QATAR UNIVERSITY

COLLEGE OF HEALTH SCIENCES

ANTI-CANCER ACTIVITY OF SANGUINARINE ON HUMAN PAPILLARY THYROID CANCER CELL LINES BY TARGETING JAK/STAT3 PATHWAY

BY

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A Thesis Submitted to

the College of Health Sciences

in Partial Fulfillment of the Requirements for the Degree of

Masters of Science in Biomedical Sciences

January 2021

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ABSTRACT

MOHAMED,ELHAM ,A. NASSER , Masters of Science : January :2021, Biomedical Sciences

Title: ANTI-CANCER ACTIVITY OF SANGUINARINE ON HUMAN PAPILLARY THYROID CANCER CELL LINES BY TARGETING JAK/STAT3 PATHWAY

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Background: Papillary thyroid carcinoma is the furthermost common type of thyroid cancer, representing 85 % of all thyroid cancer cases. It occurs more frequently in women and usually affects 20–55 year age group. **Aim**: This study intended to explore the potential underlying mechanisms of sanguinarine (SNG) mediated anti-cancer actions in papillary thyroid cancer (PTC) cell line, BCPAP and TPC-1. **Methods**: PTC cell lines were cultured, and cell viability experiments were performed following treatment with SNG for 24 hours using cell counting kit-8 (CCK-8) assay. Apoptosis was measured using fluorescent AnnexinV/PI stain and investigated by flow cytometry. Western blot was done following treatment with SNG and other compounds to identify different proteins associated with apoptosis and autophagy. Further, LC3 transfection was done using GFP-LC3 plasmid to transfect LC3 protein. To explore the underlying mechanisms and sensitizing potential of SNG, PTC cells were treated with N-Acetyl cysteine ,VAD and cisplatin respectively, alone and in combination with SNG and expression of protein markers were done. Functional assay such as: scratch assay and colony assay were performed to assess the cell's migration and colony formation ability after SNG treatment. Furthermore, effect of SNG on stemness markers were also studied. **Results**: SNG inhibits proliferation/cell viability of PTC cells dose

dependently. Real time cell analyzer (RTCA) findings supports that SNG mediated growth inhibition of PTC cells is time and dose dependent. SNG treatment downregulates constitutive expression of JAK/STAT3 in PTC cells. SNG mediated cell death occurs through activation of caspases and induction of double strand DNA breaks. z-VAD-FMK, a pan-inhibitor, partially prevents SNG mediated cell death suggesting involvement of caspases-cascades in SNG mediated apoptosis. SNG induced autophagy demonstrated by enhanced LC3 expression, and LC3 transfection resulted in cytoplasmic puncta formation. SNG induced molecular changes leading to apoptosis or growth inhibition is due to ROS involvement. Furthermore, our data also indicates that IL6 play important role in SNG mediated downregulation OF STAT3. Interestingly, we also observed that SNG potentially sensitizes PTC cells to anticancer drug cisplatin. **Conclusion**: SNG activates apoptosis in PTC cells through the inactivation of STAT3 survival pathway. SNG demonstrated caspase dependent apoptosis. In addition, drug combination of SNG with cisplatin enhances anti-cancer effect on PTC, which makes it a potential therapeutic.

DEDICATION

This research is dedicated to my lovely daughter, who suffered a lot during my absence accomplishing my master's degree.

I hope someday I will make it up for her.

ACKNOWLEDGMENTS

First of all I thank Almighty, The Allah for helping and giving me strength to complete this work. I want to thank my thesis supervisor Dr. Hatem Zayed and co-supervisor Dr. Shahab Uddin for helping me in finding research subject to fulfill my graduation from the MSc program and for their continuous support. Another thanks to Dr. Abdul Quaiyoom Khan and Dr. Kirti Sathyananda Prabhu for their continuous support during performing experiment in the laboratory, and Dr. Zafar Nawaz for his support during the research project work.

Many thanks to my husband Emad, who motivated me and supported me during my study. Many thanks to my mother, without here prayer's I would have accomplished this stage of my life. Many thanks to my sisters, brothers and friends for their continuous support.

Finally, I would like to thank iTRI as well as DGD laboratory for support during my research period.

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CHAPTER1: INTRODUCTION

Background

Cancer

According to WHO, cancer is the second main cause of death globally and is responsible for an anticipated 9.6 million mortalities in 2018. Globally, among 6 deaths one will be due to cancer. The reason behind this increase is the fast-growing of the global population, as well as the exposure to risk factors that make some people are more prone to develop cancer than others (WHO, 2018). The cause of cancer had triggered many minds since the early 1950s, when the first cancer international symposium was held and cases of cancers from around the globe was presented and discussed. Soon after the 1900s symposium, led to the construction of the worldwide Agency that is intended for cancer research (IARC) in 1965 which was instructed to conduct inclusive researches of the causes of human cancers (Blackadar, 2016). Nowadays IARC is considered a reference to many healthcare practitioners for the identification of cancers.

