**Linköping University Medical Dissertations
Thesis No. 1436**

**Cancer and cancer stem cell targeting agents: A
focus on salinomycin and apoptin**

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Linköping 2015**

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Printed in Sweden by LiU-Tryck, Linköping, Sweden, 2015

ISBN: 978-91-7519-153-9

ISSN: 0345-0082

All the 10 years of passionate hard work that I put in research is dedicated to my mother and a promise kept to my father, who considered education and character are the highest priorities.

**“The whole of science is nothing but a refinement of everyday thinking”
- Albert Einstein**

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Abstract

Current cancer treatments involving surgery, radiotherapy, and chemotherapy target the vast majority of cancer cells, but they are only partially effective in eliminating the disease. Failure to eliminate cancer with conventional treatments can lead to recurrence, which usually kills patient. This often occurs when cancer cells develop resistance to cancer drugs or when cancer-initiating cells (cancer stem cells), unaffected by existing treatment procedures, are present. Here, we studied two drugs, salinomycin and apoptin, that exhibit great potential in the future of cancer treatment not only for restricting malignancy, but also in preventing tumor recurrence. Salinomycin is an antibiotic that was used in poultry farming that is now used clinically to target cancer stem cells, and apoptin is a chicken anemia virus-derived protein that is capable of detecting and killing transformed cells. In this study, we delved into the molecular mechanism of salinomycin action leading to cancer cell death. We showed that salinomycin induces autophagy in both cancer and normal primary cells. We further demonstrated that salinomycin promotes mitochondrial fission, thus increasing mitochondrial mass and mitochondria-specific autophagy, mitophagy. Salinomycin-induced cell death was both necrotic and apoptotic as determined by increased release of HMGB1 and caspase-3, -8 and -9 activation. We also found that stress responses of normal and cancer cells to salinomycin differ and this difference is aggravated by starvation conditions. We proposed that a combinational treatment with glucose starvation, or glucose analogues such as 2DG or 2FDG, might enhance the effects of salinomycin on cancer cells while protecting normal cells. We previously reported that apoptin interacts with BCR-ABL1, a protein that is expressed in patients with chronic myeloid leukemia (CML). We located a minimal region on the apoptin protein that triggers inhibition of downstream BCR-ABL1 signaling effects. This deca-peptide region was tested on patient samples and was shown to effectively kill cancer cells derived from patients, similar to the drug Imatinib. We further show that the apoptin deca-peptide is cytotoxic to Imatinib-resistant patient-derived cancer cells. Thus, we identified a novel therapeutic targeting agent that can not only overcome drug resistance, but it can also induce cancer cell death without affecting normal cells.

Abbreviations

HPV	Human Papilloma Virus
EBV	Epstein-Barr Virus
HHV	Human Herpes Virus
EGFR	Epithelial Growth Factor Receptor
VEGFR	Vascular Endothelial Growth Factor Receptor
BCR	Breakpoint Cluster Region
MDR	Multidrug Resistance
MRP	Multidrug Resistance associated Protein
ABC	ATP Binding Cassette
ALDH1	Aldehyde Dehydrogenase1
CLL	Chronic Lymphocytic Leukemia
mTOR	mammalian Target of Rapamycin
NCX	Sodium/Calcium Exchanger
LRP6	Low-density lipoprotein Receptor-related Protein 6
CAV	Chicken Anemia Virus
NLS	Nuclear Localization Signal
NES	Nuclear Export Sequence
DEDAF	Death Effector Domain-Associated Factor
APC	Anaphase Promoting Complex
PML	Promyelocytic Leukemia Protein
NDV	Newcastle Disease Virus
PI3K	Phosphoinositide 3-Kinase
TAT	Transactivator of Transcription
PTD4	Protein Trasduction Domain 4
CML	Chronic Myeloid Leukemia
NOK	Normal Oral Keratinocytes

MEF	Mouse Embryonic Fibroblasts
GFP	Green Florescent Protein
RFP	Red Florescent Protein
ATG	Autophagy related Gene
7-AAD	7-Aminoactinomycin D
PGC1 α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
HMGB1	High Mobility Group protein B1
DRP1	Dynamin-related Protein 1
TCA	Tricarboxylic acid
DSR	Differential Stress Response
2DG	2-Deoxy-D-glucose
2FDG	2-Fluoro-2Deoxy-D-glucose
SV40	Simian vacuolating Virus 40

Manuscripts

Included in the thesis

1. **Jaganmohan R. Jangamreddy**, Saeid Ghavami, Jerzy Grabarek, Gunnar Kratz, Emilia Wiechec, Bengt-Arne Fredriksson, Rama Krishna Rao Pariti, Arthur Cieřlar-Pobuda, Soumya Panigrahi, Marek Los. Salinomycin induces activation of autophagy, mitophagy and affects mitochondrial polarity: Differences between primary and cancer cells. (*Biochim Biophys Acta Mol.Cel.Res.* June 2013).
2. **Jaganmohan R. Jangamreddy** Soumya panigrahi, Marek Los. Monitoring of autophagy is complicated: Salinomycin as an example. (*Biochim Biophys Acta Mol.Cel.Res.* Dec 2014).
3. **Jaganmohan R. Jangamreddy**, Mayur V. Jain, Anna-Lotta Hallbeck, Karin Roberg, Marek Los. Glucose starvation mediated inhibition of autophagy promotes cancer cell specific multiplicity of cell death by salinomycin. (*Manuscript to be communicated*).
4. **Jaganmohan R. Jangamreddy***, Soumya Panigrahi*, Kourosh Lotfi, Manisha Yadav, Subbareddy Maddika, Anil Kumar Tripathi, Sabyasachi Sanyal and Marek J. Łos. Mapping of Apoptin-interaction with Abl/BcrAbl, and development of apoptin-inspired targeted therapies (*Oncotarget.* August 2014) * indicates equal contribution.

Other manuscripts during PhD - Not included in thesis

5. Soumya Panigrahi, Jörg Stetefeld, **Jaganmohan R. Jangamreddy**, Soma Mandal, Sanat K Mandal, Marek Los. Modeling of molecular interaction between Apoptin, BCR-Abl and Crkl - an alternative approach to conventional rational drug design. (*PLoS one*, Jan 2012).
Contributions: Protein production and purification
6. Saeid Ghavami, Shahla Shojaei, Sudharsana R. Ande, **Jaganmohan R. Jangamreddy**, Behzad Yeganeh, Maryam Mehrpour, Jonas Christofferson, Wiem Chaabane, Adel R. Moghadam, Mohammad Hashemi, Ali A. Owji, Marek J. Los. Autophagy and Apoptosis Dysfunction in Neurodegenerative Disorders; autophagy and apoptosis-directed experimental treatments. (*Progressive neurobiology* January 2014).
Contributions: Wrote introduction, autophagy and regulation part of review
7. Ensieh A. Farahani, Hira K. Patra, **Jaganmohan R. Jangamreddy**, Iran Rashedi, Marta Kawalec, Rama K. Rao, Petros Batakis, Emilia Wiechec. Cell adhesion molecules and their relation to (cancer) cell stemness (*Carcinogenesis*, April 2014)
Contributions: Wrote Cancer stem cells, differentiation and cell surface markers part of review

8. **Jaganmohan R. Jangamreddy**, Los MJ. Mitoptosis, a novel mitochondrial death mechanism leading predominantly to activation of autophagy. (*Editorial, Hepat mon, August 2012*).
Contributions: Co-wrote the editorial
9. Mayur V. Jain, **Jaganmohan R. Jangamreddy**, Jerzy Grabarek, May Griffith, Frank Schweizer, Thomas Klonisch, Arthur Cieslar-Pobuda and Marek Los. Nuclear localized Akt enhances breast cancer stem-like cells through counter-regulation of p21Waf1/Cip1 and p27kip1. (*Manuscript communicated*).
Contributions: Study design, representation of data, analysis of the results and co-wrote the manuscript.
10. **Jaganmohan R. Jangamreddy**, Marek Los. Salinomycin induced cell death: Apoptosis, Necrosis or necroptosis. (*Manuscript under preparation*).
Contributions: Original idea, study design, execution, representation of data, analysis of the results and wrote the manuscript.

