of these pathways leads to phosphorylation of AKT and MAPK, which, in turn, phosphorylate BAD and BCL2 and inhibit apoptosis. *In vitro* overexpression of these receptors is associated with activation of the signalling pathways and resistance to cisplatin chemotherapy [138, 139]. In some clinical studies, there seems to be a correlation between overexpression of ERBB2 and poor prognosis in ovarian cancer [136]. Other general stress response pathways or poorly characterized mechanisms have been linked to cisplatin resistance in ovarian cancer include autophagy and dual-specificity Y-phosphorylation regulated kinase 1B (DYRK1B, also known as MIRK). Autophagy is an evolutionary conserved catabolic pathway that involves the sequestration and lysosomal degradation of organelles and portions of the cytoplasm [140]. Ovarian

cancer cells have been shown to progressively acquire cisplatin resistance while upregulating components of the autophagic pathway [141, 142]. DYRK1B is upregulated in multiple solid tumors [143] and exerts prosurvival functions by increasing the expression of antioxidant enzymes such as ferroxidase, superoxide dismutase 2 and superoxide dismutase 3 [144]. In ovarian cancer cells, DYRK1B depletion has been shown to potentiate the effects of subapoptotic cisplatin concentrations by favoring the establishment of lethal oxidative stress [145, 146].

Cisplatin is an important therapeutic tool in the combat against ovarian cancer. Unfortunately, cancer cells either intrinsically are or relatively rapidly become resistant to cisplatin, leading to relapse and therapeutic failure. The mechanisms responsible for cisplatin resistance are several, and contribute to the multifactorial nature of the problem. The elucidation of the mechanisms by which tumors become refractory to cisplatin will lead not only to optimal chemosensitization strategies, but also to the discovery of new prognostic and predictive biomarkers.

1.4 Salinomycin and cancers

Salinomycin (Sal) is a monocarboxylic polyether ionophore isolated from *Streptomyces albus* and has been shown to exhibit antimicrobial activity against gram-positive bacteria including mycobacteria and *Staphylococcus aureus*, some filamentous fungi, *Plasmodium falciparum*, and *Eimeria spp.*, protozoan parasites responsible for the poultry disease coccidiosis [147-149]. Thus salinomycin has been used for more than 30 years as an effective anticoccidial drug in poultry [150] and is also fed to ruminants and pigs to improve nutrient absorption and feed efficiency [151-153]. Very recently, salinomycin has been shown to kill human cancer stem cells and to inhibit breast cancer growth and metastasis in mice [154]. Salinomycin is also able to induce massive apoptosis in human cancer cells of different origins that display multiple mechanisms of drug and apoptosis resistance [155]. Therefore, at present salinomycin is considered to be a potential anticancer drug for cancer chemoprevention and cancer therapy.

1.4.1 Structure of salinomycin

In 1974, a new biologically active substance from the culture broth of *Streptomyces albus* (strain No. 80614) was isolated and termed salinomycin [148]. Salinomycin is a 751 Da monocarboxylic polyether antibiotic that constitutes a large pentacyclic molecule with a unique tricyclic spiroketal ring system and an unsaturated six-membered ring (Fig.5). It is a lipophilic, anionic and weakly acidic compound with the molecular formula $C_{42}H_{70}O_{11}$ [148, 156].

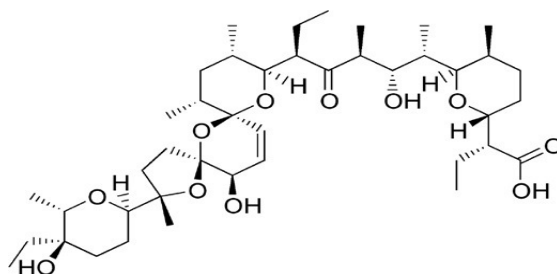


Fig.5 Structural formula of salinomycin. The pentacyclic molecule with a unique tricyclic spiroketal ring system has a mass of 751 Da, a molecular formula of $C_{42}H_{70}O_{11}$, a melting point of 113°C and a UV absorption at 285 nm. [Adapted from Miyazaki, Y., *et al.*: *J Antibiot*, 1974.]

Salinomycin and its salts exist in a pseudo-cyclic structure because of the formation of hydrogen bonds between the carboxylic group on the one side of the molecule and two hydroxyl groups on the opposite side (Fig.6) [157]. Owing to its lipophilic surface, polar inner core containing oxygen atoms and one carboxylic group, it is well suited for transporting monovalent cations, especially H^+ , Na^+ and great preference for potassium K^+ across lipid cytoplasmic and mitochondrial membranes [157]. The mechanism by which salinomycin interacts with coccidia and rumen microflora is well known. The normal physiological steady state of most living cells is dependent on the establishment of intracellular and extracellular level of Na^+ and K^+ . Intracellular concentration of K^+ is higher than that of Na^+ , and extracellular concentrations are respectively reversed. Salinomycin as a polyether ionophore can easily penetrate cellular membranes owing to its lipophilic properties and disrupt the Na^+/K^+ ion balance across cell membranes, which finally leads to the cell death [157].

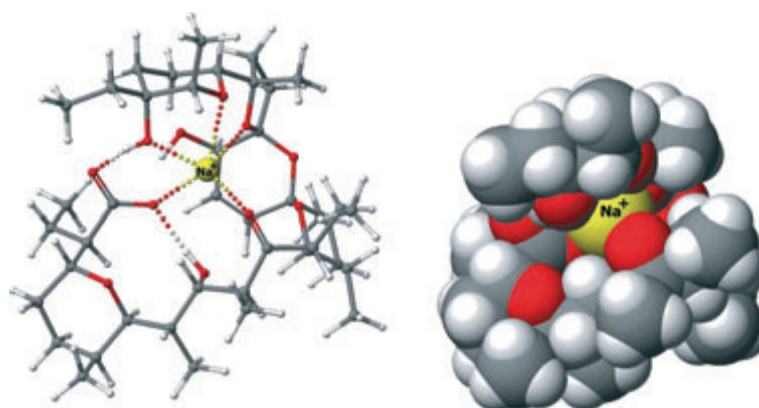


Fig.6 Structure of salinomycin sodium salt complex.
[Adapted from Huczynski, A.: *Chem Biol Drug Des*, 2012.]

1.4.2 Anticancer action of salinomycin

Cancer stem cells (CSCs) have been defined as cells within tumor that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor [158-160]. Cancer stem cells have been identified in a variety of human neoplasias, including cancers of the blood, breast, brain, bone, skin, liver, bladder, ovary, prostate, colon, pancreas and so on [161, 162]. It is often considered to be associated with chemo-

resistance and radio-resistance that lead to the failure of traditional therapies. Most therapies are directed at the fast growing tumor mass but not the slow dividing cancer stem cells. Eradicating cancer stem cells, the root of cancer origin and recurrence, has been thought as a promising approach to improve cancer survival or even to cure cancer patients [158].

In 2009, Gupta *et al.* [154] announced in the journal *Cell* that salinomycin, one of the antibiotics currently used in veterinary medicine, is a 100 times more effective killer of human breast cancer stem-like cells than paclitaxel (Taxol), a commonly used breast cancer chemotherapeutic drug. This study was very interesting and time-consuming, because about 16,000 compounds were screened for their ability to kill stem-like breast cancer cells (*i.e.* breast cancer cells passing through an epithelial-mesenchymal transition (EMT)) at a greater rate than the control cancer cells. The screen turned up 32 such compounds. The researchers winnowed the results down to the most promising and focused on one called salinomycin. Stem-like breast cancer cells treated with salinomycin were much less able to form new tumors when injected into mice [163]. Further, treatment of mice with salinomycin inhibits mammary tumor growth *in vivo* and induces increased epithelial differentiation of tumor cells. In addition, global gene expression analyses show that salinomycin treatment results in the loss of expression of breast CSC genes previously identified by analyses of breast tissues isolated directly from patients [154]. These findings strongly suggest that salinomycin is a selective killer of human cancer stem cells and a new promising agent for the elimination of cancer stem cells.

Discovery of the anticancer properties of salinomycin by Gupta *et al.* began an intensive research on these new properties. Susceptibility of CSCs to salinomycin also bolsters the possibility that this drug may target treatment-resistant advanced human cancers. A recent study revealed that salinomycin induces massive apoptosis in human cancer cells of different origin, but not in normal cells such as human T lymphocytes [155]. Moreover, salinomycin is able to induce apoptosis in cancer cells that exhibit resistance to apoptosis and anticancer agents by overexpression of Bcl-2, P-glycoprotein or 26S proteasomes with enhanced proteolytic activity. Salinomycin activates a distinct and unconventional pathway of apoptosis in cancer cells that is not accompanied by cell cycle

arrest, and that is independent of tumor suppressor protein p53, caspase activation, the CD95/CD95 ligand system and the 26S proteasome [155]. This might be one reason why salinomycin can overcome multiple mechanisms of drug and apoptosis resistance in human cancer cells. Many cancer cells harbor or acquire multiple mechanisms of apoptosis resistance mediated by the loss of p53 and overexpression of Bcl-2, P-glycoprotein or 26S proteasomes with enhanced proteolytic activity [164-166]. Salinomycin, however, appears to be capable of overcoming these mechanisms of drug and apoptosis resistance, suggesting a possible future use of salinomycin in the treatment of drug-resistant and aggressive cancers.

Recently, the *in vitro* anticancer activities of salinomycin have been validated against the lung cancer cell line A549 [167]. Expression of stem cell markers decreased significantly after 24-h treatment with salinomycin. All the results concerning salinomycin suggest that it is very promising lung cancer chemotherapeutic. Furthermore, salinomycin has also been reported to significantly inhibit stem-like gastric cancer cells with high aldehyde dehydrogenase (ALDH) activity. These findings will provide pivotal clue for selective chemotherapy on gastric carcinoma [168]. Moreover, one study found that salinomycin inhibited osteosarcoma by selectively targeting its stem cells both *in vitro* and *in vivo* without severe side effects. This finding supports the use of salinomycin for elimination of osteosarcoma stem cells and implies a need for further clinical evaluation [169].

The anticancer effect of salinomycin has been also observed by Dong *et al.* [170], who proved that salinomycin exhibited a significant toxicity toward human colorectal cancer (CRC) cell lines, HT29 (IC₅₀ ~ 8 μM ± 0.15) as well as SW480 (IC₅₀ ~ 10 μM ± 0.03). Furthermore, HT29 cells were more sensitive to salinomycin than oxaliplatin, a commonly used CRC chemotherapeutic drug. After treatment with salinomycin, the proportion of CD133⁺ subpopulations in human CRC HT29 and SW480 cells were reduced. In addition, salinomycin treatment decreased colony-forming ability and cell motility in HT29 cells. Further investigation showed that salinomycin might induce the mesenchymal-epithelial transition (MET) in HT29 cells. Therefore, this study demonstrates that salinomycin not only selectively targets CRC stem cells (*i.e.* 'CD133⁺' Cell

Subpopulations) specifically but also decreases malignant traits (invasion and migration) in CRC cell lines.

All findings from the above-mentioned studies strongly suggest that salinomycin has ability to kill cancer stem cells and apoptosis-resistant cancer cells, and would be of interest for development of anticancer drug. The number of possible questions, induced by a new discovery that a veterinary drug killed 100 times more CSCs than standard chemotherapy drugs, will be steadily growing.

1.4.3 Salinomycin-based combination cancer chemotherapy

A successful anticancer therapeutic system should eliminate both the differentiated cancer cells and the cancer stem cell population. Classical cytotoxic agents may deplete the bulk of a cancer but not the inherently chemoresistant CSCs, which ultimately recur and metastasize [171]. One ideal anticancer strategy would be to look for agents that target both the CSCs and non-CSCs within tumors. Alternatively, it may be preferable to develop combination therapies that apply agents with specific toxicity for CSCs together with agents that specifically target non-CSC populations within tumors. Therefore, the finding of targeting CSCs subpopulation should be improved the current treatments against highly aggressive, metastatic, recurrent, and lethal CSCs subpopulation [168]. Currently, salinomycin has been shown to inhibit tumor stem cells [154] and overcome drug resistance in human cancer cells [155]. These characteristics of salinomycin have the potential to be exploited to increasingly sensitize cells to anticancer drugs as part of combination chemotherapy.

The efficacy of combined treatments of salinomycin and gemcitabine in human pancreatic cancer cells were recently examined by Zhao's group, showing that salinomycin inhibited the growth of CSCs, while gemcitabine suppressed the viability of non-CSCs [172]. Furthermore, combined treatment led to a nearly complete abolishment of both CSCs and differentiated cells *in vitro*. Consistently, *in vivo* studies showed that salinomycin combined with gemcitabine could eliminate the engraftment of human pancreatic cancer more effectively than the individual agents. These data indicated that

salinomycin could be a promising agent for novel combination therapy for the treatment of human pancreatic cancers.

In 2011, Zhang *et al.* [173] developed octreotide (Oct)-modified paclitaxel (PTX)-loaded PEG-b-PCL polymeric micelles (Oct-M-PTX), which can enhance binding to somatostatin receptors (SSTR) positive human breast cancer MCF-7 cells, and salinomycin (SAL)-loaded PEG-b-PCL polymeric micelles (M-SAL), which can eradicate cancer stem cells. After the efficacy of combination therapy using Oct-M-PTX plus M-SAL were investigated *in vitro* and in the MCF-7 xenografts in mice, the results showed that the combination treatment was capable of producing a stronger inhibitory effect to the tumors by killing the breast cancer cells together with eliminating the breast cancer stem cells synchronously. Thus, this combination therapy may provide a potential strategy for the treatment of SSTR-overexpressing breast cancers by eradicating breast cancer cells together with breast cancer stem cells.

More recently, one study has demonstrated that targeting HER2 expressing tumors with anti-HER2 therapies (trastuzumab) will not necessarily eliminate cancer stem cells and may lead to a more aggressive cancer cell phenotype [174]. Combinatorial treatment of mammospheres with trastuzumab and salinomycin efficiently targets HER2^{high} cancer cells and cancer stem cell population, *i.e.*, HER2^{low} cells. Hence, this study opens a possibility for a new combinatorial treatment strategy for heterogeneous breast cancers.

During the last two years, three successive reports from Yoon's group have demonstrated that salinomycin sensitizes not only doxorubicin (DOX)-, etoposide (ETO)-, or radiation-treated cancer cells, but also antimitotic drugs-treated cancer cells [175-177]. The mechanisms underlying salinomycin sensitization to DNA-damaging compounds, radiation, and microtubule-targeting drugs could be similar and conserved. In addition, salinomycin also relatively sensitized verapamil (Ver, a well-known P-glycoprotein inhibitor)-resistant cancer cells [176]. These results may contribute to the development of Sal-based pharmacological combination therapy for cancer patients.