Cancer Risk factors

National Institute of health has illustrated that cancer risk factors could be environmental-related to exposure to chemicals, improper diet, physical inactivity, tobacco or alcohol intake (Blackadar, 2016)). Additionally, the exposure to many viruses such as human immunodeficiency virus HIV, Hepatitis HBV, or Human papillomavirus (HPV) had been proven to have a link and was linked experimentally as well (Silverberg et al., 2009).

Hematological and solid tumors

Hematological malignancies (HM) contain a collection of diverse conditions, all originating from two major groups myeloid origin of lymphoid origin. On the other hand, solid tumors (ST) are pathological mass of tissue that usually doesn't contain

cysts nor liquid. Solid tumors may be cancerous or noncancerous; benign or malignant. Different types of organ derived tumors are termed based on the type of cells they originate from. Examples of solid tumors can be sarcomas, carcinomas, and lymphomas. Sarcomas are cancers rising from connective or supportive tissues, such as bone or muscle and carcinomas are cancers originate from the body's glandular type of cells and epithelial cells, which cover body tissues. On the other hand, lymphoma's are cancers of lymphoid originating cell organs such as the lymph nodes, spleen, and thymus and it tend to be generalized since lymphoid organs are distributed all over the body (Elidrissi Errahhali, Elidrissi Errahhali, Boulouiz, Ouarzane, & Bellaoui, 2016; Rodriguez-Abreu, Bordoni, & Zucca, 2007). Compared to solid tumors, HM are extensively studied in research-based studies in major journals. Chizuka stated in his review paper that, the increased number of HM studies over ST. The reason is due to the availability of blood specimen and also due to safe non-invasive collection procedure of blood compared to solid tumors , which required invasive fine needle aspiration that could have worse patient health outcome(disease metastasis).***(Chizuka et al., 2006)***. Having frequent sampling in HM lead the scientist to study the influence of genetic make up on the clinical picture , disease complication , as well as setting up therapeutic profile for such disease***(Taylor, Xiao, & Abdel-Wahab, 2017)***.

Thyroid cancers and types

Thyroid cancers are the furthermost malignant solid tumor of the glandular system with papillary thyroid carcinoma (PTC) is one of the most important malignant thyroid cancer types. About 230,000 novel cases of were estimated in 2012 among women and 70,000 among men, with an incident of 6.10/100,000 women and 1.90/100,000 men (Ferlay et al., 2015). There are four types of thyroid follicular epithelial-derived cancers

include papillary carcinoma (PTC), follicular carcinoma (FTC), medullary carcinomas (MTC) and anaplastic thyroid carcinoma. Both PTC and FTC are well differentiated type of cancers, whereas medullary as well as anaplastic thyroid cancer are the rarest types and is poorly differentiated (Perri, Lorenzo, Scarpati, & Buonerba, 2011). Papillary thyroid carcinoma is the chief common histological type followed by follicular carcinoma in both sex. Among female, Qatar has the maximum incidence with 13.5 per 100,000 during period 1998-2002 (Al-Zahrani & Ravichandran, 2007). A more recent statistic provided by Qatar National Cancer registry (QNCR), estimated that papillary thyroid cancer graded the second most common malignant cancer among female. Globally, published data by SEER at NIH website (Surveillance, Epidemiology, and End Results) reveled that, PTC incidence is 14.5 male and females per 100,000 (in period of 1991-2006) in USA. The increase in the incidence is correlated with the increase exposure to risk factors.

PTC Risk factors

PTC risk factors could be either environmental or due to the genetic predisposition or the presence of family history of thyroid related illnesses. Although utilizing X-rays is of use to diagnose health illness, the risk factors associated with it use can't be avoided. For examples, the unnecessary exposure to neck X-ray or ultrasonography and fine-needle aspiration (FNA) of very small thyroid nodules could increase risk of having PTC (Ahn, Kim, & Welch, 2014; Davies & Welch, 2014; Vaccarella et al., 2016;Neta G et al.,2014). Additionally, dental X-rays without neck shielding could be also a risk factor, however the lack of association studies between thyroid cancer and radiation exposure assessment weaken association between PTC and radiology exposure (Neta G etal.,2014). On the other hand, Genetic predisposition to PTC is well noted and studies in literature. More precisely, all DTC (Differentiated Thyroid Cancer)

cases in patients with a first-degree family relative of thyroid cancer were cases of PTC (Xu, Li, Wei, El-Naggar, & Sturgis, 2012). DTC include follicular and papillary histology and are classified as cancers with good prognostic outcomes (Burns & Zeiger, 2010). Additionally, genetic predisposition has a role in predisposing certain individuals harboring certain mutations.