Independent collaboration works during PhD

11. Hirak K. Patra, **Jaganmohan R. Jangamreddy***, Roghayeh Imani*, et al., Selective killing of cancer cells through TiO₂ popcorn nanoarchitecture based high-throughput flash ROS generator. * indicates equal contribution).
Contributions: Involved in cell-based part of study design, representation of data, analysis of the results and helped writing the manuscript.
12. Surajit Pathak, **Jaganmohan Reddy Jangamreddy**, et al., Overexpression of microRNA 652 is an independent prognostic marker for rectal cancer patients with preoperative radiotherapy: A study of Swedish clinical trial on preoperative radiotherapy in rectal cancer patients.
Contributions: Involved in flow cytometry part of study design, representation of data, analysis of the results and helped writing the manuscript.
13. Mo-Jin Wang, Surajit Pathak, **Jaganmohan Reddy Jangamreddy** et al., MicroRNA-20a expression is an independent prognostic factor in rectal cancer patients with preoperative radiotherapy: a study in a Swedish clinical trial.
Contributions: Involved in flow cytometry part of study design, representation of data, analysis of the results and helped writing the manuscript.

Previous work

14. Wark L, Novak D, Sabbaghian N, Amrein L, **Jaganmohan R. Jangamreddy**, Cheang M, Pouchet C, Aloyz R, Foulkes WD, Mai S, Tischkowitz M. Heterozygous mutations in the PALB2 hereditary breast cancer predisposition gene impact on the three-dimensional nuclear organization of patient derived cell lines. (*Genes Chromosomes cancer*, May 2013).
Contributions: Executed and designed 3D Telomere FISH and Spectral Karyotyping.
15. Shannon A. Baxter, David Y. Cheung, Patricia Bocangel, Hae K. Kim, Krista Herbert, Josette M. Douville, **Jaganmohan R. Jangamreddy**, Shunzhen Zhang, David D. Eisenstat and Jeffrey T. Wigle. Regulation of the lymphatic endothelial cell cycle by the PROX1 homeodomain protein. (*Biochim Biophys Acta Mol.Cel.Res.* Jan 2011).
Contributions: Development of the adenoviral constructs and Prox1 constructs.
16. M. P. Czubyrt, L. Lamoureux, A. Ramjiawan, B. Abrenica, **Jaganmohan R. Jangamreddy** and K. Swan. Regulation of Cardiomyocyte *Glut4* expression by *Zac1*. (*Journal of Biological Chemistry*, May 2010).
Contributions: Western Blotting for *Zac1* and micro-array execution and analysis.
17. **Jaganmohan R. Jangamreddy**, Jeffrey T. Wigle. Activation of vascular endothelial growth factor receptor-3 expression by homeobox transcription factor, *Prox1*. (Masters thesis, 3 years of full time laboratory work).
Contributions: Study design, execution, representation of data, analysis of the results and wrote the full thesis.
18. Macoura Gadj, **Jaganmohan R. Jangamreddy***, Shubha Mathur*, Brigitte Beranger*, Josée Lamoureux, Ana- Maria Crous Tsanacis, David Fortin, Regen Drouin and Sabine Mai. Telomere dysfunction - associated time to recurrence in oligodendrogliomas and oligoastrocytomas (Manuscript under submission * indicates equal contribution)
Contributions: Executed and designed 3D Telomere FISH experiments and analyzed the results.
19. S. Hombach-Klonisch, ***Jaganmohan R. Jangamreddy**, *J Von-Vopelius-Feldt, S. Mai. Distinct chromosomal rearrangements and 3-dimensional telomere architecture in antiestrogen-resistant human breast cancer cell lines. (Manuscript under submission * indicates equal contribution).
Contributions: Executed and designed 3D telomere FISH, metaphase FISH and Spectral Karyotyping.

Introduction

Cancer exerts a death toll of ~8.2 million people every year, amounting to 12% of total human mortality. It is next only to cardiovascular and infectious diseases in human morbidity. The global economic burden of cancer is 2-3% of global GDP, or approximately \$900 billion without the cost of treatment included, making it the most financially devastating disease. With a growing number of diagnoses, the worldwide clinical costs of cancer treatments are approximated at \$300 billion per year in 2010 ¹.

The majority of cancers (~80%) are caused by environmental and lifestyle factors, while genetic predisposition constitutes the remaining 20% ¹. Lifestyle factors including tobacco and alcohol use, as well as a poor diet are the main risk factors for developing cancer worldwide. Environmental factors, including viral and bacterial infections vary geographically. Cancers caused by infectious agents account for 10% of all malignancies in technologically advanced nations, however, this number rises to 25% in tropical countries ². HPV (Human Papilloma Virus), EBV (Epstein-Barr Virus), and HHV (Human Herpes Virus or Kaposi Sarcoma associated virus) are the most common viruses that contribute to carcinogenesis in the cervix, stomach, skin, and lymphatic system ². These viruses either promote cancer cell proliferation, as in the case of Hodgkin's lymphoma (EBV), or increase cancer cell resistance to cell death, thus increasing the survival of carcinogenic cells (HPV and HHV) ². Other infectious agents such as bacteria and parasites have been proposed to increase the risk of stomach cancer but are not as well studied. In addition to the carcinogenic factors that allow abnormal cells to escape apoptosis, it is the acquisition of infinite proliferative ability that allows cancer cells to eventually develop into a tumor.

Hijacking the cancer signatures: Therapeutics

A century of research on the origins of various cancers suggests that mutations in cancer genes (tumor suppressors and oncogenes), acquired characteristics of cell survival, resistance to cell death, proliferative capacity without external stimulus, and intra-tumoral vascular development are all prominent features of cancer occurrence and progress ³. Disrupting these characteristic cancer features is vital to the development of clinically viable therapeutics.

In addition to surgical removal of solid, primary malignant tumors, radiotherapy and chemotherapy are widely used treatment options. Radiotherapy exploits several properties of cancer cells, such as their high proliferation rate, accumulation of DNA abnormalities, and generation of reactive oxygen species due to changes in their metabolic profile. Chemotherapy is even more varied, devising targets based on a tumor's molecular signature, or other hallmarks of cancer. The outcome of radiotherapy is largely dependent on the sensitivity of the cancer cells to radiation as well as the type of malignancy. Highly proliferating leukemias and lymphomas are more sensitive than glioblastoma (brain tumors) to radiation treatment. Moreover, the tolerance of different tissues and organs to radiation therapy varies widely and is a determining factor in the use of radiotherapy to treat malignancies. For example, metastasized tumors that have spread to numerous lymph nodes and tissues or tumors that are widely distributed throughout the body, such as leukemia, are rarely treated with radiation therapy. Advances in radiotherapy allow precise delivery of radioactive compounds to selectively target specific cancer cell types, causing minimal damage to the adjacent healthy tissues (eg: Zevalin, radioactive conjugated CD20 antibody, for the treatment of lymphomas). This has led to increased use of radiotherapy for sensitive and localized cancers. However, the use of radiotherapy in combination with chemotherapeutic drugs to hinder cell proliferation via inhibition of DNA repair or replication is still the most common

due to the robust sensitization of cancer cells. Thus, the use of radiation as a combined therapy is more common than standalone therapy. (Details of radiotherapy are reviewed in the following sites ^{4,5})

Unlike radiotherapy, chemotherapy is more dependent on cancer type. It is cell type-specific and can be used to treat a broad spectrum of cancers, depending on the mechanism of drug action and its specificity. The possibility of tailoring drugs based on the molecular profiling of specific cancers has increased current screening studies to identify “wonder drugs” that may effectively result in customized or even personalized cancer treatment, and it has led to the generation of novel drug delivery mechanisms to efficiently target cancer cells. Drugs used for cancer therapy can broadly be classified into two groups:

I. Standard chemotherapy: including alkylation and platinum-based drugs that trigger DNA damage and interrupt the cell cycle. Other drugs that are also used in standard therapy include topoisomerase inhibitors (Topotecan, Etoposide), anthracyclines (Doxorubicin, Daunorubicin) that hamper DNA replication, and cell cycle inhibitors (Paclitaxel, Docetaxel, Vinblastine) ⁶.