1.4.4 Mechanism of anticancer activity of salinomycin

The molecular mechanisms of anticancer action induced by salinomycin would be a potential interest for a development of anticancer drug. A more complete understanding of the salinomycin's anticancer mechanism could facilitate the therapeutic use of salinomycin in cancer patients. It was shown in a high-throughput screen that salinomycin was a highly effective agent in the elimination of CSCs and could be used as an anticancer drug, but scientists had to admit that the mechanism of action of this compound is unclear [154]. Recently, Fuchs *et al.* reported that salinomycin induces apoptosis and causes growth inhibition in diverse types of apoptosis- and chemotherapeutic-resistant cancer cells [155]. Salinomycin-mediated apoptosis in these cells is independent of known mediators of the cell death signal pathway, such as the p53 tumor suppressor protein, the 26S proteasome and the CD95/CD95 ligand system. Further study has showed that salinomycin triggers apoptosis by overcoming ATP-binding cassette (ABC) transporter-mediated multidrug resistance, as was observed in the case of KG-1a human leukemia stem cell-like cells [178]. One of the most important mechanisms of drug resistance in leukemia stem cells and other cancer stem cells is the expression of ABC transporters belonging to a highly conserved superfamily of transmembrane proteins capable of exporting a wide variety of molecules and structurally unrelated chemotherapeutic drugs from the cytosol, thereby conferring multidrug resistance, which is a major obstacle to the success of cancer chemotherapy [179-181]. As shown in the study, KG-1a cells which are expressed functional ABC transporters such as P-glycoprotein (P-gp), BCRP and MRP8, are highly sensitive to apoptosis induction by salinomycin. Moreover, salinomycin does not permit long-term adaptation of KG-1a cells to apoptosis-inducing concentrations. Thus, salinomycin should be regarded as a novel and effective agent for the elimination of leukemia stem cells and other tumor cells exhibiting ABC transporter-mediated multidrug resistance.

Studies performed in 2011 [182] showed that salinomycin induces apoptosis of prostate cancer cells by elevating oxidative stress through intracellular reactive oxygen species (ROS) production, which is accompanied by decreased mitochondrial membrane potential (MMP), translocation of BAX protein to mitochondria, cytochrome c release to the cytoplasm, activation of the caspase-3 and cleavage of PARP-1 in androgen-independent, chemotherapeutic-refractive PC-3 human prostate cancer cells. These results

are the first to link elevated oxidative stress and mitochondrial membrane depolarization to salinomycin-mediated apoptosis of prostate cancer cells. In addition, another recent report [183] also indicates that salinomycin inhibits prostate cancer cell growth and migration by reducing the expression of key prostate cancer oncogenes, inducing oxidative stress, decreasing the antioxidative capacity and cancer stem cell fraction. Moreover, salinomycin exhibits significant growth inhibition and induction of apoptosis in human ovarian cancer cells *in vitro* and *in vivo*. Salinomycin-induced apoptosis in human ovarian cancer cells might be associated with activating p38 MAPK [184].

Of note, salinomycin recently has been reported to inhibit Wnt signaling and selectively induce apoptosis in chronic lymphocytic leukaemia cells [185]. The Wnt/ β -catenin signaling pathway drives stem cell self-renewal and is involved in the pathogenesis of various types of cancer. Aberrant activation of the Wnt signaling pathway in normal stem cells can promote their transformation into CSCs [171, 186, 187]. In this study, salinomycin potently inhibits proximal Wnt/ β -catenin signalling and blocks the phosphorylation of the Wnt coreceptor lipoprotein receptor related protein 6 (LRP6) and induces its degradation. These results suggest that salinomycin as an inhibitor of Wnt/ β -catenin signaling acts on the Wnt/Fzd/LRP complex and the anticancer effects of salinomycin may be at least partially attributable to Wnt inhibition. More recently, it has also been found that Wnt/ β -catenin signaling pathway may be involved in the targeting of salinomycin on osteosarcoma stem cells [169].

Identifying mechanisms underlying salinomycin sensitization of cancer cells would also be an important step in developing Sal-based pharmacological combination cancer therapy. Recently performed studies have shown that salinomycin can function as a P-gp inhibitor to overcome apoptosis resistance in human cancer cells, including leukaemia stem cell-like cells [178, 188]. Efflux pump P-gp can increase the removal of anticancer drugs from the cell to reduce cellular damage. Treatment of the multidrug resistance (MDR) cell lines with salinomycin restored a normal drug sensitivity of these cells. Another study [176] demonstrates that salinomycin could utilize another type of P-gp substrate independent of Verapamil (Ver, a well-known P-gp inhibitor), and salinomycin more effectively inhibits

P-gp in cancer cells than Ver. In addition, salinomycin sensitized Ver-resistant cancer cells, indicating that salinomycin may be useful for treating Ver-resistant cancers.

The data from three successive reports [175-177] have demonstrated that salinomycin can sensitize DOX-, ETO-, radiation-, or antimetabolic drugs-treated cancer cells through a similar and conserved mechanism, *i.e.* increasing DNA damage and reducing p21 protein levels. Conservation of a sensitization mechanism assumes that salinomycin targets the same molecules, even when it is used in different combinations with various anticancer treatments. Identifying molecules consistently targeted by salinomycin would be beneficial for developing effective anticancer treatments [176]. In addition, salinomycin also can sensitize cancer cells to antimetabolic drugs by increasing apoptosis, preventing both G2 arrest and aneuploidy, and reducing cyclin D1 protein levels [177]. These findings may contribute to the development of Sal-based combination therapies for cancer patients.

Overall, the mechanism of anticancer activity of salinomycin is probably complicated but most likely it will soon be explained. The studies about the anticancer activity and mechanisms of salinomycin will increase in the near future.

1.4.5 Toxicity of salinomycin

Although salinomycin has been widely used as coccidiostat poultry and livestock to improve feeding efficiency for more than 30 years [151, 189], its use as a feed additive is no longer authorized in the European Union and was never approved in the United States [190]. Salinomycin can cause severe neural and muscular toxicity when accidentally fed to animals in relatively high doses, as described for chickens [191], turkeys [192], cats [193], pigs [194], alpacas [195], and horses [196]. Salinomycin as a positive inotropic and chronotropic agent has been reported to increase cardiac output, left ventricular systolic pressure, heart rate, mean arterial pressure, coronary artery vasodilatation and blood flow, and plasma catecholamine concentrations as demonstrated in dogs receiving an intravenous injection of 150 $\mu\text{g}\cdot\text{kg}^{-1}$ salinomycin [197]. Besides, salinomycin intoxication in human beings has been reported recently [190, 198]. Accidental ingestion of an estimated 1 mg/kg of salinomycin resulted in a 6-week hospital admission with prolonged rhabdomyolysis, pain, and disability [198]. Other clinical manifestations included dizziness, nausea,

vomiting, stomachache, diarrhea, limb anesthesia, weakness, and dark red to brown urine [190].

Salinomycin has never been used as a drug in humans, probably due to the considerable toxicity observed in mammals [193, 194, 198, 199]. Additionally, up to now, the precise mechanism of salinomycin-mediated toxicity is unknown. Effects of salinomycin on the human organisms have also not been examined [157]. In 2011, one study [200] proved that salinomycin in concentrations effective against CSCs exerts profound toxicity towards nervous cells (dorsal root ganglia as well as Schwann cells). This toxic effect is mediated by elevated cytosolic Na^+ concentrations, which in turn cause an increase in cytosolic Ca^{2+} by means of $\text{Na}^+/\text{Ca}^{2+}$ exchangers (NCXs) in the plasma membrane as well as the mitochondria. Therefore, salinomycin strongly reduces cell viability by means of calpain and cytochrome c-mediated caspase 9 and subsequent caspase 3 activation [200]. These findings expand the knowledge of the mechanisms involved in the pathogenesis of salinomycin-induced peripheral neuropathy and provide a mechanism for neuroprotection through inhibition of mitochondrial NCXs. Thus new strategies for a clinical translation of salinomycin therapy may be developed [200]. In view of the severe toxicity of salinomycin, future studies with salinomycin in humans should be designed carefully.

In summary, recently the potassium ionophore antibiotic salinomycin has been shown to kill human cancer stem cells, sensitize cancer cells to anticancer drugs, and induce apoptosis in drug-resistant cancer cells. One important caveat for the potential clinical use of salinomycin is its severe toxicity. All the aforementioned studies will prompt scientists to search for a new group of salinomycin derivatives which will be more effective in coordination of biologically important metal cations and less toxic especially for humans. A new chapter in chemistry and biology of ionophores has been opened, and the investigation of the structure, safety, toxicity, pharmacology and anticancer activity of this group of compounds in humans is a challenge for the coming years.

2. AIM OF THE STUDY

Ovarian cancer remains a leading cause of death from gynecological malignancy [201]. The incidence of ovarian cancer increases with age and 70% of patients present with advanced disease. Current standard of care including surgery and chemotherapy has had very limited success in treatment of the patients diagnosed with late stage disease [202, 203]. Long-term administration of cisplatin has been shown to result in the development of chemotherapeutic drug resistance in the cancer cell population [204, 205]. Therefore, searching for alternative agents to overcome chemoresistance during the treatment of ovarian cancer is essential. Salinomycin, a polyether ionophore antibiotic that has recently been shown not only to kill human breast cancer stem cell-like cells [154], but also to induce apoptosis and overcome multiple mechanisms of resistance to apoptosis in human cancer cells [155]. These results strongly suggested that salinomycin should be regarded as an anticancer compound.

The major aims of this study were:

- To evaluate *in vitro* anti-tumoral properties of salinomycin in human ovarian cancer cell lines (especially in cisplatin-resistant human ovarian cancer cell line C13 and its parent cisplatin-sensitive OV2008 cells) and to investigate the effects of salinomycin on tumor cell growth, apoptosis and cell cycle parameters.
- To establish human ovarian cancer cell lines (OV2008 or C13) xenograft tumor animal model, to observe the therapeutic effect of salinomycin in human ovarian cancer cell line xenotransplanted cancer *in vivo*, and to detect apoptosis in tumor tissue *in situ*.
- To investigate the effect of salinomycin on phosphoproteins levels in ovarian cancer cell lines (OV2008 and C13), to better understand the signal pathways involved in salinomycin-induced growth-inhibitory effect and apoptosis in ovarian cancer cell lines, and to derive mechanistic insights into the action of salinomycin.

Materials and methods used in this study were described in detail in each manuscript. The experimental work were mainly based on: 1) *in vitro* human ovarian cancer cell lines culture, including cisplatin-resistant human ovarian cancer cell lines; 2) *in vivo* human ovarian cancer cell lines (OV2008 or C13) murine xenograft model.

3. PUBLISHED MANUSCRIPTS AND SUPPLEMENTARY DATA

3.1 Published research manuscript

Title: Effects of salinomycin on human ovarian cancer cell line OV2008 are associated with modulating p38 MAPK

Authors: Bei Zhang, Xueya Wang, Fengfeng Cai, Weijie Chen, Uli Loesch, Johannes Bitzer and Xiao Yan Zhong

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Summary: Ovarian cancer remains a leading cause of death from gynecological malignancy. The therapeutic effect of ovarian cancer is undesirable. Salinomycin is a polyether ionophore antibiotic that has recently been reported as a selective inhibitor of cancer stem cell and is considered to be a potential anticancer compound for cancer chemoprevention and cancer therapy. In this study, we investigated the anticancer effect and mechanism of salinomycin on human ovarian cancer cell line OV2008 *in vitro* and *in vivo*. The results of this research demonstrated that salinomycin is a potent compound against human ovarian cancer cell line OV2008 *in vitro* and indicates significant *in vivo* efficacy in tumor (OV2008) xenograft model. Salinomycin can inhibit the growth of ovarian cancer cell line OV2008 efficiently through induction of apoptosis, which is not accompanied by cell cycle arrest, but possibly is associated with activating p38 MAPK and merits further investigations.

Author contributions:

Bei Zhang was involved in experimental design, performing the experiments, data analysis and writing the manuscript.

Effects of salinomycin on human ovarian cancer cell line OV2008 are associated with modulating p38 MAPK

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Abstract

Objective. To investigate the anticancer effect and mechanism of salinomycin, a selective inhibitor of cancer stem cell, on human ovarian cancer cell line OV2008 *in vitro* and *in vivo*.

Methods. The growth inhibitory effect of salinomycin on ovarian cancer cell line OV2008 was determined by measuring cell viability using the resazurin reduction assay. Apoptotic nuclear morphology was visualized by DAPI staining technique. The percentages of apoptotic cells and cell cycle parameters were detected by flow cytometry. The activation of p38 MAPK was analyzed by Bio-Plex phosphoprotein assay. *In vivo* activity of salinomycin was assayed through tumor growth.

Results. Salinomycin caused concentration- (0.01 μ M-200 μ M) and time-dependent (24-72hr) growth inhibitory effects in OV2008. Cell nuclear morphology observations showed that salinomycin-treated OV2008 cells displayed the typical apoptotic characteristics. Salinomycin significantly increased the percentages of apoptotic cells in OV2008, showing a concentration- and time-dependent manner. There was no cell cycle arrest in the G1/G0, S and G2/M phases between salinomycin-treated cells and control cells. Salinomycin also enhanced the phosphorylation of p38 MAPK. Moreover, salinomycin significantly inhibited the growth of the ovarian xenograft tumors.

Conclusion. Salinomycin exhibited significant growth-inhibition and induction of apoptosis in human ovarian cancer cell line OV2008. The data suggested that salinomycin-induced apoptosis in OV2008 might be associated with activating p38 MAPK and merits further investigations.

Keywords: Salinomycin; Ovarian cancer; Growth inhibition; Apoptosis; p38 MAPK; Tumor xenografts

Introduction

Salinomycin is a 751 Da monocarboxylic polyether antibiotic belonging to the group of ionophores that isolated from *Streptomyces albus* (strain No. 80614) [1]. Salinomycin acts in different biological membranes, including mitochondrial and cytoplasmic membranes, as an ionophore with a stringent selectivity for monovalent cations and a considerable preference for potassium ions [2,3]. Salinomycin exhibits a large-spectrum antimicrobial activity including anticoccidial property [4,5]. It is commonly used as a coccidiostat in poultry and other livestock and is fed to ruminants to improve nutrient absorption and feed efficiency [6]. Recently, salinomycin has been reported to selectively deplete human breast cancer stem cells from tumorspheres and to inhibit the mammary tumor growth and metastasis *in vivo* [7]. Another recent report showed that salinomycin induces apoptosis in human cancer cells, including those that display wild-type p53 or p53 mutation and multi-drug resistance due to overexpression of Bcl-2, P-glycoprotein or 26S proteasomes with deregulated proteolytic activity [8]. These results strongly suggested that salinomycin should be regarded as an anticancer compound. The mechanism of anticancer action of salinomycin is not completely understood. One study showed salinomycin activates a particular apoptotic pathway not accompanied by cell cycle arrest and independent of tumor suppressor protein p53, caspase activation, the CD95/CD95L system and the proteasome [8]. More recently, salinomycin was reported to overcome ABC transporter-mediated multidrug and apoptosis resistance [9] and act as a potent inhibitor of multidrug resistance gp170 [10]. Furthermore, a recent study uncovered that salinomycin inhibits the activity of the Wnt signaling pathway, recently appointed as an essential regulator of CSC (cancer stem cell) properties in chronic lymphocytic leukemia cells [11].