Molecular Genetics of PTC Cancer

For thyroid cells to grow , it requires a mutual effects of Thyroid Stimulating Hormone, working via cAMP, and growth factor signaling (e.g. IGF-I) acting mainly through MAP kinase with phosphatidylinositol-3-kinase (Kimura et al., 2001). Molecular mutation the occurs in these pathways play an essential role in thyroid neoplasms. For instance, continuous activation of thyroid adenomas are associated with triggering mutations of the *thyrotropin (TSH) receptor* (Kohn, Grasberger, Lam, Ferrara, & Refetoff, 2009; Parma et al., 1993; Parma et al., 1995; Xing et al., 2003) or leading to constitutive activation of adenylyl cyclase (Kalinec, Nazarali, Hermouet, Xu, & Gutkind, 1992). These benign tumors infrequently progressing to malignancy. By contrast, in papillary carcinomas , the most common form of thyroid carcinoma, there is growing experimental evidence for a role of mutations leading to ongoing activation of effectors signaling along the MAPK signaling pathway in the pathogenesis of such cancer diseases.(Fagin, 2005)

One the gene level, it has been studied that innovative long intergenic intronic RNA gene termed papillary thyroid cancer susceptibility candidate 2 (*PTCSC2*) was repressed in PTC cases. Truncated expression of *PTCSC2 gene was also correlated with existence of genomic variant* g.29720641A>G (rs965513), and such polymorphism is a common risk factor in PTC (Wang F, Yan D, Ji X et al.2016; e H,

Li W, Liyanarachchi S et al.2015). In addition, most of PTC are linked to gene mutations involved in signaling of the MAP kinase signaling pathway such as:RAS, BRAF, or RET/PTC rearrangement. lastly, having thyroid illness increase the risk to have PTC. This is supported by a met-analysis was done to investigate the relationship between Hashimoto thyroiditis (HT) and PTC , and yielded that patient with HT are often more predisposed to develop PTC (Lee J, Kim Y, Choi J, Kim Y.2013).Although the incidence of PTC is increasing, having a good prognosis factor seems to balance this increase and made this type of cancer curable and manageable as well.

Chemoprevention and Natural products as potential anti-cancer agents

Chemoprevention

Although there are various cancer types, yet all share common cancer features that make them targetable with various anti-cancer agents. Anti-cancer drugs are used to target some pathways that are either up-regulated or downregulated in different cancers, but normal cells are also affected because drugs can't obviously differentiate between normal and cancerous cells. To overcome or minimize the complications due to conventional chemotherapeutic drugs various approaches has been adopted. Cancer chemoprevention states to the utilizing of agents for the reserve, postponement, or reverse of carcinogenesis before tissue invasion happened. These chemo preventive agents are classified in four key classes—hormonal, medications, diet-related agents, and vaccines(Benetou, Lagiou, & Lagiou, 2015).

Plant Alkaloids

Plant derived alkaloids have been a rich source for anti-cancer drugs (Atashrazm, Lowenthal, Woods, Holloway, & Dickinson, 2015). Sanguinarine (SNG) (13-methyl-[1,3]benzodioxolo[5,6-c]- 1,3-dioxolo[4,5-i] phenanthridinium) is a biological active benzophenanthridine alkaloid found in flowers plants of the Papaveraceae family,

blood root plant Sanguinaria canadensis, Chelidonium majus, and Argemone Mexicana (Mackraj, Govender, & Gathiram, 2008). Theses alkaloids are used extensively in in vitro to test its anti-cancer activity on various cells lines, because they can be an effective alternative to chemical anti-canceric agents to avoid or minimize the toxicity (Atashrazm et al., 2015; Hussain et al., 2007; Tsukamoto et al., 2011).

In this study Sanguinarine (SNG) was used to investigate its anti-cancer activity on BCPAP cells and the underlying mechanisms of SNG-mediate cell death. In addition to that this would be a novel research which will illustrate, for the first time, about the SNG-mediated cell death and underlying pathways on PTC cells.

Aim of the study

The aim of the study is to explore the outcome of PTC treatment with SNG and to identify the potential underlying mechanisms of anti-cancer pathways that inhibited by Sanguinarine in PTC cell line.

Objectives

1- To determine the activation of the JAK/STAT signaling pathway in PTC.
2- To determine whether inhibition of JAK/STAT3 activity by SNG can mediates cell cycle arrest and/or apoptosis in PTC cells.
3- To determine whether SNG mediated inhibition of JAK/STAT suppresses the expression of anti-apoptotic and enhanced the expression of pro apoptotic genes in PTC cells.
4- To determine whether IL6 activates STAT3 in PTC cells and SNG block IL6 mediated activation of STAT3.
5- To determine whether SNG mediated its anti-tumor effect via inhibiting the motility and invasion of PTC Cells.
6- To determine whether SNG mediated its cytotoxic action via generation reactive

oxygen species (ROS) in PTC Cells.

7- To determine whether SNG synergistically potentiates the anticancer potential of conventional anti-cancer drugs (cisplatin).

Research hypothesis

Sanguinarine can effect cell proliferation of PTC cells through the JAK/STAT3 pathway.

CHAPTER 2: LITRETURE REVIEW

Epidemiology of PTC

Among Arab countries , PTC was the commonest type of cancer in thyroid cancer profile. Although there is variation in the number of cases studied, one can conclude the PTC incidence was high in Qatar with 13.5/100,000 person, and followed by Kuwait (7.7), Bahrain (7.6), Algeria (7.1) , Morocco(6.7) UAE (6.0). Furthermore the Amman (3.3) recorded the least incidence reported (Table 1). Female showed a significant increase in the incidence than male , with 2-3 fold increase in between . This trend in was also comparable to USA were thyroid cancer incidence increased annually by 5.8 percent between males and 7.1 percent among women in the USA, a more quick increase than for any other cancer (Meinhold et al., 2010).