II. Targeted therapy employs small molecule inhibitors and monoclonal antibodies that specifically hinder the function of their respective onco-proteins, thus playing diverse roles in cell growth, proliferation, survival, and cell death, in addition to tumor angiogenesis and drug resistance ⁶. Among the most prominent small molecule inhibitors are Gefitinib (EGFR) and Imatinib (ABL1). They are widely used in the treatment of breast cancer and CML patients that are positive for the Philadelphia chromosome (translocation of chromosomes 9 and 22 leading to the formation of BCR-ABL1) (for a complete list of approved drugs, their specific targets, and types of cancers, refer to www.mycancergenome.org ⁷). Monoclonal antibodies like trastuzumab (herceptin) and bevacizumab target HER2/neu, an EGFR-related kinase that is commonly expressed in breast cancers, and VEGFR, a crucial receptor required for intra-tumoral vascularization, respectively ⁸.

Since targeted therapy depends on the expression of the protein target, and most cancers are heterogeneous, the efficacy of small molecules and monoclonal antibodies is limited. For example, Her2/neu is highly expressed in only a quarter of breast cancer patients and therefore the use of Trastuzumab (Herceptin) is limited to these patients⁸. EGFR is highly expressed in most cancers, but the use of the small molecule inhibitor Gefitinib or monoclonal antibody Cetuximab is restricted to only a few cancers because EGFR is also expressed on normal cells and such use may trigger toxic effects^{8,9}. A problem commonly encountered with the use of small molecules inhibitors (or drugs in general) is the development of drug resistance by the cancer cells. This can occur through acquiring mutations in targeted proteins or through the adaptation of alternate cancer cells survival strategies. Monoclonal antibodies do not generate the same adaptive responses; however, their immuno-therapeutic capability depends on antibody humanization to avoid immune responses⁸.

Cancer stem cells and cancer recurrence:

The recurrence of a tumor after radiation and/or chemotherapy is often due to the presence of self-renewing cancer stem cells that are resistant to treatment. Cancer cells may also acquire resistance to drug treatment. The acquisition of resistance, as previously mentioned, is caused by the development of alternate cancer cell survival strategies. Cancer cells expressing p-glycoprotein and Multidrug-resistance-associated protein (MRP) develop resistance to chemotherapy, and the identification of these resistance-mediating proteins has initiated vast interest in the field⁶. Further comparison of cancer stem cells, causing tumor recurrence, and normal stem cells that are relatively quiescent reveals that cancer stem cells possess a heightened ability to repair themselves and they express high levels of active ABC drug transporters. These differences allude to the possible origin of drug-resistant cancer cells from normal stem cells that have been transformed (Hierarchical hypothesis)¹⁰⁻¹². The

hierarchical hypothesis supports the notion that the altered pluripotent or multipotent cell is the source of cancer initiating cells. This leads to the existence of small population of cells that possess self-renewal capacity and can generate terminally differentiated cells. The alternative stochastic model proposes the random acquisition of stem cell-like characteristics in differentiated cancer cells (the de-differentiation hypothesis)^{13, 14}. The authenticity of both models is widely debated, but the existence of the tumor initiating cells or cancer stem cells is more profoundly accepted¹⁵⁻¹⁷.

The ambiguity in cancer stem cell theories arises mainly due to the lack of a complete set of markers that clearly define the hierarchy of cancer stem cells and reliable techniques to assess clonal expansion¹⁸. A popular screening method for cancer stem cells involve flow cytometric estimation of side population (SP) cells based on reduced uptake of Hoechst 33342 DNA binding dye, the aldefluor assay, and the cell surface protein markers (CD34, CD44, CD133 etc.)^{13, 18, 19}. SP cells express higher levels of the ABC cassette family of drug transporters, leading to higher efflux of Hoechst 33342 dye and thus less intense labeling compared to other cell populations. The aldefluor assay exploits the high level of aldehyde dehydrogenase1 (ALDH1) expression by cancer stem cells compared to differentiated mature cells. Both ABC transporters and ALDH1 have a functional role in the maintenance of both stem cell and cancer stem cell “stemness,” and thus, the origin of cancer stem cells from stem cells is compared as mentioned earlier^{20, 21}. However, in some cancers, ALDH1 promoted the differentiation of cancer stem cells, and in other cancers inhibition of its activity promoted stemness, demonstrating that the use of the aldefluor assay is precarious depending on the cancer type^{13, 22}. The ABC transporter ABCB5 is a marker for colorectal cancer stem cells, thus encouraging the use of tissue and cancer type-specific cancer stem cell markers²⁰.

The identification of several cancer cell-specific surface markers such as CD34⁺ (leukemia), CD44⁺ (breast cancer, prostate cancer, and head and neck cancers), and CD133⁺ (pancreatic, brain, liver, and lung cancers) in several cancer types in

conjunction with lineage markers specific to their tissue of origin supports the hierarchical model ^{10, 13, 23}. Most studies describing cancer-specific cancer stem cell markers employ tumor xenotransplantation functional studies that are immuno-dependent and/or orthologous regeneration-capable selection of cancer stem cells. Not taking into account these important factors, confounding support for the hierarchical model comes from cancer stem cell lineage tracing performed by following the expression of a fluorescent marker linked to stem cell specific genes such as Nestin ²⁴. More recently, a mathematical model correlating the probability of developing cancer to the number of stem cell divisions (which depends on the tissue type) shows convincing support of the stem cell origin of cancer stem cells ²⁵. However, because cancer cells undergo both genetic and epigenetic transformations, the de-differentiation of differentiated cancer cells to acquire stem cell-like properties (Clonal evolution hypothesis) is also a possibility ¹³⁻¹⁷. Regardless of the complexities surrounding stem cell origin, their identification, or the terminology used, metastatic recurrence of cancers caused by surviving cancer stem cells are a widely accepted mechanism.

Figure 1. Defying the odds—three stages of cancer: (A) Transformation of a normal cell to a cancer cell is kept in check through various homeostatic mechanisms within cells and tissues. Repair and degradation mechanisms are in place to address cellular damage at the DNA and protein levels; however, if a cell acquires multiple aberrations in these crucial mechanisms, the cell can transform into a cancer cell. (B) Even after acquiring cellular abnormalities the body eliminates the malfunctioning cell through an immune attack or phagocytosis, or it keeps the cell in a quiescent state. If the cell eventually escapes these barriers, it can then be tested for its ability to adapt to hypoxic and starvation conditions. (C) If successful, these cells enter the blood stream where these malignant cells undergo another test for survival, involving the mechanical stresses of vascular transport and eventual adaptation in a new tissue.