Ovarian cancer remains a leading cause of death from gynecological malignancy, with more than 204,000 new cases and 125,000 deaths each year, accounting for 4% of all cancer cases and 4.2% of all cancer deaths in women around the world [12]. The high mortality rate of women with ovarian cancer has been attributed both to lack of early detection and to development of chemoresistance during treatment [13,14]. Current standard of care including surgery and chemotherapy has had very limited success in

treatment of the patients diagnosed with late stage disease [15,16]. The aims of this study were (1) to determine the anticancer biological activity of salinomycin toward human ovarian cancer cell line OV2008; (2) to derive mechanistic insights into the action of salinomycin; and (3) to determine whether salinomycin would significantly inhibit tumor growth in an *in vivo* model of ovarian cancer. The studies were conducted using human ovarian cancer cell line OV2008 and its murine xenograft model. The results showed salinomycin inhibited cell-growth and induced apoptosis in ovarian cancer cell line OV2008 *in vitro* and suppressed tumor growth *in vivo* as well. The salinomycin-induced apoptosis in ovarian cancer cell line OV2008 could be mediated through an increase in the activation of p38 MAPK.

Materials and Methods

Cell line and culture

The OV2008 human epithelial ovarian cancer cell line was kindly supplied by Dr. Gaetano Marverti (University of Modena and Reggio Emilia, Italy) and routinely grown in humidified condition at 5% CO₂ and 37°C, incubated with RPMI 1640 standard medium supplemented with 10% fetal bovine serum (FBS), antibiotics (100IU/ml penicillin and 100µg/ml streptomycin) and L-glutamine (2mM). Exponentially growing cells were used throughout the study. All these reagents were from Invitrogen (Carlsbad, CA).

Growth inhibition assay

The growth inhibitory effect of salinomycin on OV2008 was determined by measuring cell viability using the resazurin reduction assay. Briefly, Cells were seeded in 100µl media in 96-well microtitre plates at a density of 5000/well. Following overnight incubation, cells were exposed to a range of different concentrations of salinomycin (Sigma-Aldrich, S4526 and 0.1% DMSO as solvent control) and grown at 37°C under a 5% CO₂ atmosphere for 24-72hr. 5µl of 0.02% (w/v) Resazurin (Sigma-Aldrich, R7017) in phosphate buffered saline (PBS) was then added to each well and incubation was continued for an additional 2hr. Finally, fluorescence was read using a spectramax GEMINI XS microplate reader ($\lambda_{exc}=544nm$, $\lambda_{em}=590nm$).

Cell nuclear morphology observations

Exponentially growing cells were incubated with salinomycin for 12hr, 24hr and 36hr, respectively, and equal volumes of solvent (0.1% DMSO) as control. Apoptotic nuclear morphology was visualized by DAPI staining technique. Cells (1×10^5) were collected on the slide using cytospin, then fixed with 3.7% of paraformaldehyde (#28906, Pierce) for 15min at room temperature, washed three times with PBS and immersed in 0.1% of Triton X-100 (Sigma-Aldrich, T8787) for 15min. Thus, paraformaldehyde-fixed cells were stained using 1:1000 DAPI (4, 6-diamino-2-phenylindole; 1 mg/ml in ddH₂O; Invitrogen, D29410) in ddH₂O for 5 minutes under dark at room temperature. After three times of washing with PBS, cells were coverslipped with a fluorescence mounting medium (Dako, S3023) and visualized using fluorescence microscope (Olympus B×51, Japan).

Cell apoptosis detection

Cell apoptosis was studied by using the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit (BD pharmingen) in combination with flow cytometry (CyAn ADP, Dako). After cells were incubated with salinomycin for 12hr, 24hr and 36hr, respectively, and with solvent control (0.1% DMSO) as well, they were harvested by quick trypsinization to minimize potentially high annexin V background levels in adherent cells. Cells were then washed twice with cold PBS and re-suspended in binding buffer at a concentration of 1×10^6 cells/ml. 100µl cells were taken to stain with 5µl annexin V/FITC and 5µl PI and incubated in dark at room temperature for 15min. Then 400µl binding buffer was added before cells were analyzed by flow cytometry. Cells negative for both annexin V and PI are viable, annexin V⁺/PI cells are in early apoptosis, and annexinV⁺/PI⁺ cells are necrotic or in late apoptosis. The percentages of apoptotic cells were analyzed by Flowjo software.

Cell cycle distribution analysis

To evaluate cell cycle profile, cells (about 1×10^6 cells), pretreated with salinomycin for 12hr and 24hr (0.1% DMSO as a solvent control), were harvested, washed twice with PBS, then fixed and stored in ice-cold 70% (v/v) ethanol at -20°C. Prior to analysis, samples

were washed again with PBS and then incubated in propidium iodide/Rnase staining buffer (BD pharmingen) at room temperature in the dark for at least 15 min. After filtration to remove cellular debris, the single-cell suspensions were analyzed on a flow cytometer. Cell cycle parameters were analyzed using Flowjo software.

Phosphoprotein assay

Phosphoprotein was measured in duplicate using a bead-based multiplex assay (Bio-Plex Phosphoprotein Detection, Bio-Rad, Hercules, USA), according to the manufacturer's instructions [17,18]. After OV2008 cells were cultured with salinomycin or with solvent control (0.1% DMSO) for the indicated time interval, cells were rinsed with ice-cold cell wash buffer and then lysed in lysing solution. The lysate was collected and centrifuged at 4500g for 20 min at 4°C. The protein concentration was measured and calculated with a DC (detergent compatible) protein assay (Bio-Rad). The Bio-Plex assay was applied to detect and quantify phosphoproteins of p38 mitogen-activated protein kinase (p38 MAPK). The prepared first antibody with coupled beads was captured under 96-well plates, and then samples (15 µg proteins each) were incubated with the coupled beads overnight at room temperature. On the next day, after washing, the samples were incubated with biotin-labelled detection antibodies followed by further incubation with the PE-labelled streptavidin reporter. The level of phosphoproteins bound to the beads was indicated by the intensity of the reporter signal. The signal was acquired and analyzed using Bio-Plex Manager software (Bio-Rad) interfaced with a Bio-Plex 200 system (Bio-Rad). In this assay, the lysates of Phosphatase-Treated HeLa cells and UV-Treated HEK293 cells, provided by the Bio-Plex phosphoprotein assay, were used as the background control and Phospho-p38 MAPK (Thr180/Tyr182) positive control, respectively. This experiment was repeated in duplicate.

Ovarian cancer tumor xenografts in mice

Female mice of NOD/SCID were in-house breeding from the Animal Center (Tierversuchsstation) at the Department of Biomedicine, University Hospital of Basel and used at 6 weeks of age. All mouse procedures were approved by Cantonal Veterinary

Office (Kantonales Veterinäramt) and performed in accordance with the regulations concerning animal experiments. For *in vivo* salinomycin treatment study, cultured OV2008 cells (2×10^6 cells per mouse in 0.1ml saline) were subcutaneously injected into the back of NOD/SCID mice. On the day after tumor cells injection, mice were divided into two groups of 5 mice each. Treatment was initiated 24hr after injection. The two experimental groups were administrated with salinomycin (5mg/kg) [7] and 5% ethanol (vehicle), respectively, by intraperitoneal injection on every other day for 3 weeks. The size of the tumor was measured every 2 days using a digital vernier caliper. Tumor volume was estimated by the following formula: $\text{volume} = (a \times b^2) \times \pi/6$, where a and b are major and minor axes of the tumor.

Statistical analysis

All data were expressed as mean values \pm standard deviation. Growth-inhibitory curve were analyzed using GraphPad Prism 5.01 Software. Comparisons among groups were performed by Student's t-test. The significance level was set at $P < 0.05$.

Results

Growth-inhibitory effect of salinomycin on ovarian cancer cell line OV2008

The growth-inhibitory effect of salinomycin against OV2008 cell line is shown in Fig.1. The effect of incubation time and concentration on viability of OV2008 cells by salinomycin was studied. Cells were exposed for 24, 48 or 72hr to salinomycin at (0.01 μ M-200 μ M) concentration range, and cell viability measured by the resazurin reduction assay. In this study, salinomycin inhibited the growth of OV2008 cells in a concentration- and time-dependent pattern. IC₅₀ (95% confidence interval) of salinomycin on OV2008 cell line for 24hr, 48hr and 72hr was 7.44 (6.80 to 8.14), 4.78 (4.12 to 5.55) and 3.20 (2.90 to 3.53), respectively.

Effect of salinomycin on cell morphology and nuclear change

Treating OV2008 cells with 20 μ M or 50 μ M salinomycin for 12hr, 24hr and 36hr, respectively, resulted in the detachment of cells from the tissue culture plates as well as cell death. In order to examine whether cells died due to apoptosis mechanisms, the nuclear morphological changes were observed under fluorescence microscope. The nuclei in control cells exhibited equal distribution of the chromatin, while salinomycin-treated cells showed the characteristic morphologic changes of apoptosis, such as condensed chromatin, nuclear fragmentation and blebbing (Fig.S1). Therefore, these morphological changes suggested the occurrence of apoptosis in OV2008 cells after treated with salinomycin.

Effect of salinomycin on tumor cell apoptosis and cell cycle

Salinomycin-treated OV2008 cells were analyzed by flow cytometry which can distinguish between early apoptosis or late apoptosis of cells after cells were stained simultaneously with annexin V and PI. Compared to control, salinomycin treatment significantly increased the percentages of apoptotic cells in OV2008, showing a concentration- and time-dependent manner (Fig.2A). In control culture, 3.36 \pm 0.04% cells were in early apoptosis stage whereas 10.09 \pm 0.16% cells were in late apoptosis stage. After cells were treated with 50 μ M salinomycin for 12hr, the percentages of apoptotic cells at early phase increased to 6.95 \pm 1.82% and that of late phase increased to 13.03 \pm 0.38%. Whereas, when cells treated with salinomycin for 24 and 36hr, 9.70 \pm 1.77% and 9.35 \pm 1.79% cells were in early apoptosis, and 24.5 \pm 1.71% and 30.53 \pm 1.55% cells were in late apoptosis. A time-dependent increase in the number of apoptotic cells was observed (Fig.2B). These results clearly indicate that salinomycin evoked apoptosis in OV2008 cells.

Effect of different concentrations of salinomycin (20 and 50 μ M) on the cell cycle phases was investigated in OV2008 cells cultured over different times (12 and 24hr) by DNA content analysis, by flow cytometry. The results showed that the percentages of the cell population in the sub-G1 phase were significantly higher in salinomycin-treated OV2008 cells with a concentration-dependent fashion, whereas the percentages of cells in other phases (G1/G0, S and G2/M phases) were almost reduced, in comparison with control (Fig.3). These effects were similar at 12 and 24hr (Fig.3). The dramatic accumulation of cells in sub-G1 phase was another marker for apoptosis, which further

confirmed the results of annexin V/PI assay. Additionally, there was no cell cycle arrest in G1/G0, S and G2/M phases between salinomycin-treated cells and control cells, suggesting that salinomycin inhibits the cellular proliferation of OV2008 cells not accompanied by cell cycle arrest.

Effect of salinomycin on phosphorylation of p38 MAPK in OV2008 cells

To investigate the effect of salinomycin on p38 MAPK activity in ovarian cancer cell line OV2008, the regulation of p38 MAPK phosphorylation by salinomycin was examined using Bio-Plex phosphoprotein assay. The results showed phosphorylation of p38 MAPK in OV2008 cells was enhanced by salinomycin (20 μ M) after 12, 24 and 36hr of incubation (Fig.4A), while a marked concentration-dependent increase in the p38 MAPK phosphorylation was observed following salinomycin exposure for 24hr (Fig.4B). These findings suggest that salinomycin-induced growth-inhibitory effect and apoptosis in OV2008 could be mediated through the alteration of phosphorylation of p38 MAPK.

Evaluation of antitumor activity of salinomycin in vivo

Based on the *in vitro* results, which showed significant cytotoxicity of salinomycin to human ovarian cancer cell line OV2008, the *in vivo* antitumor efficacy of salinomycin was further evaluated in a human ovarian tumor xenograft grown in the back of mice. The mice were treated with salinomycin and the change in tumor volume after first injection was followed for 21 days (Fig.5A). Compared with vehicle-treated controls, a significant reduction in the tumor volume was observed in the mice treated with salinomycin (Fig.5C). When the test came to the end, in OV2008 tumor model, the tumor volume of salinomycin therapy groups and controls was 122.3 ± 41.4 mm³ and 252.2 ± 55.29 mm³, respectively ($P<0.01$; Fig.5B).

Discussion

The present study demonstrated that salinomycin inhibited the growth of human ovarian cancer cell line OV2008 *in vitro* and *in vivo*. The growth inhibition effects of salinomycin

and salinomycin-induced apoptosis in human ovarian cancer cell line OV2008 could correlate with modulating p38 MAPK.

Apoptosis, or programmed cell death, is an important homeostatic mechanism that balances cell division, cell death and maintains the appropriate cell number in the body [19]. Therefore, searching for agents which trigger apoptosis of tumor cells has become an attractive strategy in anticancer drug discovery [20]. Apoptosis is characterized morphologically by cell shrinkage and loss of contact with neighboring cells, formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing, chromatin condensation, and formation of apoptotic bodies [21]. In the present investigation, after OV2008 cells were treated with salinomycin, the fluorescence microscopic observations clearly indicated these apoptotic characteristics (Fig.S1). Furthermore, flow cytometry results, from both annexin V/PI assay (Fig.2) and sub-G1 populations in cell cycle analysis (Fig.3), further evidenced a concentration- and time-dependent increase in the percentage of apoptotic subpopulations after salinomycin treatment. These results provided evidence that salinomycin triggered apoptosis in OV2008 cells, which resembles previous report on various human cancer cells such as leukemia cells [8,9].

Cell cycle control plays a critical role in the regulation of tumor cell proliferation. Many anticancer agents and DNA-damaging agents arrest the cell cycle at the G0/G1, S, or G2/M phase and then induce apoptotic cell death [22,23]. From the results of present study, it appeared that no cell cycle arrest in G1/G0, S and G2/M phases was observed between salinomycin-treated OV2008 cells and control cells (Fig.3), which confirms previous finding that salinomycin activates a particular apoptotic pathway not accompanied by cell cycle arrest [8].

To better understand the signal pathways involved in salinomycin-induced growth-inhibitory effect and apoptosis in OV2008, we investigated the possible involvement of p38 MAPK activity. To address this issue, phosphorylation of p38 MAPK was determined by Bio-Plex assays with Luminex technology, which contain dyed beads conjugated with monoclonal antibodies specific for a target protein or peptide such as a cytokine or a phosphoprotein. The antibodies used in these assays undergo rigorous optimization to

ensure the highest degree of sensitivity, specificity, and reproducibility. Recently, using optimized standard operating procedures regarding sample size and total protein concentration range and monoclonal antibodies used for immunoanalysis, and on the basis of the US Food and Drug Administration guidelines, Bio-Plex phosphoprotein array intra-assay and inter-assay coefficients of variation revealed good reproducibility of the technique and the results achieved using Bio-Plex phosphoprotein array analyses significantly correlated ($P < 0.001$) with those obtained with numerized western blot analyses [18]. Furthermore, Bland-Altman analyses clearly demonstrated that Bio-Plex phosphoprotein array could be used instead of western blot providing a unique way of analyzing multiple phosphoprotein expression in small specimens.