Table 1. *Incidence of PTC in MENA region.*

Country	Incidence/100,000 person	Female : male	Study period/study type)	Reference
Algeria	Incidence e rates are of1.2(male)and7.1(female)/100,000	5.9 : 1	2008-2012(retrospective study)	(Guidoum, M,2015)
Bahrain	7.6/100,000 person	6.1:1	1998-2002(retrospective study)	(Al-Zahrani AS, Ravichandran K,2007)
Country	Incidence/100,000 person	Female : male	Study period/study type)	Reference

Iran	3.5(females) and 1.0 (male) per 100,000 per year,	3:1	1998 - 2001(retrospective study)	(Larijani, B., 2005)
Jordan	(3.3 per 100,000) in Amman and (2.2 per 100,000) in Jarash	3:1	1996 - 2008(retrospective study)	(Sharkas, 2015)
Kuwait	7.7/100,000 person	2.6:1	1998-2002(retrospective study)	(Al-Zahrani AS, Ravichandran K,2007)
Morocco	6.7/100,000	-	2005-2007(retrospective study)	(Bouchbika et al., 2013)
Oman	5.9/100,000 person	2.6:1	1998-2002(retrospective study)	(AlZahrani AS,Ravichandran K,2007)
Country	Incidence/100,000 person	Female : male	Study period/study type)	Reference
Qatar	13.5 per 100,000	2.6:1	1998-2002(retrospective study)	(Al-Zahrani AS,

				Ravichandran K,2007)
	5.0/100,000 person	2.6:1	1998-2002(retrospective study)	(Al-Zahrani AS, Ravichandran K,2007)
Saudi Arabia	Total 4.4/100,000 (6.8 for female and 2/100,000 for males)	1:0.3	2000 to 2010 (retrospective study)	(Hussain et al., 2013)
	-	3.7:1	2006-2013	(Albasri, Sawaf, Hussainy, & Alhujaily, 2014)
United Arab Emirates (UAE)	6.0/100,000 person	2.6:1	1998-2002(retrospective study)	(Al-Zahrani & Ravichandran, 2007)

Cell cycle

Cell grow in a formulated sequence of harmonized steps that is known as cell division cycle. Cell cycle composed of four phases: The cell cycle includes four stages including G1 (gap phase 1), S (DNA synthesis), G2 (gap phase 2), and M (mitosis) Figure 1. The duration cell spent in each phase varies among different cell type. The consistent order in cell cycle is important, as cells can't start cell division until the DNA is replicated otherwise the cells will fail to have the parent DNA. Normally in human cells their check point system that manage cells in cell cycle. Mostly, when cells grow through their controlled cells cycle no error occur, but problems start to occur when control of cell cycle is distrusted. Eventually, cancer will occur, and it is sometimes referred as the disease of cells cycle (Hausman, 2000))

Hallmarks of cancer

Sustaining proliferative signaling

A common feature that all types of cancer share is, their ability to sustain consistent uncontrolled growth, in contrary to normal tissue where it is the opposite. Normal body tissues cautiously regulate production and release of growth signals that instruct entry of cells to go to G1 and cell remained adherent to signals provided to them in this Gap phase(G1)(L.-H. Yu, Huang, & Zhou, 2019). The control of cell proliferation is mastered in G1 phase , and there is a wide variety of tigers ranging from growth factors to DNA damage that make the transition to S phase(L.-H. Yu et al., 2019) . Transition of cells from G1 phase to S phase is a point of no return in the cell cycle. The main pathway that control G1 phase is pRB pathway, were it is tightly regulated by cyclins and CDK inhibitors (CKIs)(Dyson, 1998). In pRB(Retinoblastoma) pathway, the master component is the pRB tumor suppressor proteins and is

responsible for a major G1 checkpoint ,which is the first tumor suppressor identified thereby blocking the cells from entering to the S phase and cells growth(L.-H. Yu et al., 2019). The Retinoblastoma family contains Rbp105, p107 and Rb2/p130, all together denoted to as pocket proteins. The key function of these proteins is to suppresses the gene transcription that aid the cells to go to S-phase (Giacinti & Giordano, 2006) . The major kinases that phosphorylate pRB proteins in mammals are three cyclin-dependent kinases: cyclin-D-dependent CDK4 , CDK6 as well as cyclin-E-dependent CDK2(Duronio & Xiong, 2013). The critical role of pRB pathway was well studied in tumor DNA viruses: adenovirus, human papillomavirus (HPV), simian virus 40 (SV40). While completely distinct virus types, Each one produce a protein that suppresses pRB protein and thus continuous proliferation and viral replication will be maintained (Duronio & Xiong, 2013).