Targeting cancer stem cells

The field of cancer research focused on cancer stem cell-targeting agents was initiated with the development of inhibitors against ABC cassette family members. Now cancer stem cell targeting is employed to treat the disease, and it is based on cell surface marker recognition through immuno-therapeutics, small molecule inhibitors against intrinsic signaling pathways like hedgehog, Wnt/ β -catenin, and notch, and tumor microenvironment targeting agents. The discovery of verapamil, a calcium channel antagonist, bolstered the search for MDR inhibitors over the last three decades. However, this search has resulted in only partial success with the development of third generation inhibitors such as tariquidar, inhibiting P-glycoprotein with least side effects²⁶⁻²⁹. Even though much earlier generations of MDR inhibitors showed potential in vitro, the clinical translation of these drugs lagged due to their toxicity towards healthy tissues. Now alternate mechanisms are being explored to target MDRs, such as the development of small peptides that have high affinity to the transmembrane domains of ABC transporters, and thereby block their activity, transcriptional suppression of MDR genes, and the development of novel drugs that act as substrates for ATP transporters and evade efflux²⁶.

Xenotransplantation of tumor cells into immunocompromised mice to understand the clonogenicity or stemness properties of these cells has revealed that immune responses play a vital role in tumorigenesis and provide a counter argument for the hierarchical model of cancer stem cell origin. Differentiated oral squamous carcinoma cells are more resistant to the actions of natural killer cells compared to their stem cell counter parts that are positive for CD44 and CD133³⁰. On the other hand, circulating leukemic cells and human bladder cancer initiation cells express CD47, hampering their phagocytosis by macrophages in a similar manner to normal hematopoietic stem cells before entering the blood circulation³¹⁻³³. These studies show that cancer stem cells avoid immune attack and that targeting the cancer stem cell microenvironment to directly induce

cancer stem cell death or promote the differentiation of cancer stem cells to prevent metastatic recurrence should be further evaluated. VEGF inhibitors that block angiogenesis are shown to alter the tumor vasculature when used with chemotherapeutics that modulate the pH of the microenvironment, promoting cancer stem cell differentiation and death³⁴⁻³⁷.

FDA recently approved vismodegib for the treatment of basal cell carcinoma; this drug targets the protein smo because these tumor cells possess an active hedgehog signaling pathway³⁸⁻⁴¹. This has initiated interest in blocking similar signaling pathways to treat other cancers. Vismodegib's success in treating basal cell carcinoma is only partial, however, some resistant tumors develop. This drug was further tested for use in treating medullablastoma and pancreatic tumors⁴⁰. Similarly, inhibitors of the Wnt/ β -catenin and notch signaling pathways were also explored, however, the abysmal results obtained from treating ovarian and colorectal cancers with vismodegib made researchers cautious when using inhibitors of crucial signaling pathways that are important for normal tissue and stem cell function⁴⁰. In this study, we focused on the newly identified cancer stem cell-targeting agents salinomycin and apoptin.

Salinomycin:

Salinomycin was originally used as an anti-coccidial drug in poultry feed and for efficient nutrient absorption in farmed pigs. Gupta and colleagues (2009) first described the preferential toxicity of salinomycin toward cancer-stem cells *in vitro*, using E-cadherin-targeted HMLER cells (HMLER^{shEcad}), which show increased CD44⁺/CD24⁻ phenotypes with high mammosphere formation capabilities⁴². In the same study, they went on to further show that salinomycin is 300 times more effective in targeting cancer stem cell-like cells than paclitaxel, and salinomycin pre-treated cells show a 100-fold decrease in seeding capacity, or the ability to form tumors upon xenotransplantation into immunocompromised mice⁴². This study was followed by several reports confirming salinomycin's

toxicity among cancer stem cells in gastrointestinal sarcoma, osteosarcoma, pancreatic, colorectal cancer, and breast cancer ⁴³⁻⁴⁷. Cell death mechanisms induced by salinomycin still remain elusive even though they are thought to be largely dependent on the impairment of mitochondrial function, excessive ROS generation, and caspase-dependent or independent pathways based on cell type ⁴⁸⁻⁵².

The major pathways that are affected by salinomycin are the Wnt/ β -catenin and Akt/mTOR ⁵³⁻⁵⁵. Lu et al., 2011, showed that salinomycin targets cancer stem cells by inhibiting the Wnt/ β -catenin pathway. Nanomolar concentrations are sufficient to block Wnt, whereas higher concentrations (micromolar) hamper LRP5/6 phosphorylation and β -catenin activation ⁵⁶. Salinomycin action on Wnt signaling is further reported among cancer stem cells of CLL patients and gastric tumor mouse models ^{55, 56}. Salinomycin's action on Akt and mTOR is dependent on cancer cell type. While salinomycin treatment of non-small cell lung carcinoma and ovarian cancer cells led to reduced Akt and mTOR activity, cancer stem cells from head and neck squamous cell carcinoma showed elevated Akt activity ⁵⁷⁻⁵⁹.

Another vital function of salinomycin is its ability to inhibit MDR protein function, leading to the increased susceptibility of ABC protein expressing drug resistant cells to treatment. Salinomycin inhibits the function of MDR1, ABCG2, and ABCC11 among both naturally expressing cells and cells that over express these respective proteins after drug treatment ^{60, 61}. Salinomycin is also reported to be non-toxic to primary normal cells ⁵⁶.

Salinomycin as anti-cancer stem cell therapeutic drug:

Thus far, studies using both *in vitro* and *in vivo* models show that salinomycin is a potent cancer stem cell-targeting agent that can be developed for clinical treatment for a broad spectrum of cancers. The previous use of salinomycin in veterinary treatments provides evidence that the drug is well

tolerated among mice, pigs, cats, and dogs and that only very high doses lead to neural dysfunction that may cause paralysis⁶²⁻⁶⁵. So far there has been only one case of human toxicity after treatment with salinomycin where a patient reported having symptoms of weakness in the legs, tachycardia, and decreased reflexes, indicating a similar neurotoxicity as in animals^{62, 66}. Animal studies on salinomycin usage along with CGP (a benzothiazepine derivative of clonazepam), an inhibitor of NCX (sodium calcium exchanger), were shown to protect neuronal cells from the toxic effects of salinomycin without altering its anti-cancer properties⁶⁷. This further suggests that salinomycin has great potential for clinical use. The ability of salinomycin to inhibit MDR protein activity as well as Wnt and its downstream signaling cascade, LRP6 and β -catenin, at different concentrations provides substantial support for its use as an adjuvant therapy. Much of the data promoting the use of salinomycin for clinical treatment comes from pilot studies involving four metastatic breast cancer patients, a metastatic ovarian cancer patient, and a patient with head and neck squamous cell carcinoma⁶². The patients are reported to have shown symptoms of mild acute tachycardia and tremor for about an hour, but they reported no persistent long-term side effects. The symptoms did not differ among groups of different ages (40 to 80 years of age), and all of them showed reductions in tumor volume and metastasis⁶². These promising initial successes however need to be further substantiated with next phase of clinical trials. Many of the recent pre-clinical studies suggest combination of salinomycin with several commonly used chemotherapeutic drugs has a better outcome and gives the advantage of using lower doses that can be translated to even more minor side effects^{43, 68-71}.

Apoptin:

Apoptin is a chicken anemia virus (CAV)-derived nonstructural protein consisting of 121 amino acids. Of the three different proteins encoded by the virus, only VP3/apoptin is involved in induction of apoptosis by CAV. Apoptin

leads to regression of tumors in chickens carrying tumors induced by the Rous sarcoma virus ⁷². Apoptin consists of a bi-partial nuclear localization sequence (NLS) present at the c-terminal end along with a putative nuclear export sequence (NES) at amino acids 97-105. The nuclear and cytoplasmic shuttling of apoptin is driven by these sequences. Apoptin also has hydrophobic leucine-rich region (aa 33 to 46), which facilitates its self-association and binding with a leukemia protein and its other interacting partners ⁷³⁻⁷⁵.