The p38 MAPK pathway is implicated in cancer cell apoptosis and is induced by several chemotherapeutic drugs [24,25]. We found there are marked time-dependent and concentration-dependent increases in the phosphorylation of p38 MAPK following salinomycin treatment in OV2008 cells (Fig.4). This result suggests that the activation of p38 MAPK appears to contribute to the proapoptotic effect of salinomycin in OV2008 cells and that the activation of the p38 MAPK pathway might play a causal role in the salinomycin-induced apoptosis in ovarian cancer cell line OV2008. However, detailed downstream and upstream signaling molecules of p38 MAPK modulated by salinomycin are not known and warranted further investigations.

In the present study, the xenografts of human OV2008 ovarian cancer model showed very good efficacy when treated with salinomycin (Fig.5). Although, we have not yet attempted to ascertain the mechanism of cell death in the xenograft tumor model, it remains possible that cell apoptosis induced by salinomycin may account for some of the observed reduction in tumor growth rate and needs further investigations. Additionally, considering one ovarian cell line was involved in the present study, we also believe that further *in vitro* and *in vivo* studies with salinomycin in different characterized ovarian cancer cell lines, such as p53 mutation cell lines, drug-resistant (MDR overexpression) cell lines are warranted to enhance our understanding of this promising antitumorigenic compound.

Overall, the results of this research demonstrated that salinomycin is a potent compound against human ovarian cancer cell line OV2008 *in vitro* and indicates significant *in vivo* efficacy in tumor (OV2008) xenograft model. Salinomycin can inhibit the growth of ovarian cancer cell line OV2008 efficiently through induction of apoptosis, which is not accompanied by cell cycle arrest, but possibly is associated with activating p38 MAPK and merits further investigations.

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Conflict of interest statement

No potential conflicts of interest were disclosed

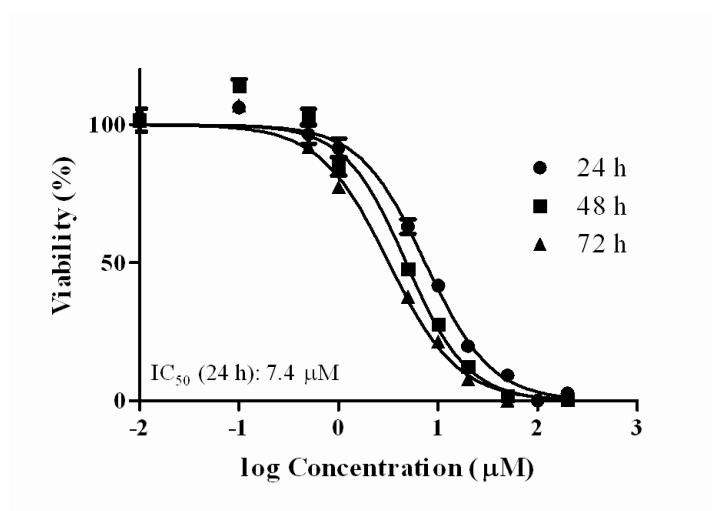


Fig.1 *Effect of salinomycin on cell viability in human ovarian cancer cell line OV2008* Cells were exposed to salinomycin at concentrations (0.01-200μM, 0.1% DMSO as solvent control) for 24, 48 and 72hours and cell viability measured by resazurin reduction assay. Results are Mean±SD of quadruplicates from one of three independent experiments

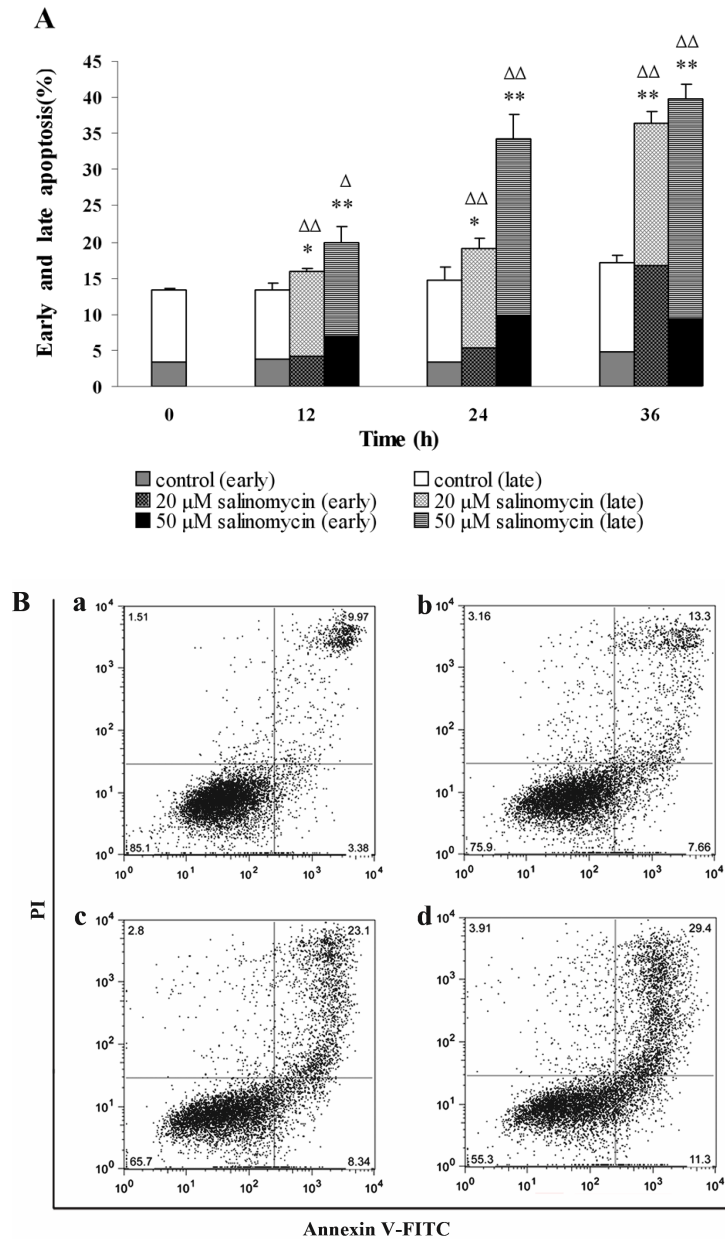


Fig.2 Effect of salinomycin on cell apoptosis in OV2008 cells After cells were treated with 20 μ M or 50 μ M salinomycin for 12hr, 24hr and 36hr, respectively, and with solvent control (0.1% DMSO) as well, the percentage of apoptotic subpopulations (A) was determined by flow cytometry analysis based on mean values obtained from three independent experiments. Results are expressed as Mean \pm SD. ‘*’ and ‘***’: Significant difference from control of time point ($P<0.05$ and $P<0.01$, respectively). ‘ Δ ’ and ‘ $\Delta\Delta$ ’: Significant difference from 0hr control ($P<0.05$ and $P<0.01$, respectively). (B) represent flow cytometry results showing a time-dependent increase in the number of apoptotic cells after cells were treated with 50 μ M salinomycin for 12hr (b), 24hr (c), 36hr (d) and 0hr as control (a)

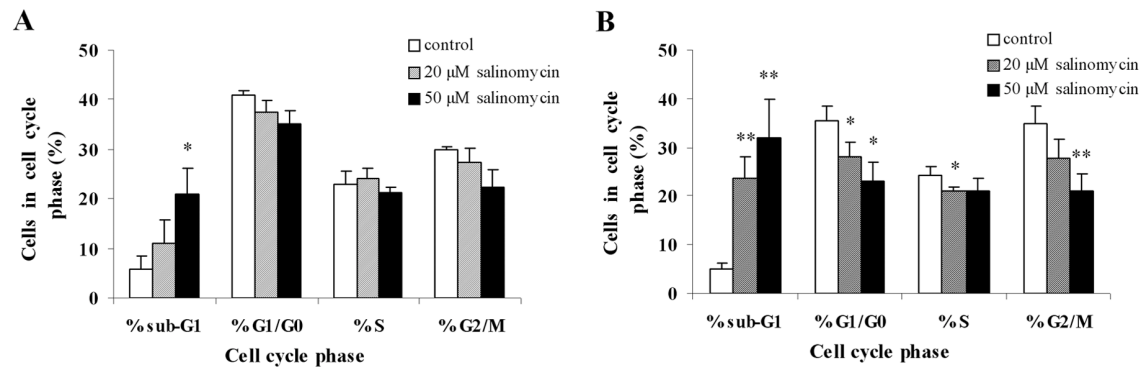


Fig.3 Effect of salinomycin on cell cycle distribution in OV2008 cells Cells were treated with 20 μ M or 50 μ M salinomycin for 12hr (A) and 24hr (B), and with 0.1% DMSO as a solvent control. The percentages of each cell cycle were evaluated by flow cytometry based on mean values obtained from three independent experiments. Results are expressed as Mean \pm SD. '*' and '**': Significant difference from control (P <0.05 and P <0.01, respectively)

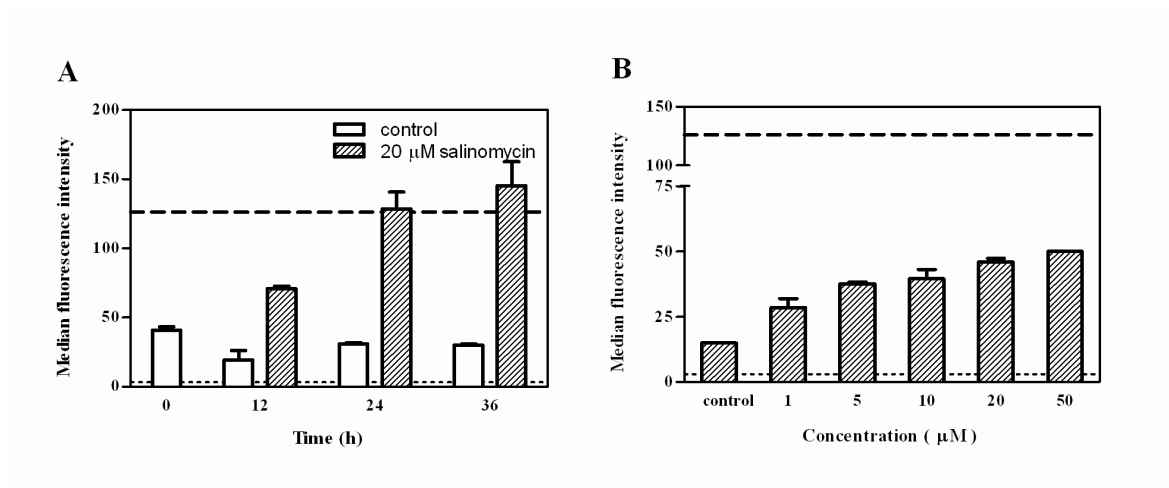


Fig.4 Effect of salinomycin on phospho-p38 MAPK level in OV2008 cells (A). The phospho-p38 MAPK level in OV2008 cells was assessed by the Bio-Plex assay at the indicated intervals after 20μM salinomycin treatment (0.1% DMSO as solvent control). After two washes with ice-cold cell wash buffer, monolayer cells were lysed. The fluorescence intensity of phospho-p38 MAPK in OV2008 was counted by Bio-Plex Suspension Array System. (B). The OV2008 cells were incubated with salinomycin (1, 5, 10, 20, 50μM) or with solvent control (0.1% DMSO) for 24hours and phosphoprotein analysis was performed as described above. The ‘....’ line shows the signal intensity of Phosphatase-Treated HeLa cells as a background control. The ‘-----’ line shows the signal intensity of positive control. Results are expressed as Mean±SD

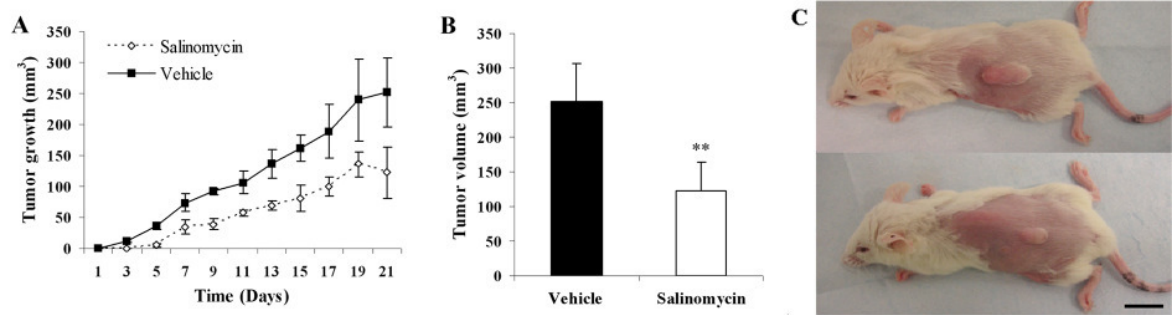


Fig.5 Antitumor activity of salinomycin on NOD/SCID mice bearing human OV2008 cells (A). Tumor-growth curves of the mice treating with salinomycin (5mg/kg) and vehicle control (5% Ethanol). (B). Final volume of tumors in salinomycin- and vehicle-treated animals on the 21th day after tumor injection. Data are presented as Mean±SD of tumor volumes (n=5). ‘**’: Significant difference from vehicle control ($P<0.01$). (C). One of the vehicle control group mice (up) and one of the salinomycin-treated group mice (down). Tumor size in salinomycin-treated mouse (down) was significantly reduced relative to tumor in vehicle-treated mouse (up). Black bar indicates 1cm