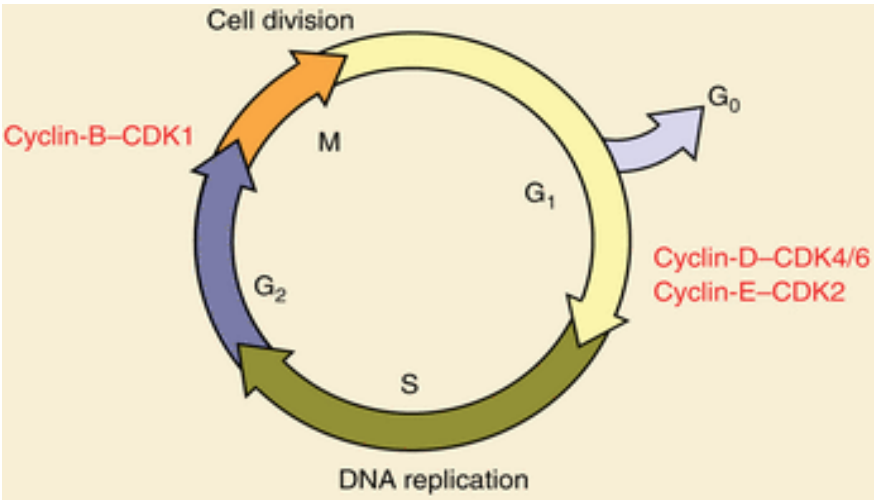


Figure 3. cell cycle and the main controllers (Duronio &Xiong,2013).

In cancer, the disruption of the G1 control is the key principle of many evolving cancers

(Sherr & Roberts, 1995). For cancer cell to maintain a sustained proliferative status, is through many alternative ways: first, cancer cells produce a growth ligand to which they response and express a conjugate receptor , resulting an autocrine circuit. The second mean is, sending out signals to normal cells in order to provide various growth factors (Hanahan & Weinberg, 2011). And third way, elevate receptor expression on the cancer cell itself , which enable the cells to sense signals even if it is at low concentration(Hanahan & Weinberg, 2011).

Resisting cell death

Normally cell death is a programmed mechanism that is termed apoptosis, and it is considered as a natural barrier to cancer development. Cell apoptosis is doubtless one of the furthermost thoroughly studied fields in cell biology. Studying cell death in disease conditions is vital as it gives insights into the disease progression as well as leaves hints on how the certain pathological condition can be treated. In cancer, there is an imbalance between mitosis and apoptosis and cells that should be dead fail to receive the signals to do so. Dysregulated cell death (apoptosis) is critical in both cancer progression and a main limitation to absolute treatment. As a result of various intracellular damage signals plus those induced by cancer treatment, cell's choice to go through apoptosis is determined by interconnection between three of the Bcl-2 protein family("The Cell: A Molecular Approach," 2000). The cell-suicide program is regulated by mean of a Bcl-2(B-cell lymphoma 2) protein family and it was first revealed in follicular B- cell lymphoma; where the name come from (Tsujimoto, Gorham, Cossman, Jaffe, & Croce, 1985) . Through bcl-2 proteins have a role in the regulation of apoptosis, it is continually related to tumor growth. In the last two centuries, the *BCL-2* gene family was discovered, and it encodes for a group of 30 family proteins that share bcl-2 homology domain (BH); amino acid unique sequence (around 20 amino acid residues) (Figure 3)(Czabotar, Lessene, Strasser, & Adams,

2014). The presence of certain protein motifs will discriminate the apoptotic from the antiapoptotic bcl-2 proteins. The pro-survival and the anti-apoptotic comprises: BCL2, BCL-XL, BCL-W, and MCL-1 proteins. BH4 protein domain is typically not present in the apoptotic proteins and for that it is considered a key factor for the antiapoptotic activity(Kelekar & Thompson, 1998). Bcl-2 protein is located on the outer membrane of the mitochondria as well as to the nuclear outer envelope and the endoplasmic reticulum membrane. There are two pathway of programmed cell death, extrinsic pathway (activated signal attach to a ligand on cell receptor) and the intrinsic (through mitochondria) pathway. The extrinsic pathway is initiated through the Fas/Fas receptor and the intrinsic will sense any intracellular signals such as: DNA damage (Hanahan & Weinberg, 2011). Normally, there is an equilibrium between the cell mitosis and cell death , but in cancer the cells fail to respond to death signals continue to divide independently and resist cell death(L.-H. Yu et al., 2019). For example, downregulation of p53; a tumor suppressor gene , result is decreased apoptosis and thus cells continue to divide uncontrollably, and this is the basis of many cancers(Fulda & Debatin, 2006; Wong, 2011).