As the case in cancer cells, apoptin can also translocate into the nucleus of normal cells, but it is promptly exported back to cytoplasm, owing to its nuclear export signal located close to the N-terminus. Nuclear localization of apoptin in normal cells may lead to senescence, whereas in cancer cells, nuclear-apoptin induces apoptosis (typically after 16-30 h) ⁷⁶. Apoptin triggers apoptosis through a mitochondrial death pathway ^{77, 78}. The proposed mechanism suggests that apoptin associates with and activates PI3K in the cytoplasm, which leads to translocation of Akt into the nucleus ⁷⁹. In the nucleus, Akt activates CDK2 both directly and indirectly, by degradation of p27^{kip1} ^{79, 80}. Activated CDK2 phosphorylates apoptin at Thr-108, leading to its nuclear accumulation ⁸⁰. Apoptin in the nucleus interacts with other proteins like DEDAF, APC, PML, and Nml, and it also leads to the phosphorylation of Nur77 and its translocation to cytoplasm where it converts anti-apoptotic Bcl2 into a pro-apoptotic molecule ^{81, 82}.

The potency of apoptin to induce cell death among minimally transformed cells and broad range of cancer cells has been established for over a decade; however, its translation to clinic has been hampered by poor stability and due to the lack of efficient tumor delivery methods ^{74, 83}. As a chicken-infecting virus-derived protein, apoptin is in itself immunogenic. Therefore, an effective mechanism to deliver apoptin to the tumor site is the main confounding factor preventing the development of apoptin-based therapies. Several delivery methods including adenoviral, oncolytic, and bacterial systems have so far been tested in preclinical studies, and the oncolytic virus NDV has shown promise ⁸⁴⁻⁸⁷.

Other non-viral, direct delivery methods using small peptide tags, such as TAT and PTD4, which assist in cellular transduction or penetration and facilitate access to the entire tumor volume, are also under consideration^{81, 88-90}. With the recent discovery of human gyroviral-derived apoptin showing similar function in cell death as its chicken homolog, apoptin-based therapies may be developed in the foreseeable future^{81, 91, 92}.

Thesis objectives:

- 1.) To investigate the molecular mechanism of salinomycin in the induction of autophagy and apoptosis in cancer cells.
- 2.) To identify the BCR-ABL1-interacting minimal region of apoptin that can impair the downstream activity of the aforementioned tyrosine kinase.

Aims of the manuscripts:

Paper 1: The aim of the paper was to understand the role of salinomycin in inducing autophagy as well as the function of autophagy in salinomycin-induced cell death. The goal was also to understand whether mitochondrial function had a role in salinomycin-induced cancer cell death.

Paper 2: The aim of the paper was to investigate autophagic flux induced by salinomycin and to explore conflicting reports in the literature.

Paper 3: The goal of the paper was to study differential stress responses of cancer and primary cells to salinomycin-treatment under starvation conditions. The aim was also to study the autophagic responses of cells after starvation and salinomycin treatment.

Paper 4: The aim of the manuscript was to identify the minimal region of apoptin necessary to bind the BCR-ABL1 protein and inhibit its function, as well as to study its ability to induce cell death among CML patient samples.

Methods

Cells and cell culture:

A wide range of human cancer cell lines, human normal primary cells (dermal fibroblasts and NOK), mouse embryonic fibroblasts (MEF), and ATG5 knockout MEF's were used in this work. We used prostate cancer (PC3), breast cancer (SKBR3 and MDAMB468), LK4013, and LK0923 cells for salinomycin studies. The K562 human CML cell line and the mouse cell line 32D^{DSMZ} as well as its BCR-ABL1 variant-expressing cell line 32D^{p210} were used for apoptin-based studies. The culture conditions were described in their respective manuscripts.

Human CML patient and normal primary samples:

After obtaining informed consent, peripheral blood samples were collected from 3 healthy donors and 6 BCR-ABL1 positive CML-patients (3 of which responded positively to imatinib treatment (imatinib responsive) and 3 that did not respond to imatinib therapy (imatinib resistant). All samples were obtained from King George's Medical University (erstwhile Chatrapati Shahu Ji Maharaj Medical University), Lucknow, Uttar Pradesh, India. Histopaque gradient centrifugation (density 1.077 g/mL; Sigma-Aldrich) was used to isolate mononuclear cells. Cells were then re-suspended in RPMI-1640 supplemented with 10% FBS after washing with PBS.

Estimation of autophagic flux by flow cytometry:

Principle: LC3I, an ortholog of yeast ATG8, is a widely used marker to estimate both the induction and completion of autophagy. LC3I is initially cleaved and then tagged with phosphatidylethanolamine (PE) to form LC3II, which is then transferred to both the internal and external membranes of autophagosomes during elongation. Autophagosomes fuse with acidic lysosomes to form mature autolysosomes that maintain a lower pH and contain proteases that degrade autophagosome contents. The LC3II that is localized to the inner membrane is degraded, while the remaining LC3II is recycled once the process is complete. Thus, fluorophore-tagged LC3 (GFP or a GFP-RFP tandem tag) is commonly used to measure autophagy and autophagic flux. As illustrated in Figure 2, under

normal conditions, due to basal autophagy, LC3II is localized to the autophagosome and undergoes partial degradation while the rest is recycled. This leads to a continuous partial decrease in fluorescence compared to the hypothetical true state where there is no autophagy. When cells are treated with inhibitors of autophagy (chloroquine or bafilomycin) LC3II is not degraded and the fluorescence intensities of GFP-LC3 more closely resemble their “true” state. Under conditions stimulating autophagy (starvation and rapamycin treatment), GFP-LC3II is degraded much more quickly and fluorescence intensity is decreased. In the case of cells pre-treated with an autophagy inhibitor that are then treated with an autophagy inducer, the fluorescence intensity should increase to reach the “true” state. mTandem GFP-RFP-LC3 produces similar results as GFP-LC3, but RFP is more resistant to the bleaching effects of that acidic conditions in the autolysosomes compared to GFP. The GFP to RFP ratio can be calculated to estimate the autophagic flux⁹³⁻⁹⁶.

Conversely, membrane-bound LC3II is estimated by permeabilization of the cells with a detergent so that the free, cytoplasmic-localized GFP-LC3 is removed and only the GFP-LC3II that is localized to the membrane remains⁹⁷. Such estimation provides similar results to LC3II quantification using the Western blotting technique, where both inducer and inhibitor treatment show increased LC3II but pre-inhibition of autophagy and treatment with an autophagy inducer shows radiometric increases.

Figure 2: LC3II turn over as a marker for autophagy: LC3I undergoes cleavage and lipidation to form LC3II and localizes to the either side of the elongating phagosome membrane. Upon completion of autophagosome formation, with collected cell debris and proteins such as p62, the lysosome fuses with the autophagosome, leading to increased acidity in the autophagolysosomal complex. After degradation of the enclosed cellular organelles and debris is complete, LC3II that is localized on the outer membrane of autophagosome gets recycled along with other membrane components to form new autophagosomes. This LC3II turnover of is quantitated to monitor autophagic flux along with p62 degradation.

Figure 3: Methods to estimate autophagy inducers and inhibitors using GFP-LC3:

The presence of basal autophagy among cells decreases green fluorescence and any further induction of autophagy increases the bleaching of green fluorescence as GFP-LC3 is converted to GFP-LC3II and localized to autophagosomes that eventually fuse with acidic lysosomes. On the other hand, inhibitors of autophagy hinder bleaching, resulting in increased green fluorescence. In the presence of both an inhibitor and inducer of autophagy, similar increases in green fluorescence are observed similar to the addition of the inhibitor alone. When cells are permeabilized, unbound GFP-LC3 is lost and the green fluorescence represents only autophagosome-localized GFP-LC3II. Therefore, in the presence of either inhibitor or inducer of autophagy, increases in green fluorescence will be observed; however, the green fluorescence increases ratiometrically in the presence of both inhibitor and inducer, unlike that observed in the presence of any one of these agents.