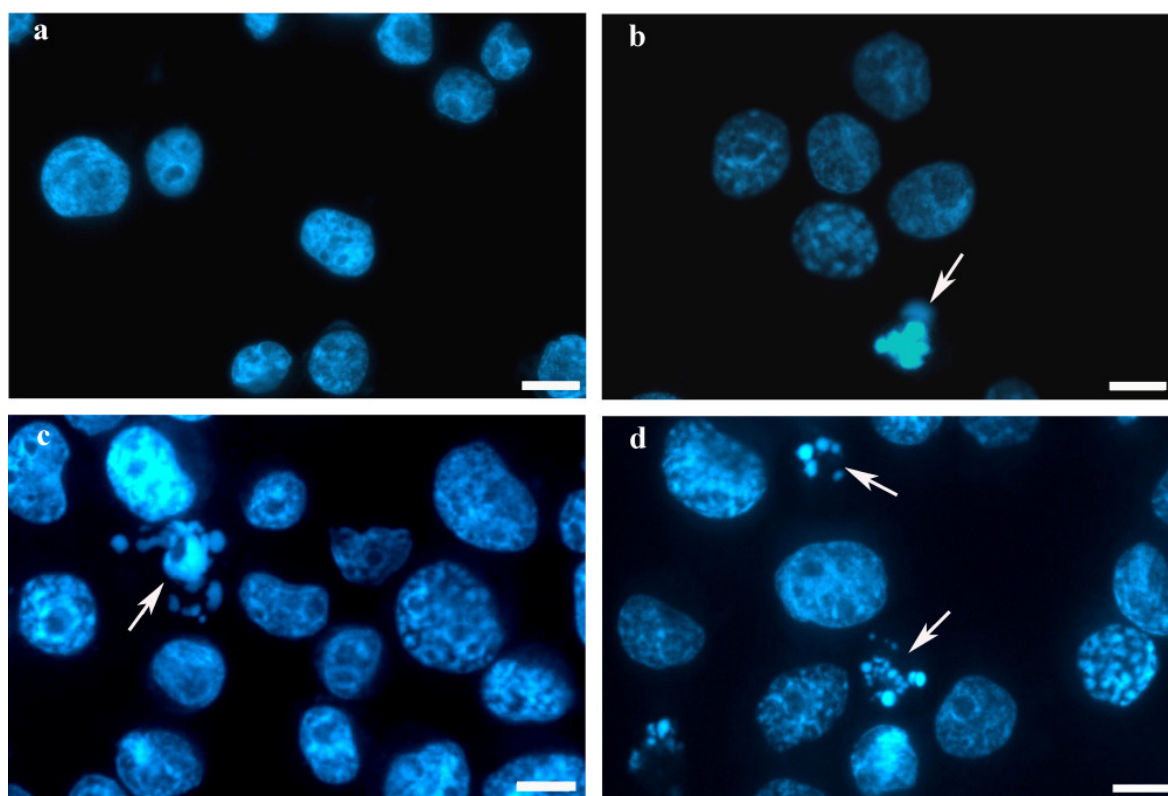


Fig.S1 Cell nucleus morphology observation after salinomycin treatment in OV2008 as indicated by DAPI staining 0hr control cells (**a**) and cells treated with 50μM salinomycin for 12hr (**b**), 24hr (**c**) and 36hr (**d**) were collected by cytopsin, fixed, permeabilized and stained with DAPI to visualize the nucleus (blue) under fluorescent microscope (400×). Apoptotic features (condensed chromatin, nuclear fragmentation and blebbing) were found (arrow). White bars indicate 10μm

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3.2 Published research manuscript

Title: Antitumor properties of salinomycin on cisplatin-resistant human ovarian cancer cells *in vitro* and *in vivo*: involvement of p38 MAPK activation

Authors: Bei Zhang, Xueya Wang, Fengfeng Cai, Weijie Chen, Uli Loesch and Xiao Yan Zhong

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Summary: The ability of salinomycin to effectively kill both cancer stem cells and apoptosis-resistant cancer cells suggests a possible use of salinomycin in the treatment of drug-resistant and aggressive cancers. The present study explored the anticancer biological activity of salinomycin toward cisplatin-resistant human ovarian cancer cell line and its tumor xenograft model, and investigated the mechanisms of anticancer action induced by salinomycin as well. The results demonstrated that salinomycin inhibits the growth and induces the apoptosis in cisplatin-resistant human ovarian cancer cell line C13 *in vitro* and *in vivo*. The proapoptotic effects of salinomycin are not mediated through Akt dependent pathways, but possibly associated with activation of p38 MAPK and demands broader investigations to address the pathway involved.

Author contributions:

Bei Zhang was involved in experimental design, performing the experiments, data analysis and writing the manuscript.

Antitumor properties of salinomycin on cisplatin-resistant human ovarian cancer cells *in vitro* and *in vivo*: involvement of p38 MAPK activation

Short title: Anticancer effects of salinomycin on cisplatin-resistant ovarian cancer cells

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Keywords: salinomycin, ovarian cancer, cisplatin-resistant, growth inhibition, apoptosis, p38 MAPK, tumor xenografts

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Abstract.

The therapeutic effect of ovarian cancer is undesirable. In order to search for alternative agents to overcome chemoresistance during the treatment of ovarian cancer, this study aims at exploring the anticancer effects and mechanism of salinomycin, a selective inhibitor of cancer stem cell, on cisplatin-resistant human ovarian cancer cell line *in vitro* and *in vivo*. Concentration- (0.01 μ M-200 μ M) and time-dependent (24-72 h) growth inhibitory effects of salinomycin were observed in 6 ovarian cancer cell lines (OV2008, C13, A2780, A2780-cp, SKOV3 and OVCAR3) by measuring cell viability using the resazurin reduction assay. IC₅₀ (24 h) range of salinomycin on these 6 cell lines was 1.7-7.4 μ M. After cisplatin-resistant C13 cells were treated with salinomycin, the percentages of apoptotic cells determined by flow cytometry were significantly increased, showing a concentration- and time-dependent manner. But no cell cycle arrest in the G1/G0, S and G2/M phases was detected between salinomycin-treated cells and control cells. Bio-Plex phosphoprotein 5-plex assay (Akt, I κ B- α , ERK1/2, JNK and p38 MAPK) revealed a marked time-dependent and concentration-dependent increase in the phosphorylation of p38 MAPK following salinomycin treatment. Moreover, salinomycin significantly suppressed the growth of tumors in tumor xenograft model. These findings suggest that salinomycin can inhibit the growth of cisplatin-resistant human ovarian cancer cell line efficiently through induction of apoptosis, which might be associated with activation of p38 MAPK.

Introduction

Ovarian cancer remains a leading cause of death from gynecological malignancy, with more than 204,000 new cases and 125,000 deaths each year, accounting for 4% of all cancer cases and 4.2% of all cancer deaths in women around the world (1). The incidence of ovarian cancer increases with age and more than 70% of the patients are diagnosed with late stage disease after distant metastasis has occurred. The 5-year survival rate for the patients diagnosed with late stage disease is less than 20% even with extensive surgery and chemotherapy (2, 3). Chemotherapy with administration of cisplatin (cis-diamminedichloroplatinum (II)) or cisplatin in combination with taxanes is the current standard of care (4, 5). Despite the fact that most of the ovarian tumors are sensitive to chemotherapy for the first time (6, 7), long-term administration of cisplatin has been shown to result in the development of chemotherapeutic drug resistance in the cancer cell population (8, 9). Cisplatin resistance is a major hurdle to successful therapy of recurrent ovarian tumors and responsible for poor long-term overall survival (6, 7). The suggested mechanisms for cisplatin resistance include the increase in intracellular thiols in the redox pathway (10), defects in the apoptotic pathway and the altered activation of signaling pathways, such as PI3K/Akt (11), MAPK (12), or NF- κ B (13). Several groups have targeted these pathways in an attempt to circumvent the cisplatin resistance (13, 14).

Salinomycin is a 751 Da monocarboxylic polyether antibiotic belonging to the group of ionophores that isolated from *Streptomyces albus* (strain No. 80614) (15). It is commonly used as a coccidiostat in poultry and other livestock and is fed to ruminants to improve nutrient absorption and feed efficiency (16). Recently, salinomycin has been reported to selectively deplete human breast cancer stem cells from tumorspheres and to inhibit the mammary tumor growth and metastasis *in vivo* (17). Another recent report showed that salinomycin induces apoptosis in human cancer cells, including those that display wild-type p53 or p53 mutation and multi-drug resistance due to overexpression of Bcl-2, P-glycoprotein or 26S proteasomes with deregulated proteolytic activity (18). These results strongly suggested that salinomycin should be regarded as an anticancer compound. The mechanism of anticancer action of salinomycin is not completely understood. One

study showed salinomycin activates a particular apoptotic pathway not accompanied by cell cycle arrest and independent of tumor suppressor protein p53, caspase activation, the CD95/CD95L system and the proteasome (18). More recently, salinomycin was reported to overcome ABC transporter-mediated multidrug and apoptosis resistance (19) and act as a potent inhibitor of multidrug resistance gp170 (20). Furthermore, a recent study uncovered that salinomycin inhibits the activity of the Wnt signaling pathway, recently appointed as an essential regulator of CSC (cancer stem cell) properties in chronic lymphocytic leukemia cells (21).

The purposes of this study were to determine the anticancer biological activity of salinomycin toward cisplatin-resistant human ovarian cancer cell line and its tumor xenograft model, and to derive mechanistic insights into the action of salinomycin as well. The results showed salinomycin inhibited cell-growth and induced apoptosis in cisplatin-resistant human ovarian cancer cell line *in vitro* and suppressed tumor growth *in vivo* as well. The salinomycin-induced apoptosis in cisplatin-resistant ovarian cancer cell line could correlate with an increase in the activation of p38 MAPK.

Materials and Methods

Cell lines and culture

The six ovarian cancer cell lines used in this study were OV2008, C13, A2780, A2780-cp (A/CP), SKOV3 (p53-negative) and OVCAR3 (p53-mutant). Two pairs of cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines (OV2008 and C13, A2780 and A/CP, respectively) were kindly supplied by Dr. Gaetano Marverti (University of Modena and Reggio Emilia, Italy). All the cell lines were routinely grown in humidified condition at 5% CO₂ and 37°C, incubated with RPMI 1640 standard medium supplemented with 10% fetal bovine serum (FBS), antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) and L-glutamine (2 mM). Exponentially growing cells were used throughout the study. All these reagents were supplied by Invitrogen (Carlsbad, CA).

Growth inhibition assay

The growth inhibitory effects of salinomycin or cisplatin on 6 ovarian cancer cell lines were determined by measuring cell viability using the resazurin reduction assay. Briefly, cells were seeded in 100 μ l media in 96-well microtitre plates at a density of 5000 cells/well. Following overnight incubation, cells were exposed to a range of different concentrations of salinomycin (Sigma-Aldrich, S4526) or cisplatin (Ebewe 0.5 mg/ml, Ebewe Pharma Schweiz AG) and grown at 37°C under a 5% CO₂ atmosphere for 24-72 h. 5 μ l of 0.02% (w/v) Resazurin (Sigma-Aldrich, R7017) in phosphate buffered saline (PBS) was then added to each well and incubation was continued for an additional 2 h. Finally, fluorescence was read using a spectramax GEMINI XS microplate reader (λ_{exc} =544 nm, λ_{em} =590 nm).

Cell apoptosis detection

Cell apoptosis was studied by using the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit (BD pharmingen) in combination with flow cytometry (CyAn ADP, Dako). After cells were pretreated with salinomycin for 12 h, 24 h and 36 h, respectively, and with solvent control (0.1% DMSO) as well, they were harvested by quick trypsinization to minimize potentially high annexin V background levels in adherent cells. Cells were then washed twice with cold PBS and re-suspended in binding buffer at a concentration of 1×10^6 cells/ml. 100 μ l cells were taken to stain with 5 μ l annexin V/FITC and 5 μ l PI and incubated in dark at room temperature for 15 min. Then 400 μ l binding buffer was added and cells were analyzed by flow cytometry. Cells negative for both annexin V and PI are viable, annexin V⁺/PI⁻ cells are in early apoptosis, and annexinV⁺/PI⁺ cells are necrotic or in late apoptosis. The percentages of apoptotic cells were analyzed by Flowjo software.

Cell cycle distribution analysis

To evaluate cell cycle profile, cells (about 1×10^6 cells), pretreated with salinomycin for 12 h and 24 h (0.1% DMSO as the solvent control), were harvested, washed twice with PBS, then fixed and stored in ice-cold 70% (v/v) ethanol at -20°C. Prior to analysis, samples were washed again with PBS and then incubated in propidium iodide/Rnase staining buffer

(BD pharmingen) at room temperature in the dark for at least 15 min. After filtration to remove cellular debris, the single-cell suspensions were analyzed on a flow cytometer. Cell cycle parameters were analyzed using Flowjo software.

Phosphoprotein assay

A panel of phosphoproteins was measured in duplicate using a bead-based multiplex assay (Bio-Plex Phosphoprotein Detection, Bio-Rad, Hercules, USA), according to the manufacturer's instructions (22, 23). Briefly, cells were treated with salinomycin or with solvent control (0.1% DMSO) for the indicated time interval and then the cell lysates were collected with Bio-Plex Cell Lysis Kit. The protein concentration was measured with a DC (detergent compatible) protein assay (Bio-Rad) and adjusted to 600 µg/ml. Fifty microliters of coupled beads, which recognize phosphorylated Akt, IκB-α, ERK1/2, JNK and p38 MAPK, respectively, were added to the 96-well filter plate, followed by washing twice. Same volume of the cell lysates were added and incubated with the beads for 15–18 h (overnight). Next, 25 µl of biotin-labelled detection antibodies were added after washing and incubated for 30 min. Fifty microliters of streptavidin-PE was added followed washing and incubated in the dark for 10 min. After rinsing, 125 µl of resuspension buffer was added, and the phosphoproteins were analyzed by a Bio-Plex 200 system and Bio-Plex Manager software (BioRad). In this assay, the lysates of Phosphatase-Treated HeLa cells, TNF-α-Treated Hela cells, UV-Treated HEK293 cells and EGF-Treated HEK293 cells, provided by the Bio-Plex phosphoprotein assay, were used as the background control and the positive control of phospho-IκB-α (Ser32/Ser36), phospho-p38 MAPK (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), phospho-Akt (Ser473) as well as phospho-ERK1/2 (Thr202/Tyr204, Thr185/Tyr187). This experiment was repeated in duplicate.

Ovarian cancer tumor xenografts in mice

Female mice of NOD/SCID were in-house breeding from the Animal Center (Tierversuchsstation) at the Department of Biomedicine, University Hospital of Basel and used at 6 weeks of age. All mouse procedures were approved by Cantonal Veterinary

Office (Kantonales Veterinäramt) and performed in accordance with the regulations concerning animal experiments. For *in vivo* salinomycin treatment study, cultured ovarian cancer cells (2×10^6 cells per mouse in 0.1 ml saline) were subcutaneously injected into the back of NOD/SCID mice. On the day after tumor cells injection, mice were divided into two groups of 5 mice each. Treatment was initiated 24 h after injection. The two experimental groups were administrated with salinomycin (5 mg/kg) (17) and 5% ethanol (vehicle), respectively, by intraperitoneal injection on every other day for 3 weeks. The size of the tumor was measured every 2 days using a digital vernier caliper. Tumor volume was estimated by the following formula: $\text{volume} = (a \times b^2) \times \pi/6$, where a and b are major and minor axes of the tumor.

Statistical analysis

All data were expressed as mean values \pm standard deviation. Growth-inhibitory curve were analyzed using GraphPad Prism 5.01 Software. Comparisons among groups were performed by Student's t-test. The significance level was set at $p < 0.05$.