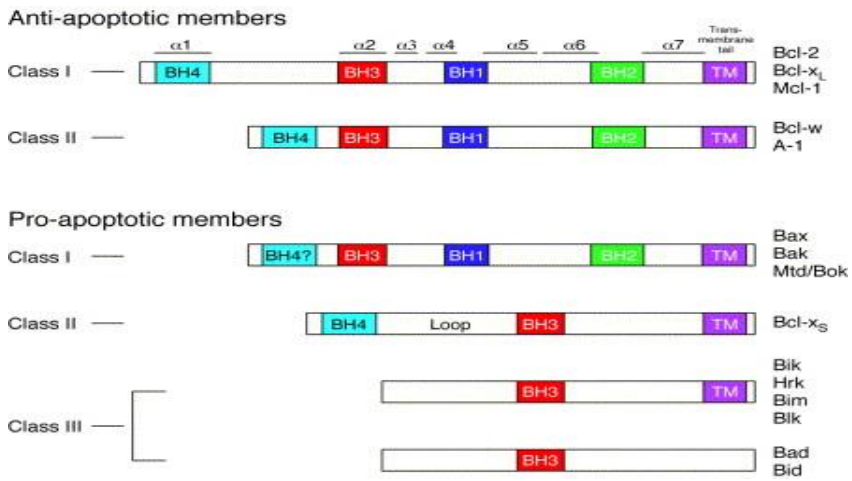


Figure 4. BCL-2 Classification of the Bcl-2 family of proteins based on protein domain organization *(Kelekar & Thompson, 1998)*.

Germline and Somatic mutations

The genome is guard with gate keepers that maintain normal cellular function and healthy signaling, and when these keeper are disrupted by stress that cause mutation then cancer can evolve. *TP53* (OMIM #191117) is a known tumor suppressor controlling gene that codes for transcription factor protein p53, which has an antiproliferative function. Somatic mutations are mostly single base pair substitution change that is scattered along the coding sequence of *TP53* gene and can prime to the loss of function(Olivier, Hollstein, & Hainaut, 2010). On the other hand passing of a *TP53* mutation through generations causes individual more prone to early start of cancers including breast cancer, sarcomas, brain tumors, and adrenal carcinomas (Malkin, 2011; Olivier et al., 2010). The IARC p53 database listed all the known somatic and germline mutations of *TP53* gene ,along with the prevalence, base change and 3D visual illustration of proteins site affected

(http://p53.iarc.fr/TP53SomaticMutations.aspx). Somatic Mutations also can occur in signaling pathways, where it affects growth factor, which make a constitutive activation of signaling circuits. More ever, Around 50 % of all melanomas occurs due to an activating mutation in the B-Raf proteins; V600E. BRAFV600E point mutation has been related to the stimulation of the downstream MEK/ERK signaling pathway, and thus inhibiting of the apoptosis (Ascierto et al., 2012).

Disruption of TGF-beta pathway

Transforming growth factor- beta is a multifunctional regulatory type of polypeptide from a large family of cytokine which govern many pieces of cellular metabolism, including cellular proliferation, differentiation, migration, apoptosis, immune regulatory surveillance, and existence. They function that such cytokine depends on the type of the cell. An important biological function of TGF β cytokine is the decrease of growth of almost all epithelial cells through it's autocrine mood of action, and this proposes a tumor suppressor role for TGF β. Loss of TGF-β action give some of the epithelial cells the advantage to be malignant tumor cells. This give insight to a pro-oncogenic important job of TGF β cytokine in addition to its tumor suppressor role (Jakowlew, 2006).

Deregulated signaling pathways and Thyroid cancer

ERK signaling pathway

Another important kinase pathway is ERK pathway, and also refered to the 42-/44-kDa mitogen-activated protein kinase (MAPK) pathway, were it is tiggered in a variety of cell types by various extracellular stimuli and it is one of the most throughly ivestigated signaling pathways that connect different membrane receptors to the nucleus (Lewis, Shapiro, & Ahn, 1998; Pagès et al., 1993).Activation of the ERK pathway involves the guanosine triphosphate protine (GTP)-filling of Ras at the plasma surface membrane, and ultimatly a sequential protien kinase activation will be truned on. At start, truned

on Ras protiens suffuls Raf kinases such as Raf-1 to the surface of plasma membrane, akey activation step that is not fully explored. Raf-1 acts as the MAPK kinase kinase and tiggers MAPK/ERK kinase 1 and 2 by protine serine single phosphorylation. MEK1/2, double-specificity protein kinases, leads to priming to the phosphorylation ERK1/ERK2 (p44 MAPK and p42 MAPK, respectively) specifically on tyrosin kinase protein residues. When swiched on, ERK1/2 lead to phosphorelate many downstream seconday protiens involved in a many of the cellular responses such as cell proliferation, cell differentiation, cell survival and the cell movements(Kohno & Pouyssegur, 2006).