Method: To mimic the normal conditions MEF cells were used to discard the plausible genetic aberrations in monitoring autophagy as in the case of cancer cells. We also used PC3 cell line as a follow up to our initial studies. MEF cells when infected with GFP-LC3 expressing adenovirus at a concentration of 50 MOI showed moderate expression by 24 h but PC3 cells showed similar expression by 48 h at the same MOI. In the case of mTandem GFP-RFP-LC3 over expression both cells were infected with 200 MOI of respective adenovirus for 48 h. The cells are optionally pretreated with 50 μ M of Chloroquine for 30 min for comparison of flux and then treated with different concentrations of Salinomycin (0.1 μ M, 1 μ M and 10 μ M), 0.25 μ M Rapamycin, or serum starvation media as per experimental set up for 6 h. The cells are then washed with PBS, trypsinized and resuspended in 500 μ l of PBS. For the studies where cells are permeabilized, the resuspended cells are incubated with 0.05% Saponin in PBS for 10 min and centrifuged for 5 min at 1000 RPM. The pellet is then re-suspended in PBS and is subjected for flow cytometry. For all the experiments 10^6 cells were plated in each well of a 6-well plate and cultured for 24 h before they were infected with the adenovirus. Mean Florescence Intensities were measured using FL1 and FL4 filters of Gallios flow cytometer equipped and excited using 488-nm laser. Analysis of the data and representative images were developed using FlowJo software.

Live Cell Imaging:

Cells plated and infected as mentioned above with adenovirus expressing GFP-LC3 for 24 h were treated with 100 nM lysotracker Red for 30 min. The cells were treated with salinomycin for 30 min at 0.1 μ M concentrations and the cell chamber is fixed on to the microscope stage. For the Z-stacking upper and lower limit of the cell is defined for each cell and 8 slices were taken for each cell. Series of time-lapse images were then acquired using Leica DMI6000 fluorescence microscope equipped with a DFC365 monochrome camera and 63X oil objective (NA 0.6-1.4) for 1 h at a rate of 10 sec interval per frame. The images were further analyzed using ImageJ and Bitplane Imaris software.

Results and Discussion

Manuscript 1: Salinomycin induces activation of autophagy, mitophagy and affects mitochondrial polarity: Differences between primary and cancer cells

In this article, we focused on gaining mechanistic insight into salinomycin toxicity. Using immunoblotting techniques, we observed that salinomycin triggered an increase in autophagy in both cancer cell lines (prostate and breast cancer cells) and primary human fibroblasts. Salinomycin-induced autophagy acted as a cell-protective mechanism in cancer cells, as autophagy blockage, either with an autophagic inhibitor (Chloroquine) or murine fibroblasts deficient for ATG5 (ATG5^{-/-}), showed increased cell death. Interestingly, the increase in salinomycin toxicity upon autophagy inhibition was observed in breast and prostate cancer cells but not in primary fibroblasts.

To explore the role of mitochondria in salinomycin-induced cell death, we studied mitochondrial-specific autophagy, abnormalities in mitochondrial morphology, and the dynamic mitochondrial processes of fusion and fission. We observed that the mitochondrial tubular network displayed fragmentation and disintegration, resulting from mitochondrial fission and fusion, upon salinomycin treatment. We also showed that treating cancer cells with salinomycin decreased the total number of mitochondria with intact inner membrane potential ($\Delta\Psi$) and concomitantly increased mitochondrial mass. To understand the role of nuclear signaling in mitochondrial biogenesis, we looked at changes in PGC1 α protein expression in cancer cell lines upon treatment with salinomycin and found no significant changes. These results suggested that mitochondrial fusion and fission mechanisms contributed to the alteration in mitochondrial mass and the maintenance of mitochondrial function in salinomycin treated cells. Supporting this hypothesis, cancer cells treated with salinomycin showed depletion of total cellular ATP in a time and concentration dependent manner. However, primary human fibroblasts did not show similar ATP depletion upon salinomycin treatment.

The cancer cell-specific deleterious effects observed after salinomycin treatment could be explained by the fact that cancer cells harbor various abnormalities within mitochondria, such as hyperpolarized mitochondria or defective mitochondrial fission and fusion mechanisms due to the lack of functionally intact mitofusin proteins, DRP1, and other proteins involved in mitochondrial dynamics^{98, 99}. Compromised mitochondrial function in cancer cells may be the major factor for cancer cell susceptibility to ionic fluctuations induced by salinomycin.

We took a closer look at the mechanism of salinomycin-induced cancer cell death. In this study, we demonstrated that salinomycin triggered activation of executioner caspase-3 along with initiator caspase-9, among cells with hyperpolarized mitochondria. Inhibition of caspases using the pan-caspase inhibitor Q-VD-OPh partially protected against salinomycin-induced cell death, but it was unable to fully reverse the toxic effects of salinomycin. In addition to the activation of apoptotic signaling pathways, we also observed necrotic/necroptotic cell death, especially among cancer cells treated with higher concentrations (10 μ M) of salinomycin. A subpopulation of cells showed increased 7-AAD staining, the preferential method used for identifying necrotic cells, without co-staining with the apoptotic marker (Po-Pro). Necrosis/necroptosis induction was also confirmed by detection of the HMGB1 protein, a marker for necrosis^{100, 101}, in the supernatant of cells treated with higher concentrations of salinomycin.

Manuscript 2: Monitoring of autophagy is complicated: Salinomycin as an example.

Concurrent with our publication on salinomycin-induced autophagy, Yue et al. proposed that salinomycin acts as an inhibitor of autophagic flux¹⁰². We further evaluated this possibility by examining LC3II expression, a marker for autophagosomes, by Western blotting analysis of protein lysates from mouse embryonic fibroblasts and PC3 cell lines after treatment with various concentrations of salinomycin or chloroquine. The results confirmed that, at lower concentrations, salinomycin treatment does lead to ratiometric increases in LC3II, compared to cells treated with the autophagic inhibitor chloroquine in conjunction with salinomycin. However, at higher concentrations, even though a linear increase in LC3II levels was detected upon chloroquine treatment, there was no significant ratiometric increase. We also observed that when the concentration of chloroquine was increased from 25 μ M to 50 μ M, LC3II accumulation was increased after treatment with the same salinomycin concentration (10 μ M). We further investigated autophagic flux using GFP-LC3 and tandem mRFP-GFP-LC3-expressing adenoviral vectors with traditional immunocytochemistry as well as novel flow cytometry and advanced microscopy techniques. We employed robust live-imaging techniques to carefully monitor autophagy after salinomycin treatment. Consistent with our previous findings, we observed that salinomycin is an inducer of autophagic flux at lower concentrations, and it can act as an inhibitor of functional autophagy at high concentrations.

Manuscript 3: Glucose starvation-mediated inhibition of autophagy promotes cancer cell-specific multiplicity of cell death after salinomycin treatment.

To further understand the mechanism of salinomycin-induced autophagy and enhance cell death induced by salinomycin treatment specifically in cancer cells, while protecting primary cells, we looked into whether salinomycin treatment modulated cancer cell metabolism (i.e. the Warburg effect). Our initial studies showed that salinomycin treatment in coordination with glucose starvation rapidly killed cancer cells (90% cell death in 24 h) while showing no toxicity to primary fibroblasts under non-proliferative conditions. When the same experiments were carried out under hypoxic conditions to mimic the tumor environment further increase in cancer cell death was observed. These results indicated that salinomycin induced more robust cell death among cancer cells that has switched their metabolic dependency toward glycolysis and lactic acid production rather than being dependent on the TCA cycle for ATP production. In this study, we also tested the ability of primary and cancer cells to recover after salinomycin treatment. Interestingly, cancer cells (LK0412 cells) treated with (10 μ M) for 6, 12, or 24 h did not show any sign of recovery, even after 24 or 48 h incubation with normal media. This indicated that salinomycin-treated cells lose the ability to proliferate, leading to inhibition of cancer metastasis. Similar experiments performed on normal primary keratinocytes (NOK) showed recovery of cellular proliferation suggesting that salinomycin has minimal effects on normal cells.