Results

Growth-inhibitory effect of salinomycin on ovarian cancer cell lines

The growth-inhibitory effect of salinomycin against OV2008, C13, A2780, A/CP, SKOV3 and OVCAR3 cell lines is shown in Fig.1. The effect of incubation time and concentration on viability of the ovarian cancer cell lines by salinomycin was studied. Cells were exposed for 24, 48 or 72 h to salinomycin at (0.01 μM -200 μM) concentration range, and cell viability measured by the resazurin reduction assay. In all the six cell lines studied, the inhibition ratio of cell viability showed a concentration- and time-dependent pattern. IC_{50} of salinomycin or cisplatin on the six ovarian cancer cell lines is reported in Table 1. This shows that salinomycin was slightly more potent in A2780 than in the rest of cell lines and was almost equipotent in the rest five cell lines, including cisplatin-resistant ovarian cancer cells such as C13, A/CP and SKOV3. IC_{50} (24 h) range of salinomycin on the six ovarian cancer cell lines was 1.7-7.4 μM . In addition, salinomycin was more potent in C13 cells, about 9-fold resistance to cisplatin, than its parent OV2008 cells (cisplatin-sensitive cells).

So, the C13 cisplatin-resistant human epithelial ovarian cancer cell line was attracted more attention and used for most parts of the study.

Effect of salinomycin on tumor cell apoptosis and cell cycle

Salinomycin-treated C13 cells were analyzed by flow cytometry which can distinguish between early apoptosis or late apoptosis of cells after cells were stained simultaneously with annexin V and PI. Compared to control, salinomycin treatment significantly increased the percentages of apoptotic cells in C13, showing a concentration- and time-dependent manner (Fig.2). In control culture, $4.25\pm 0.46\%$ cells were in early apoptosis stage whereas $9.31\pm 0.12\%$ cells were in late apoptosis stage. After cells were treated with $20\ \mu\text{M}$ salinomycin for 12 h, the percentages of apoptotic cells at early phase increased to $16.2\pm 0.68\%$ and that of late phase increased to $13.7\pm 1.17\%$. Whereas, when cells treated with salinomycin for 24 and 36 h, $25.0\pm 0.70\%$ and $22.1\pm 1.91\%$ cells were in early apoptosis, and $23.3\pm 1.08\%$ and $27.6\pm 1.13\%$ cells were in late apoptosis. These results clearly indicate that salinomycin evoked apoptosis in C13 cells.

Effect of different concentrations of salinomycin (10 and $20\ \mu\text{M}$) on the cell cycle phases was investigated in C13 cells cultured over different times (12 and 24 h) by DNA content analysis, by flow cytometry. The results revealed that the percentages of the cell population in the sub-G1 phase were significantly higher in salinomycin-treated C13 cells with a concentration-dependent fashion, whereas the percentages of cells in other phases (G1/G0, S and G2/M phases) were almost reduced, in comparison with control (Fig.3). These effects were similar at 12 and 24 h (Fig.3). The dramatic accumulation of cells in sub-G1 phase was another marker for apoptosis, which further confirmed the results of annexin V/PI assay. Additionally, there was no cell cycle arrest in G1/G0, S and G2/M phases between salinomycin-treated cells and control cells, suggesting that salinomycin inhibits the cellular proliferation of C13 cells not accompanied by cell cycle arrest.

Effect of salinomycin on phosphoproteins levels in OV2008 and C13 cells

To investigate the effect of salinomycin on phosphoproteins levels in ovarian cancer cell lines (OV2008 and C13), the regulation of phosphorylation by salinomycin in five proteins

was examined using Bio-Plex phosphoprotein assay. The results showed the basal levels of phosphorylation of Akt and I κ B- α were higher in untreated C13 cells than in untreated OV2008 cells (\sim 1.7 fold and \sim 1.6 fold, respectively) (Fig.4A, 4B). An increase in phosphorylation of Akt in response to salinomycin was observed in OV2008 cells (\sim 2.2 fold) and C13 cells (\sim 1.3 fold) (Fig.4A). ERK was phosphorylated in OV2008 cells (\sim 2 fold) and C13 cells (\sim 1.4 fold) by addition of salinomycin, but the level was independent of salinomycin dose and treatment time (Fig.4C). There was no clear alteration of phosphorylation of I κ B- α (Fig.4B) and JNK (Fig.4D) after salinomycin treatment in either type of cell lines. But a marked concentration-dependent increase in the p38 MAPK phosphorylation was observed in both cell lines following salinomycin exposure for 24 h (Fig.4E). Phosphorylation of p38 MAPK was also enhanced by salinomycin (10 μ M) after 12, 24 and 36 h of incubation with OV2008 (Fig.4F) or C13 (Fig.4G), showing a time-dependent fashion. These findings suggest that salinomycin-induced growth-inhibitory effect and apoptosis in both cell lines could be mediated through the alteration of phosphorylation of p38 MAPK.

Evaluation of antitumor activity of salinomycin in vivo

Based on the *in vitro* results, which showed significant cytotoxicity of salinomycin to human ovarian cancer cell lines, the *in vivo* antitumor efficacy of salinomycin was further evaluated in a cisplatin-resistant human ovarian tumor (C13) xenograft grown in the back of mice. The mice were treated with salinomycin and the change in tumor volume after first injection was followed for 21 days (Fig.5A). Compared with vehicle-treated controls, a significant reduction in the tumor volume was observed in the mice treated with salinomycin (Fig.5C). When the test came to the end, in C13 tumor model, the tumor volume of salinomycin therapy groups and controls was 84.2 ± 30.8 mm³ and 252.5 ± 63.4 mm³, respectively ($p < 0.01$; Fig.5B).

Discussion

In view of recent finding that salinomycin not only kills human breast cancer stem cell-like cells (17), but also induces apoptosis and overcomes multiple mechanisms of resistance to

apoptosis in human cancer cells, mainly human haematological tumor cells (18), in the present study we investigated the effects of salinomycin on human ovarian cancer cell lines, including cisplatin-resistant cell lines. As shown in Fig.1, salinomycin demonstrated a strong growth-inhibitory effect on ovarian cancer cell lines at a concentration- and time-dependent manner. The six ovarian cancer cell lines were chosen for this study, including cisplatin-resistant cell lines such as C13, A/CP and SKOV3, which are characterised as advanced and refractory ovarian cancer. Salinomycin showed almost equipotent in these cisplatin-resistant cells, and even displayed more growth-inhibitory activity toward cisplatin-resistant C13 cells than its parent cisplatin-sensitive OV2008 cells (Table.1). These results are consistent with previous study on breast cancer cell line which indicated that paclitaxel-resistant cells remain sensitive to salinomycin treatment (17).

Apoptosis, or programmed cell death, is an important homeostatic mechanism that balances cell division, cell death and maintains the appropriate cell number in the body (24). Therefore, searching for agents which trigger apoptosis of tumor cells has become an attractive strategy in anticancer drug discovery (25). In the present investigation, after C13 cells were treated with salinomycin, flow cytometry results, from both annexin V/PI assay (Fig.2) and sub-G1 populations in cell cycle analysis (Fig.3), showed a concentration- and time-dependent increase in the percentage of apoptotic subpopulations. These results provided evidence that salinomycin triggered apoptosis in C13 cells, which resembles previous report on various human cancer cells such as leukemia cells (18, 19).

Cell cycle control plays a critical role in the regulation of tumor cell proliferation. Many anticancer agents and DNA-damaging agents arrest the cell cycle at the G0/G1, S, or G2/M phase and then induce apoptotic cell death (26, 27). From the results of present study, it appeared that no cell cycle arrest in G1/G0, S and G2/M phases was observed between salinomycin-treated C13 cells and control cells (Fig. 3), which confirms previous finding that salinomycin activates a particular apoptotic pathway not accompanied by cell cycle arrest (18).

To better understand the signal pathways involved in salinomycin-induced growth-inhibitory effect and apoptosis in cisplatin-resistant ovarian cancer cells, we investigated

the activity of Akt, I κ B- α , ERK1/2, JNK and p38 MAPK in cisplatin-resistant C13 cells, compare to cisplatin-sensitive OV2008 cells. To address this issue, multiple phosphoproteins were determined by Bio-Plex assays with Luminex technology. The recent report showed that the results achieved using Bio-Plex phosphoprotein array analyses significantly correlated ($P < 0.001$) with those obtained with numerized western blot analyses (23). Furthermore, Bland-Altman analyses clearly demonstrated that Bio-Plex phosphoprotein array could be used instead of western blot providing a unique way of analyzing multiple phosphoprotein expression in small specimens.

The PI3-kinase/Akt pathway contributes to the tumor formation by elevating the activity of the anti-apoptotic action of Akt. Akt inhibits apoptosis through phosphorylation of Bad, GSK3, and caspase-9 and activation of transcriptional factors such as Forkhead (FOXO1) and NF- κ B (28). It has been reported that cisplatin resistance is associated with the altered activation of PI3K/Akt signaling pathways in an ovarian cancer cell line (11). Suppression of Akt activation could lead to the activation of pro-apoptotic signaling pathways (29, 30). The present study showed basal levels of phospho-Akt in untreated cisplatin-resistant C13 cells were higher compared to cisplatin-sensitive OV2008 cells, and salinomycin enhanced the phospho-Akt levels in both C13 and OV2008 cells (Fig.4A). However, the growth inhibition effect of salinomycin on C13 cells was not significantly different from that of OV2008 cells. Moreover, I κ B- α is a downstream Akt substrate. Via the phosphorylation of I κ B kinase, Akt activates NF- κ B, a transcription factor that has been implicated in cell survival (31, 32). A wealth of data has indicated that the NF- κ B has been linked with cell proliferation, invasion, angiogenesis, metastasis, suppression of apoptosis and chemoresistance in multiple tumors (33). In ovarian cancer cells, it has been reported that increased phosphorylation of I κ B- α and constitutive activation of NF- κ B mediates cisplatin resistance and inhibition of NF- κ B activation sensitizes the ovarian cancer cells to cisplatin (13). In the current study, basal levels of phospho-I κ B- α in untreated cisplatin-resistant C13 cells were higher than those in untreated cisplatin-sensitive OV2008 cells. But no phosphorylation of I κ B- α was induced by salinomycin (Fig. 4B). These results indicate that in cisplatin-resistant C13 cells, salinomycin induces

apoptosis through non-Akt/I κ B- α dependent pathways. The exact mechanism of the enhanced phospho-Akt by salinomycin is not yet known and warrants further investigation.

MAPKs are essential parts of the signal transduction machinery and play central roles in cell growth, differentiation, and programmed cell death (34). Recent studies have suggested that apoptotic stimuli are transmitted to caspases through the activation of MAPKs, such as p38 MAPK and JNK (35). Therefore, we tested whether MAPK activation is involved in salinomycin-induced apoptosis in ovarian cancer cell lines (OV2008 and C13). According to our data, only p38 MAPK, and not JNK or ERK, is associated with the proapoptotic activity of salinomycin (Fig.4C-4G). The p38 MAPK pathway is implicated in cancer cell apoptosis and is induced by several chemotherapeutic drugs (36, 37). It was also reported that the loss of the capacity to activate p38 MAPK in response to cisplatin treatment may be one of the mechanisms of chemoresistance (38). We found there are marked time-dependent and concentration-dependent increases in the phosphorylation of p38 MAPK following salinomycin treatment in both cell lines (Fig.4E-4G). This result suggests that the activation of p38 MAPK appears to contribute to the proapoptotic effect of salinomycin in ovarian cancer cell lines and that the activation of the p38 MAPK pathway might play a causal role in the salinomycin-induced apoptosis in ovarian cancer cell lines. However, detailed downstream and upstream signaling molecules of p38 MAPK modulated by salinomycin are not known and should be further investigated.

In the present study, the xenografts of human cisplatin-resistant ovarian cancer (C13) model showed very good efficacy when treated with salinomycin (Fig.5). Although, we have not yet attempted to ascertain the mechanism of cell death in the xenograft tumor model, it is likely that cell apoptosis induced by salinomycin may account for some of the observed reduction in tumor growth rate and needs further investigations. Additionally, further studies with salinomycin alone in different characterized ovarian cancer cell lines or in combination with other conventional drugs *in vitro* and *in vivo* are still warranted to enhance our understanding of this promising antitumorigenic compound.

In summary, the present study demonstrated that salinomycin inhibits the growth and induces the apoptosis in cisplatin-resistant human ovarian cancer cell line C13 *in vitro* and exhibits significant *in vivo* efficacy in tumor (C13) xenograft model. The proapoptotic effects of salinomycin are not mediated through Akt dependent pathways, but possibly associated with activation of p38 MAPK and demands broader investigations to address the pathway involved.

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Conflict of interest statement

No potential conflicts of interest were disclosed

Table 1. IC₅₀ of cisplatin or salinomycin on ovarian cancer cell lines

Cell lines	Cisplatin			Salinomycin		
	24 h	48 h	72 h	24 h	48 h	72 h
OV2008						
IC ₅₀ (μ M)	8.08	2.49	0.60	7.44	4.78	3.20
%95 CI	6.15 to 10.63	2.09 to 2.97	0.52 to 0.68	6.80 to 8.14	4.12 to 5.55	2.90 to 3.53
C13						
IC ₅₀ (μ M)	77.10	24.29	9.69	4.42	3.10	1.86
%95 CI	65.95 to 90.13	21.85 to 27.01	8.19 to 11.46	3.62 to 5.39	2.67 to 3.59	1.67 to 2.06
A2780						
IC ₅₀ (μ M)	6.48	1.60	1.03	1.70	0.43	0.27
%95 CI	5.34 to 7.86	1.30 to 1.96	0.53 to 2.00	1.40 to 2.07	0.39 to 0.48	0.21 to 0.33
A/CP						
IC ₅₀ (μ M)	26.09	3.35	1.84	5.56	1.02	0.51
%95 CI	23.53 to 28.91	2.75 to 4.09	1.32 to 2.55	4.75 to 6.51	0.91 to 1.13	0.44 to 0.58
SKOV3						
IC ₅₀ (μ M)	54.55	11.39	2.09	7.08	4.17	2.83
%95 CI	38.97 to 76.35	7.17 to 18.10	1.69 to 2.59	5.33 to 9.40	3.47 to 5.01	2.14 to 3.76
OVCAR3						
IC ₅₀ (μ M)	13.23	2.12	0.63	5.56	2.50	1.87
%95 CI	10.90 to 16.06	1.83 to 2.46	0.55 to 0.72	4.30 to 7.18	2.09 to 2.98	1.32 to 2.67