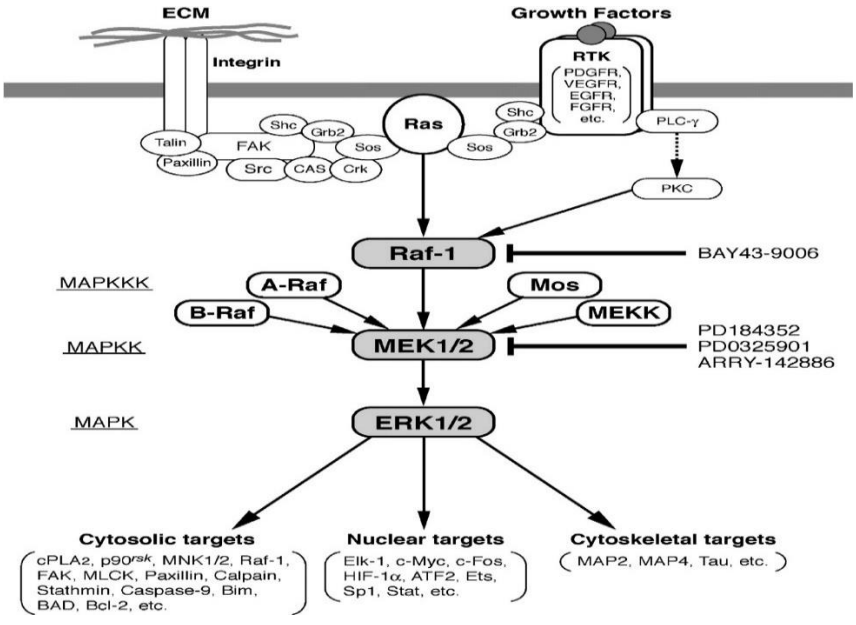


Figure5. Schematic graphical representation of the ERK pathway (Kohno & Pouyssegur, 2006)

PI3K/Akt signaling pathway

Phosphatidylinositol-3 lipid kinases, PI3Ks, a cluster of kinase group characterized by their fucntion to cause lipid phosphorylation of inositol hydroxyl ring group in inositol phospholipids to form another effector cellular messenger phosphatidylinositol-3,4,5-trisphosphate. The Akt-PI3K signaling pathway is crucial for various cell existence as truned on Akt effect many factors that is involved in apoptosis, either by transcription modulation or straight phosphorylation RPTK initiation that would results in PI(3,4,5)P(3) and PI(3,4)P(2) production by PI3K at the inside layer of the plasma cell membrane. Akt interconnects with these types of phospholipids, which will lead to changing its location to the internal membrane side, where it is will phosphorylated and turned on PDK1 and PDK2. Triggered Akt modulates contribute to regulation of cell sustainability, cell life cycle development and also cellular growth. Recently, in research it has been explored that PI3K/Akt signaling are most frequently converted in human cancers. Cancer therapy using powerful drug related chemotherapy and irradiation kills targeted cells through primarily activation of the programmed cell death (apoptosis). However, the development of chemoresistance is an important scaring clinical obstacle. A common feature of resistance tumor cells, when apoptotic machinery fails to be activated. Survival triggering signals that are induced by various receptors are facilitated by mainly by PI3K/Akt, henceforth this pathway circuit may definitively will contribute to the cancer resistant phenotype. Many of the activated signaling pathways involved in cellular alteration have been explained extensively and scientists are continuously working on uncovering better treatment options that will target such essential signaling pathways or it’s related effector proteins.

The PI3K/Akt pathway is linked to variety of the cellular mechanisms that targeted by these new drugs, therefore a better understanding of this crosslinking can benefit to fully uncover the potential benefits of these newly found agents.(Fresno Vara et al., 2004)

JAK/STAT3 pathway

JAK/Stat3 is an essential component downstream a cytokine receptor, and it is involved in many pathological conditions such as cancer (Toh, Lim, Hooi, Rashid, & Chow, 2020; Xin et al., 2020), immunological, infectious disease (Clere-Jehl et al., 2020). JAK/Stat3 is activated in many cancers such as: PTC cells and considered a plausible therapeutic target for researchers.

JAK/Stat3 pathway has three main key players: JAK (Janus kinases) ,STAT (signal transducers with activators of the transcription) , and the receptor binding ligand. In mammals, JAK consists of four main (non-receptor) tyrosine kinases members, JAK1, JAK2, JAK 3 and Tyk2 (D. A. Harrison, 2012). JAK is present in the cytoplasmic domains, were exists attached to a pairs of polypeptides, therefore displaying two intracellular signal-transducing domains. Structurally, JAK's N-terminal FERM (amino terminal (NH2) end (JH4-JH7)) , SH2, pseudokinase , and a kinase domains, which facilitates the binding to the receptors (Ferrao & Lupardus, 2017). Upon ligand-mediated receptor binding, such as interlukin-6 to IL-6 Receptor , the two JAKs parts became in a close proximity were they brought into close juxtaposition, allowing of trans-phosphorylation (Sacktor, 2012). Phosphorylated JAKs targets mainly the receptor it binds to and another major target, STAT3 transcription factor (TF). STAT3 TF present in an inactive form inside the cytoplasm (Rawlings, Rosler, & Harrison, 2004) . Then it is phosphorylated and translocate to nucleus ,by certain mechanisms relay on nucleoprotein interactor importin α-5 and Ran nuclear shuffle pathway, to

enhances repression of transcription of certain proteins depending to the ligand binding initially(Rawlings et al., 2004). In mammals, STAT3 TF comprise a family of seven STAT family members (STAT1-4, STAT5 alpha, STAT5βeta, and STAT6), where they have a common well-conserved tyrosine sequence in the C-terminal residues, which is ultimately phosphorylated by JAKs (Laudisi, Cherubini, Monteleone, & Stolfi, 2018). This link between JAKs and STAT3 allows activation of JAK/STAT3 pathway, through extracellular signals to develop a potential transcriptional activity (Rawlings et al., 2004). Thus, the JAK/STAT circuits offer a straight mechanism to dictate an extracellular stimulatory signals into a and effector functional response response.