We further explored the mechanism of salinomycin-induced robust cell death under starvation conditions. Since both starvation and salinomycin induce autophagy, we expected to find increases in the autophagic process. Surprisingly, we observed decreased expression of the autophagic marker LC3II in cancer cells treated with salinomycin under starvation conditions. This may be due to the heightened impact of both salinomycin and starvation on cells, increasing the severity of cell death by overriding the protective measures conferred by the

autophagic process. In addition to the aforementioned experiments, we also investigated the possible role of the PI3K/Akt/mTOR pathway in LC3II upregulation. We found that inhibition of Akt, similar to starvation, decreased LC3II expression and increased cell death in the presence of higher concentration of salinomycin.

Manuscript 4: Identification of the apoptin minimal region and its requirement for interaction with BCR-ABL1 to inhibit downstream signaling.

In our previous study, we identified a putative BCR-ABL1 binding site on the apoptin protein using in silico methods ¹⁰³. For the first time we showed that apoptin interacts with SH3 domain of BCR-ABL1 and inhibits phosphorylation of Crkl, a downstream target of BCR-ABL1 ¹⁰³. In this study we investigated apoptin derivatives that could be directed towards inhibiting multiple hyperactive kinases including BCR-ABL1. We found that a proline-rich segment of apoptin interacts with the SH3 domain of a BCR-ABL1 (p210) fusion protein and acts as a negative regulator of BCR-ABL1 kinase activity, preventing phosphorylation of its downstream targets. In addition, we determined the effects of a deca-peptide that spanned the apoptin-BCR-ABL1 interaction domain and identified its relative toxicity towards BCR-ABL1-positive and negative cells. Lastly, we tested this deca-peptide for its inhibition of BCR-ABL1-downstream signaling pathways and for its ability to kill primary CML-cells resistant to imatinib (Gleevec). These data provide the foundation for the development of peptide-based tyrosine kinase inhibitors as novel anti-cancer agents.

General Discussion

Both the polyether compound salinomycin and chicken anemia viral protein apoptin exhibit toxicity towards a broad range of cancer cells, irrespective of p53 function, with minimal to no lethality among normal cells^{82, 104}. However, these anti-tumoral agents differ in their cellular localization and mechanism of action. Salinomycin is an ionophore, forming ion channels (with a preference for but not restricted to K⁺ ions) in the cellular membrane and the membranes of other cellular organelles such as mitochondria^{105, 106}. Apoptin, on the other hand, functionally induces cell death among transformed cells and cancer cells as it is transferred to the nucleus, while normal cells effectively restrict apoptin to the cytoplasm^{75, 81}. However, both salinomycin and apoptin trigger mitochondrial dysfunction, leading to activation of the caspase-signaling cascade. Our work provides further insight into the role of autophagy in salinomycin-induced cell death, as well as hints the possible roles of necrosis and apoptosis in this process. We further show that glucose starvation, or mimicking glucose starvation using competitive inhibitors enhances salinomycin-induced cancer cell death and so does the hypoxic conditions of tumor microenvironment. In this study, we also evaluated the toxicity of an apoptin-derived decapeptide, which was able to inhibit BCR-ABL1 signaling in imatinib-resistant patient-derived samples.

Salinomycin: Autophagy, mitochondrial polarity, and differential stress responses.

Based on our initial experiments demonstrating that salinomycin treatment induces autophagy, we continued to explore the role of autophagy in salinomycin-induced cell death. We used autophagy inhibitors, chloroquine and bafilomycin, and ATG5-deficient cells to show that inhibition of autophagy enhances salinomycin-induced cell death in prostate and breast cancer cells as well as in normal cells. Similar protective effects mediated by autophagy were

also observed in cells that exhibited cancer stem cell like properties (ALDH⁺) and other cancer cells treated with salinomycin^{102, 107}. Moreover, Yue et al., 2013, showed that ALDH⁺ cells are more sensitive to salinomycin treatment compared to ALDH⁻ cells due to inhibition of autophagic flux¹⁰². Contrary to our results, the study showed that salinomycin is an inhibitor of autophagic flux by using densitometric quantification of protein bands obtained from Western blots and confocal image analysis of LC3 puncta¹⁰². Concomitantly several studies published with some showing salinomycin as an inhibitor of autophagic flux and others as autophagy inducer^{49-51, 107}. To further clarify the effects of salinomycin treatment on autophagic flux, we used flow cytometry to monitor fluctuations in the median green fluorescence of GFP-LC3 or the median green and red fluorescence of mRFP-GFP-LC3 with and without salinomycin treatment. We showed that salinomycin is primarily an inducer of autophagic flux; however, this is dependent on cellular resistance to its toxicity. Since salinomycin is an effective inhibitor of Wnt, even at nanomolar levels, and a previous study showed that cancer stem cells are more susceptible to lower doses of salinomycin, using micromolar concentrations of salinomycin to study autophagy may have led to secondary effects, such as the inhibition of autophagic flux^{56, 102, 108}.

In this study, we also evaluated the effects of salinomycin on mitochondrial function, the disruption in of mitochondrial inner membrane potential, and the dynamics of mitochondrial fission and fusion. We observed an increased staining for mitochondrial dyes (JC1 and Mitotracker), indicating hyperpolarization of mitochondrial membranes in surviving cells after treatment with salinomycin. Surprisingly, we saw similar results with staurosporine and another cytotoxic drug, SM40 (data not shown). However, our later studies show that the hyperpolarization of mitochondria that occurs initially is a transient phenomenon. Similarly, both initial studies on the effects of salinomycin on isolated mitochondria and as well as more recent studies on erythrocytes show concentration- dependent and solvent solvent-dependent polarization changes with lower concentrations, leading to mitochondrial hyperpolarization due to the

specific efflux of K^+ ions ^{105, 106}. Treatment with high concentrations of salinomycin, however, does not show any such sensitivity for ion transitions and thus leading to membrane depolarization ⁹⁶. We further showed that salinomycin treatment decreased ATP production, and increased mitochondrial fission and mitochondrial-specific autophagy (mitophagy).

Similar to its dual role in autophagy and mitochondrial polarity, salinomycin-treatment is also divergent with respect to cell death, driving both necrosis and apoptosis. Even though mitochondrial depolarization, the release of cytochrome c, and the caspase 3 axis of cell death were previously reported in neuronal cells exposed to salinomycin, our study was the first to report both the activation of caspases 3, 8, and 9 as well as HMGB1-release indicating necrosis. Our most recent studies suggest that inhibition of caspase activity using pan-caspase inhibitors partially rescues cells from salinomycin-induced cell death (data not shown). Other recent studies show that salinomycin sensitizes cancer cells to TRAIL-induced cell death through upregulation of the cell death receptors, DR5 and DR6, in coordination with the downregulation of c-FLIP through a proteasomal pathway ^{109, 110}. From this data, we can infer that salinomycin-induced cell death is cell type-dependent, and salinomycin employs heterogeneous cell death mechanisms to promote cancer cell death. However, we cannot rule out the possibility that salinomycin-induced cell death may be a secondary effect of severe cell vacuolization, leading to cytoskeletal disruptions and mitotic catastrophe.

Lastly, with regards to salinomycin, we studied the differential stress response (DSR) of cancer cells under starvation conditions to salinomycin toxicity ^{111, 112}. Similar to previous findings involving DSR, cancer cells showed increased susceptibility to salinomycin toxicity-triggered stress while primary, normal cells showed a protective effect ^{111, 112}. We then used 2DG and 2FDG to block the glycolytic pathway, mimicking glucose starvation conditions as well as the Akt inhibitor triciribine that mimics amino acid starvation. Salinomycin, in the presence of either 2DG (or 2FDG) or triciribine, showed increased toxicity to

cancer cells. Furthermore, we showed that salinomycin is most toxic to cancer cells under hypoxic conditions. Therefore, a combination of starvation and hypoxia will further enhance salinomycin-induced cancer cell death.