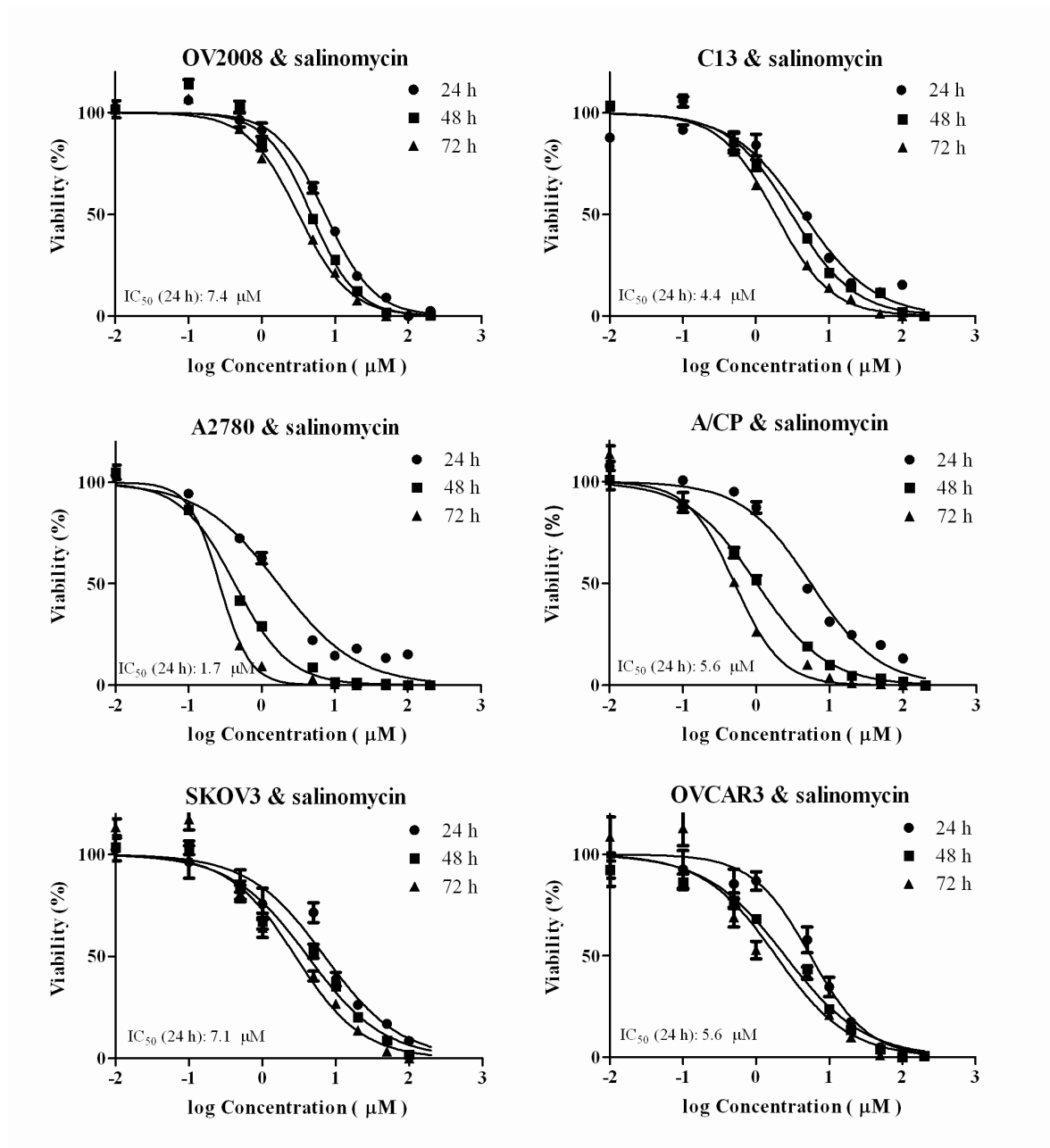


Figure 1. Effect of salinomycin on cell viability in human ovarian cancer cell lines. Cells were exposed to salinomycin at concentrations (0.01-200 μM) for 24, 48 and 72 hours and cell viability measured by resazurin reduction assay. Results are Mean \pm SD of quadruplicates from one of three independent experiments.

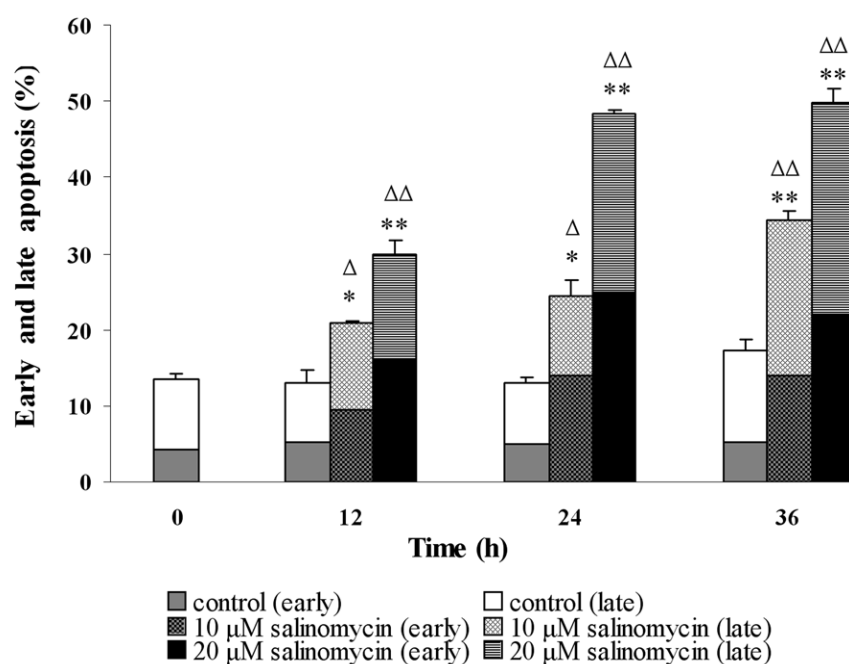


Figure 2. Effect of salinomycin on cell apoptosis in C13 cells. After cells were treated with 10 μM or 20 μM salinomycin for 12 h, 24 h and 36 h, respectively, and with solvent control (0.1% DMSO) as well, the percentage of apoptotic subpopulations was determined by flow cytometry analysis based on mean values obtained from three independent experiments. Results are expressed as Mean±SD. ‘*’ and ‘**’: Significant difference from control of time point ($p<0.05$ and $p<0.01$, respectively). ‘Δ’ and ‘ΔΔ’: Significant difference from 0 h control ($p<0.05$ and $p<0.01$, respectively).

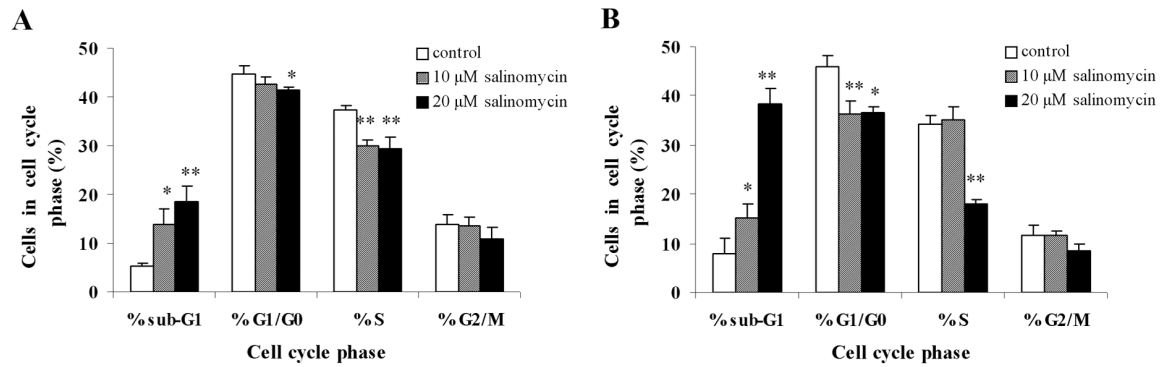


Figure 3. Effect of salinomycin on cell cycle distribution in C13 cells. Cells were treated with 10 μ M or 20 μ M salinomycin for 12 h (A) and 24 h (B), and with 0.1% DMSO as a solvent control. The percentages of each cell cycle were evaluated by flow cytometry based on mean values obtained from three independent experiments. Results are expressed as Mean \pm SD. * and **: Significant difference from control ($p < 0.05$ and $p < 0.01$, respectively).

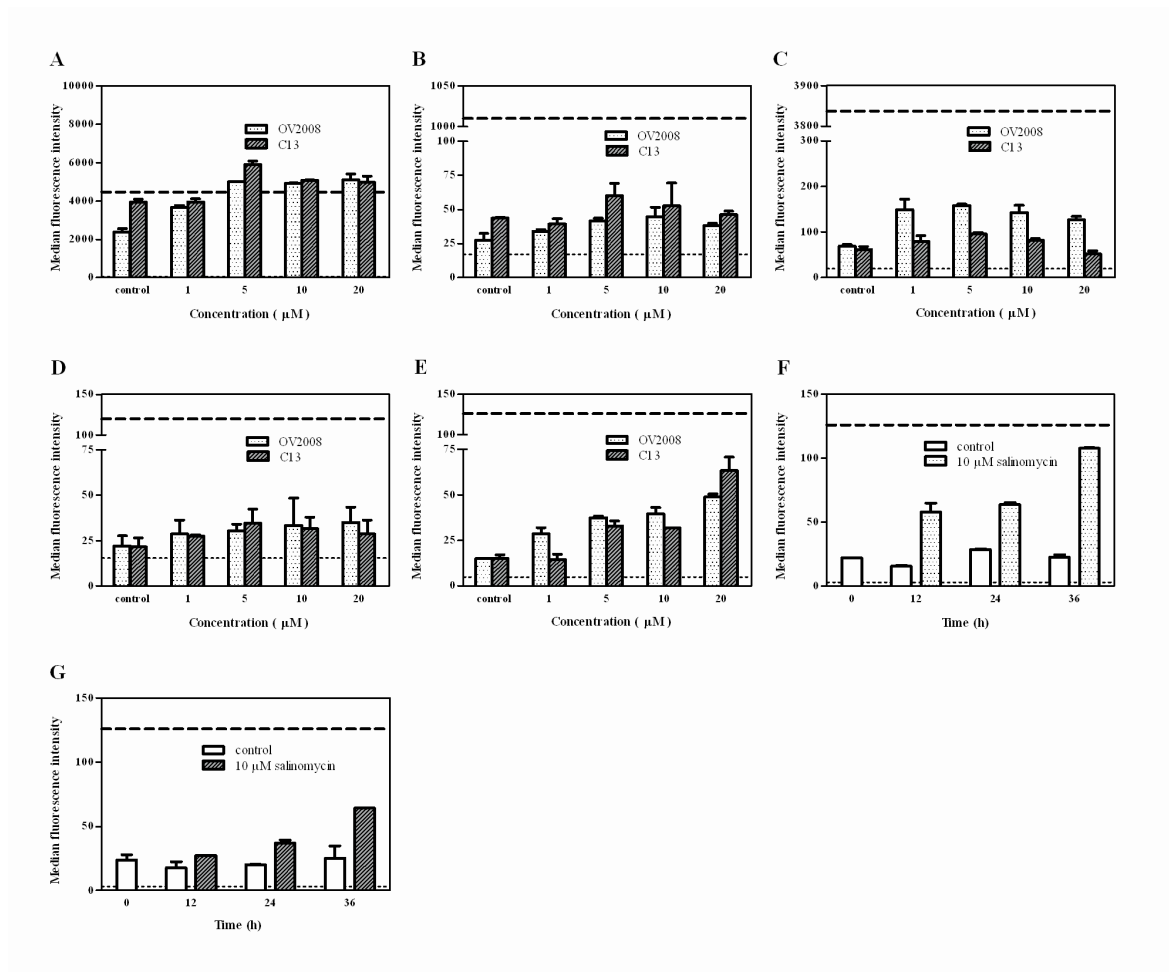


Figure 4. Effect of salinomycin on phosphoproteins levels in OV2008 and C13 cells. The phosphoproteins in OV2008 and C13 cells were assessed by the Bio-Plex assay at 24 h after different doses (1-20 μM, 0.1% DMSO as solvent control) of salinomycin treatment. After two washes with ice-cold cell wash buffer, monolayer cells were lysed. The fluorescence intensity of phosphoproteins in cells was counted by Bio-Plex Suspension Array System: (A) phospho-Akt; (B) phospho-IκB-α; (C) phospho-ERK1/2; (D) phospho-JNK; and (E) phospho-p38 MAPK. The time-dependent phospho-p38 MAPK levels in OV2008 (F) and C13 (G) were analyzed by the Bio-Plex assay at the indicated intervals after 10 μM salinomycin treatment (0.1% DMSO as solvent control). The ‘....’ line shows the signal intensity of Phosphatase-Treated HeLa cells as a background control. The ‘----’ line shows the signal intensity of positive control. Results are expressed as Mean±SD.

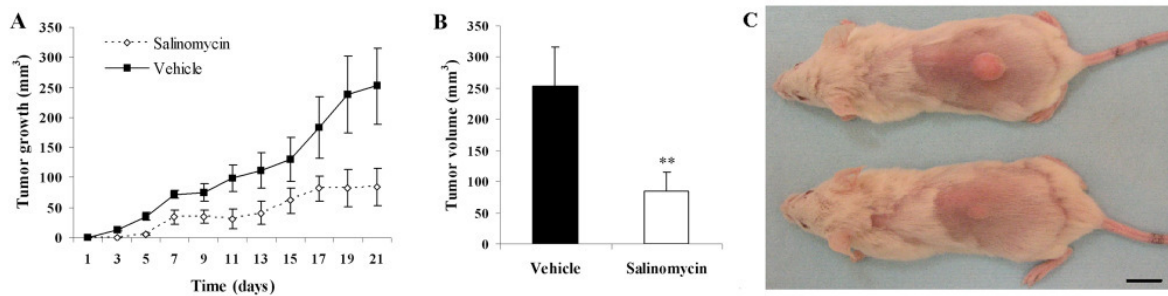


Figure 5. Antitumor activity of salinomycin on NOD/SCID mice bearing human C13 cells. (A). Tumor-growth curves of the mice treating with salinomycin (5 mg/kg) and vehicle control (5% Ethanol). **(B).** Final volume of tumors in salinomycin- and vehicle-treated animals on the 21th day after tumor injection. Data are presented as Mean±SD of tumor volumes (n=5). ‘**’: Significant difference from vehicle control ($p<0.01$). **(C).** One of the vehicle control group mice (up) and one of the salinomycin-treated group mice (down). Tumor size in salinomycin-treated mouse (down) was significantly reduced relative to tumor in vehicle-treated mouse (up). Black bar indicates 1cm.