Apoptin derived peptides: Anti-cancer stem cell agent:

The ability of apoptin to specifically target cancer cells from a variety of tumors has been studied for over a decade. However, the potential of apoptin to inhibit cancer cells that are clonogenic has not yet been reported. Our previous study was the first to identify the ability of apoptin to specifically interact with the SH3-domain of BCR-ABL1¹⁰³. This study shows the precise mapping of apoptin's proline rich region (aa 81-90) using a GST-apoptin pull-down assay and deletion mutagenesis, identifying an apoptin minimal region that specifically binds to the SH3 domain of BCR-ABL1. This apoptin-derived decamer peptide had the ability to kill BCR-ABL1-positive cells (K562 and 32Dp210) and CML patient-derived cells with comparable efficiency as wild type apoptin and imatinib. Further, the apoptin decamer peptide showed toxicity even among imatinib-resistant, BCR-ABL1-positive patient samples. Even though, BCR-ABL1-positive CML stem cells that are capable of initiating tumors are reported, the function of BCR-ABL1 in the survival of these cancer stem cells has not been clearly documented^{113, 114}. Previous findings show that BCR-ABL1 expression among CML stem cell-like cells hinders their long-term self-renewal capability and promotes their differentiation to mature cancer cells¹¹⁵. This suggests that the capacity of the apoptin decamer peptide to kill BCR-ABL1-positive, imatinib-resistant cells does not indicate its cancer stem cell-targeting ability but its ability to target cells independent of ATP-binding pocket hotspots. However, since apoptin is able to induce cell death among cells that are transformed by a single event (such as SV40 virus-mediated transformation of cells) or even "temporarily transformed" by UV-irradiation, its ability to identify and target the initial transformed cancer stem cell from a normal stem cell could be further explored

¹¹⁶. In conclusion, this study initiates serious inquiry into the cancer cell type-specific targeting ability of apoptin-derived small peptides that could be used clinically either alone or as an adjuvant to current therapeutics. These small peptides, because of their inability to form tertiary structures, give them the advantage of avoiding the initiation of immune responses, unlike wild type apoptin. Thus, we have demonstrated that further exploration into apoptin-based biosimilar synthetic peptides, to be used for the treatment of drug-resistant and cancer-initiating cells, is vitally important.

Conclusions

The major findings of this study are as follows:

1. Salinomycin induces cell death among a variety of cancers including breast cancer and prostate cancer as well as head and neck carcinomas with minimal toxicity towards healthy normal primary cells.
2. Salinomycin-induced cell death is more profound among CD44-expressing cells compared to CD44 negative cells.
3. Salinomycin is an inducer of autophagy and autophagic flux. However, at higher doses, salinomycin inhibits autophagic flux.
4. The inhibition of autophagic flux by high concentrations of salinomycin does not occur through the inhibition of lysosome fusion with autophagosomes.
5. Autophagy triggered by salinomycin treatment is a cellular protection mechanism. Conditions that compromised autophagic responses promote cell death by salinomycin.
6. Salinomycin inhibits mitochondrial function by promoting mitochondrial fission and initial hyperpolarization of mitochondria, thus hindering ATP production through oxidative phosphorylation.
7. The mechanism of salinomycin-induced cell death is both apoptotic and necrotic.
8. Differential stress responses under low glucose starvation conditions and serum levels amplify cancer cell death in response to salinomycin but offer protection to primary normal cells.
9. 2-Deoxy D-Glucose and 2-fluoro D-Glucose, analogues of glucose, promote cell death after salinomycin exposure, but not by sodium oxamate that inhibits the conversion of pyruvate to lactate, suggesting that hampered oxidative phosphorylation promotes salinomycin-induced cell death rather than the inhibition of aerobic glycolysis.
10. An apoptin deca-peptide spanning the proline rich region (amino acids 81-90) is crucial for binding of apoptin to the BCR-ABL1 SH3 motif. This decapeptide alone has similar toxicity to both imatinib-resistant and imatinib-sensitive cancer cells as the wild-type apoptin protein.

Acknowledgements

I thank every one who has given me support and friendship without which it would have been more difficult.

First, I thank my parents and family members for all the love and support; I thank my best friends Naren and Rajeev for everything during the last 10 years. If it were not for your motivation, emotional support and intellectual discussions on Skype every week, I would have been brain dead dealing everyday routine alone.

I am very grateful to Prof. Jeffrey T. Wagle for training me in scientific approaches, techniques, and setting highest standards to rationalize and question for scientific truth. I thank him for all the support, encouragement and friendship over the last decade.

I thank Prof. Peter Phålsson for his down to earth persona, general discussions over coffee or lunch and support. I also thank Prof. May Griffith for all the discussions, and introducing me to latest technologies and concepts in stem cell advances.

Thanks to all the people on the floor 10, administration (Camilla and Anna), autoclave personnel (Lena and Anna) and people from Peter's lab (Eva and Patric) for their support and help. I am indeed very grateful to Prof. Annelie Lindström for all the help to ensure an easier transition to defense. In general, I have greatly admired the "lagom" philosophy of Swedes and approach to education.

I thank my friends in Sweden, Anand (and family) and Ramana for the fun times and discussions. Hirak, it was great to collaborate with you and you are the first one I came across here where I had a symbiotic intellectual association. The last two years would have been terrible if we didn't collaborate. I am glad at least in the last few months I came to know more about Miraz and Joel. It is always great to find some more similar minds.

It was a great opportunity to train several masters project students from the lab, Mayur (eventually guide him in his PhD projects also), Sahana, Florence, Ramakrishna, Petros, Alex, Mikael and several other students who were constantly asked to bother me for expertise inputs and help.

Finally, I have to thank my supervisor Prof. Marek Los for the opportunity to get my degree. During this time in his lab, I got opportunities to do various things such as

establish the laboratory, train students right from day one (directly and indirectly), work and collaborate independently, and to review several articles for journals helped me in shaping myself as an independent researcher.

I thank my Co-Supervisors Prof. Karin Roberg and Prof. Anna-Lotta Hallback for valuable suggestions and support.

Concluding remarks and Time line

Having had an extensive training (6 years) in very good profiled laboratories and had prolifically worked on the several techniques I used in the thesis, it was a very smooth flow both technically and scientifically. Training several students, being responsible for the successful completion of their thesis and implementation of techniques gave me a great confidence and got the opportunity to learn the business aspects of science.

2011 Feb – 2012 Feb

Establishing equipment and protocols in the lab from cell culture, electrophoresis, restriction digestion, cloning to protein expression and purification, Western blotting and immuno-cytochemistry. Trained 4 master students to complete their projects. Started work on Paper 1 at the later part of the year. Gained experience in flow cytometry and Co-wrote a review and editorial.

2012 March – 2013 March

Exclusively worked independently on my PhD projects. Finished experiments for paper 1 and partly for paper 3. Trained 2 masters students and 2 project students. Started independent collaboration with Hirak. Co-Wrote a review.

2013 March – 2014 March

Published paper 1, Finished work for Paper 2, 3 and 4. Co-wrote Paper 4. Trained 1 master student. Finished independent collaboration project with Hirak. Teaching assistant for Masters degree course. Reviewed papers for European Journal of Pharmacology

2014 March – 2015 to date

Published Paper 4, Wrote Paper 2 (published) and 3 (communicated). Started and finished independent collaboration projects with Surajit and Mojin. Guiding Mayur's project and co-wrote the manuscript. Writing thesis and wrap up.

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