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3.3 Supplementary data

3.3.1 Supplementary data 1: p38 MAPK inhibition assay

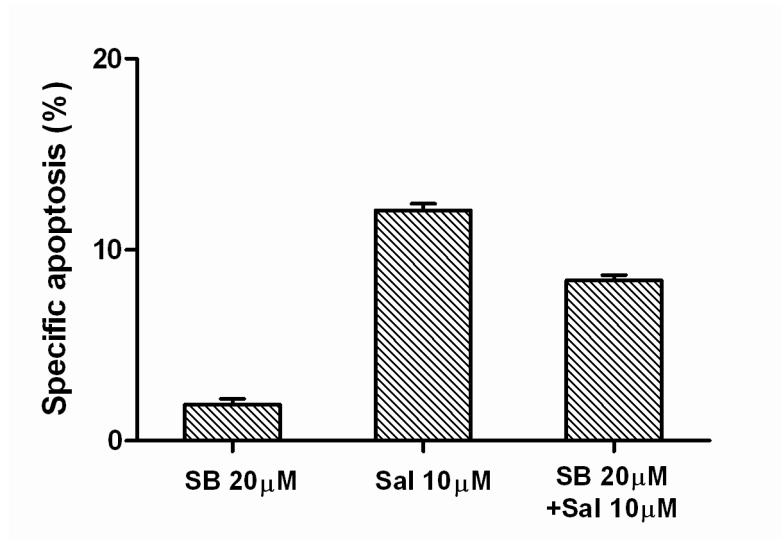


Fig.S1 *Salinomycin-induced apoptosis is markedly inhibited by SB202190 (p38 MAPK inhibitor)* C13 cells were serum-starved overnight and then preincubated with or without 20 μM SB202190 for 1 h, and subsequently treated with 10 μM salinomycin for 24 h (0.1% DMSO as solvent control). After salinomycin treatments, cells were harvested and the percentages of apoptotic cells (%sub G1) were determined by flow cytometry. Specific apoptosis (SA) was calculated using the following formula: $SA (\%) = 100 \times (A_E - A_C) / (100 - A_C)$, where A_E equals % of apoptotic cells in the experimental group and A_C equals % of apoptotic cells in the control group. Data are given as mean values \pm SD.

3.3.2 Supplementary data 2: cell apoptosis *in situ*

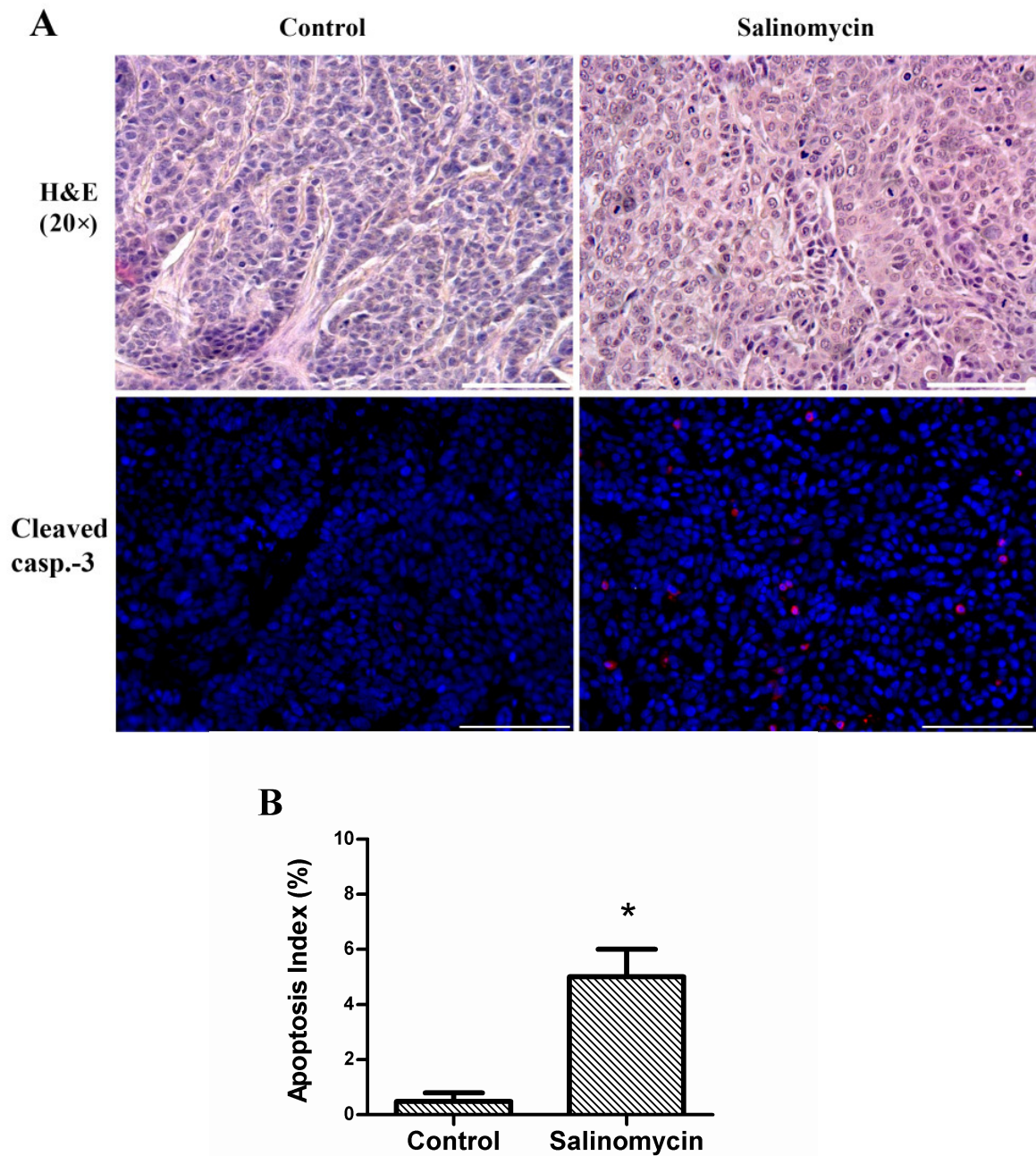


Fig.S2 *Salinomycin induces tumor cell apoptosis in situ* (A) Illustrated are representative tumor sections prepared from the mice treating with vehicle control or salinomycin after euthanasia. Tumor sections were stained with hematoxylin eosin (HE) to observe morphology (upper row) or with anti-cleaved caspase-3 antibody to view apoptotic cells (lower row). (B) The cleaved caspase-3 positive cells were counted to calculate the apoptosis index. A significant difference in apoptosis index between tumors treated with salinomycin versus control is denoted by “*”. White bars indicate 100µm.

4. SUMMARY AND OUTLOOK

Ovarian cancer is the most frequent cause of death from gynecological cancer [201]. Even though there are a lot of options in treating gynecological malignancies, the therapeutic effect of ovarian cancer nowadays is still unfavourable, especially in treatment of the patients diagnosed with late stage disease [202, 203]. The development of chemotherapeutic drug resistance during treatment is thought to cause treatment failure and the high mortality rate [204, 205]. Thus, searching for alternative agents to overcome chemoresistance during the treatment of ovarian cancer is essential. Very recently, it has been shown that it is possible to selectively kill breast cancer stem cells using the ionophore antibiotic, salinomycin [154]. Its ability to kill cancer stem cells and apoptosis-resistant cancer cells may define salinomycin as a novel anticancer drug [154, 155].

In our study, we firstly evaluated *in vitro* growth-inhibitory effects of salinomycin on six human ovarian cancer cell lines, including cisplatin-resistant cell lines such as C13, A/CP and SKOV3, which are characterised as advanced and refractory ovarian cancer. Salinomycin showed a strong growth-inhibitory effect on ovarian cancer cell lines at a concentration- and time-dependent manner, and even displayed more growth-inhibitory activity toward cisplatin-resistant C13 cells than its parent cisplatin-sensitive OV2008 cells. These results are consistent with previous study which indicated that drug-resistant cancer cells remain sensitive to salinomycin treatment [154]. Salinomycin inhibiting the growth of cisplatin-resistant ovarian cells suggests a possible future use of salinomycin in the treatment of cisplatin-resistant and aggressive ovarian cancers.

Apoptosis, or programmed cell death, is an important homeostatic mechanism that balances cell division, cell death and maintains the appropriate cell number in the body [206]. As a regulated cell death process, apoptosis requires the cascaded activation and execution of a series of regulatory molecules and cysteine-aspartic proteases, known as caspases [207]. Stress agents, such as reactive oxygen species (ROS), ultraviolet radiation, viral infections, and anticancer agents are well-characterized apoptosis triggers. Recently, salinomycin has been reported to induce apoptosis in diverse types of apoptosis- and chemotherapeutic-resistant cancer cells [156]. Salinomycin-induced apoptosis has also

been detected in various human cancer cells, such as leukemia cells [171] and prostate cancer cells [182]. In the present investigation, cell nuclear morphology observations by fluorescence microscopy and flow cytometry results, from both annexin V/PI assay and sub-G1 populations in cell cycle analysis, clearly provided evidence that salinomycin evoked apoptosis in cisplatin-resistant human ovarian cancer cell line C13 and its parent cisplatin-sensitive OV2008 cells.

Cell cycle control plays a critical role in the regulation of tumor cell proliferation. Many anticancer agents and DNA-damaging agents arrest the cell cycle at the G0/G1, S, or G2/M phase and then induce apoptotic cell death [208, 209]. From the results of present study, it appeared that no cell cycle arrest in G1/G0, S and G2/M phases was observed between salinomycin-treated OV2008 or C13 cells and control cells, which confirms previous finding that salinomycin activates a particular apoptotic pathway not accompanied by cell cycle arrest [155]. However, three recent reports showed that treatment with salinomycin positively correlated with reduced cell cycle-related proteins, especially reduced p21 levels [175-177]. Salinomycin sensitized radiation-treated cancer cells by inducing G2 arrest [176], while it sensitized the cancer cells to antimetabolic drugs by preventing G2 arrest [177].

Based on the *in vitro* results, which showed significant cytotoxicity of salinomycin to human ovarian cancer cell lines, in the present study we established human ovarian cancer cell lines (OV2008 or C13) xenograft tumor mouse models and further observed the antitumor efficacy of salinomycin *in vivo*. It was shown that salinomycin significantly inhibited the tumor growth in these both human ovarian cancer cells xenograft models. The preliminary analysis of tumor cell apoptosis *in situ* by immunohistochemistry staining of cleaved caspase-3 indicated that cell apoptosis induced by salinomycin might account for some of the observed reduction in tumor growth rate. But the mechanism of cell apoptosis in the xenograft tumor model is still unclear and needs further investigations.

Identifying molecular mechanisms of anticancer action induced by salinomycin would be an important step in developing salinomycin-based pharmacological cancer therapy. A more complete understanding of the salinomycin's anticancer mechanism could

facilitate the therapeutic use of salinomycin in cancer patients. In our study, Bio-Plex assays with Luminex technology, which is a rapid, high-throughput, multiplex, bead-based, quantitative assay for protein analysis, was used to investigate the effect of salinomycin on phosphoproteins levels in ovarian cancer cell lines (OV2008 and C13). After screening the activity of five proteins (Akt, I κ B- α , ERK1/2, JNK and p38 MAPK), we found there are marked time-dependent and concentration-dependent increases in the phosphorylation of p38 MAPK following salinomycin treatment in both cell lines. These findings suggest that salinomycin-induced growth-inhibitory effect and apoptosis in both cell lines could be mediated through the alteration of phosphorylation of p38 MAPK. Subsequently, the link between p-p38 MAPK and apoptosis in salinomycin-exposed cells was also evidenced from the inhibition of apoptosis in SB202190 (p38 MAPK inhibitor)-pretreated OV2008 or C13 cells. However, detailed downstream and upstream signaling molecules of p38 MAPK modulated by salinomycin are not known and should be further investigated. Recently, two reports [182, 183] indicate that salinomycin inhibits prostate cancer cell growth via induction of oxidative stress. Reactive oxygen species (ROS) are implicated as important mediators of apoptotic cell death. MAPK is considered as one of the most important signaling molecules in ROS-mediated apoptosis in cancer cells [210, 211]. Oxidative stress has also been reported to play a role in p38 MAPK activation [211]. Our findings show a significant increase in the phosphorylation of p38 MAPK following salinomycin treatment in both cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines. Although, we have not yet attempted to measure intracellular ROS induced by salinomycin in our cell lines, it remains possible that ROS trigger is an upstream signal which may contribute to the enhanced p38 MAPK activity and initiate the series of apoptotic events induced by salinomycin, which is warranted further investigations.

In order to better enhance our understanding of the effect of salinomycin, this promising antitumorigenic compound, on human ovarian cancer cells, the future research will focus on three main aspects: One is further *in vitro* and *in vivo* studies with salinomycin alone in different characterized ovarian cancer cell lines, such as p53 mutation/null cell lines, various drug-resistant cell lines or patient-derived primary ovarian cancer cells, and investigating its mechanism of action as well. Another is evaluating

whether salinomycin could selectively target cancer stem cells in ovarian cancer. The third is research on salinomycin in combination with other conventional drugs *in vitro* and *in vivo* to improve the efficiency of therapy in ovarian cancer.

Due to the severe neural and muscular toxicity of salinomycin observed in mammals, including human beings [193, 194, 198, 199], its use as a feed additive is no longer authorized in the European Union and United States [190]. The considerable toxicity is also one important obstacle for the potential clinical use of salinomycin. Searching for a new group of salinomycin derivatives which will be more effective in coordination of biologically important metal cations and less toxic especially for humans will be a challenge for the coming years. Moreover, future studies with salinomycin in humans should be designed carefully.

In conclusion, this work demonstrated that salinomycin is a potent compound against cisplatin-resistant human ovarian cancer cell line C13 and its parent cisplatin-sensitive OV2008 cells *in vitro* and *in vivo*. Salinomycin can inhibit the growth of both cell lines efficiently through induction of apoptosis, which is not accompanied by cell cycle arrest, but possibly is associated with activating p38 MAPK and merits further investigations. Salinomycin shows substantial promise for further development as a potential agent for treating ovarian cancer, especially drug-resistant ovarian cancer.

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6. APPENDIX

6.1 Acknowledgements

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6.2 Short curriculum vitae

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6.3 List of publications and presentations

Publications during PhD work

- (1) **Zhang B**, Wang X, Cai F, Chen W, Loesch U, Zhong XY. Antitumor properties of salinomycin on cisplatin-resistant human ovarian cancer cells *in vitro* and *in vivo*: involvement of p38 MAPK activation. *Oncol Rep* 2013;29(4):1371-8.
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- (11) **Zhang B**, Zhong XY. Salinomycin and cancer: a review. (Submitted, under review). Review.

Presentations

- (1) **Stepping Stone Symposium, SSSTC Swiss Sino Science and Technology Corporation (First conference on Cancer and Neurodegenerative Diseases), 20-22 June, 2012, ETH Zurich, Switzerland.** Zhang B, Wang X, Cai F, Chen W, Loesch U, Zhong XY. *Antitumor properties of salinomycin on human ovarian cancer cells in vitro and in vivo* (Poster Presentation)
- (2) **Australia New Zealand Gynaecological Oncology Group (ANZGOG) ASM Conference 2012, 22-25 February, 2012, Gold Coast, Australia.** Zhang B, Wang X, Cai F, Chen W, Loesch U, Zhong XY. *Anticancer effects of salinomycin on human ovarian cancer cells are associated with modulating p38 MAPK* (Poster Presentation)
- (3) **Annual Conference and Symposium, Chinese Association of Life Science in Switzerland (CALSS), 12 February, 2012, Zurich, Switzerland.** Zhang B, Wang X, Cai F, Chen W, Loesch U, Zhong XY. *Anticancer activity and effects of salinomycin in human ovarian cancer cells* (Poster Presentation and won 1st Rank Award of Poster)