There are various JAK and STAT3 repressor that regulate the activation or repression of this pathway. These regulatory factors have been studied on various cell studied in mammals and Drosophila models(Duval, Duval, Kedinger, Poch, & Boeuf, 2003). Protein repressors or inhibitor of activated STAT (PIAS) suppress the sustained transcriptional activity of STAT protein by Janus kinase (JAK)/STAT signaling pathway gene transcription modulation. There are five type of PIAS and all share a conserved structure and a common structural properties: PIAS1, PIASxα and PIASxβ and PIAS3, and PIASy (Duval et al., 2003). PIAS share a common structure among all isoforms. PIAS (SAP) domain, and it has a DNA–specific binding sequence. The PINIT (a Pro-Ile-Asn-Ile-Thr) protein theme, a preserved segment region of PIAS proteins that aid in nuclear attachment. The RLD ,which is termed finger like zinc domain, is required for the SUMO-E3-enzym ligase activity, which is a cofactor needed for an additional protein binding to propagate the pathway activity (Kotaja, Karvonen, Jänne, & Palvimo, 2002). The mechanism of how this protein repress the JAK/STAT pathway was not known for long time utile Guo-Juan Niu, et.

al used knockdown PIAS protein expression in shrimp exposed to *V. anguillarum* (*Vibrio anguillarum)* infection. He found out that bacterial infection is cleared out in knockdown model and survival rate increased compared to the control model (Niu et al., 2018). This was confirmed by the Pulldown assay, which showed that PIAS interact with STAT protein to prevent STAT protein from translocating to nucleus (Laudisi et al., 2018; Niu et al., 2018; O'Shea, Gadina, & Schreiber, 2002)

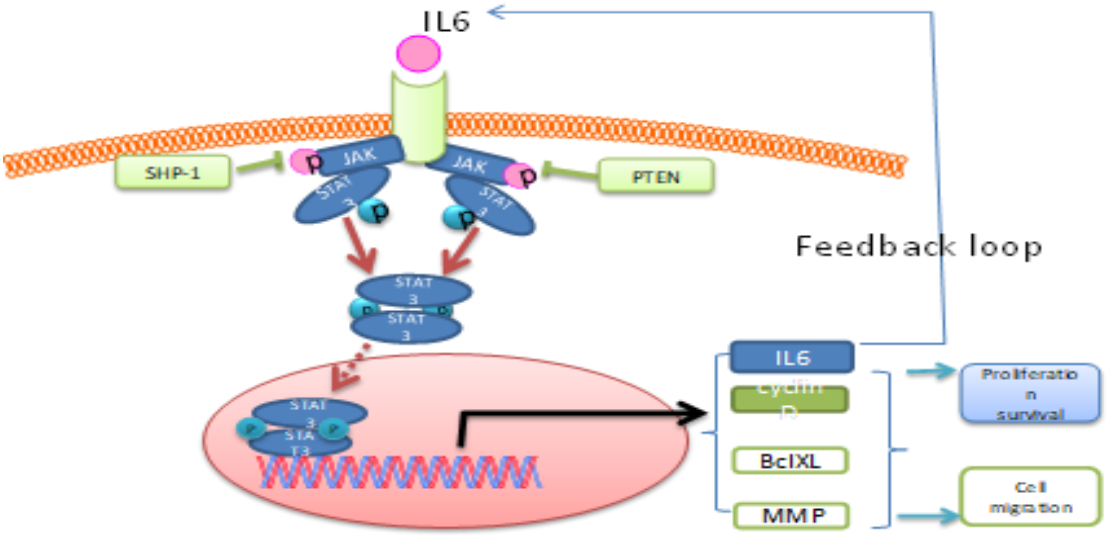


Figure 6. JAK/STAT3 signaling pathway.

STAT3 is a powerful protein molecules which functions is mainly through protein-protien dimerization and phosphorylation in certain tyrosine residues and eventually lead to translocation to cell nuclei to excerts it effector function(Lim & Cao, 2006; Schindler, Levy, & Decker, 2007). STAT3 signaling is continuously turned on and activated in many human malignant cancers types and leading in to multiple

intracellular growth with tumorigenesis (Santoni et al., 2015; Subramaniam et al., 2013).Targeting STAT3 protein in tumor treatment options has optimistically shown therapeutic assistances in both wet bench research as well as clinical studies (Bendell, et al., 2014(Bendell et al., 2014; Hong et al., 2015; Zhang et al., 2012). Oncogenic STAT3 protein over-activation has been largely observed in many various malignant human cancers (Santoni et al., 2015; Subramaniam et al., 2013). The major common phosphorylation starting sites are at residue Tyr 705 as well as Ser 727 Figure-2. JAKs are activated upon binding to cytokines to their receptor resulting in STAT3 protein phosphorylation and translocating to the nucleus to exert its effector function. In the nucleus, it plays role in transcriptional regulation of many antiapoptotic and other metastatic related genes together with cyclin D1, survivin, c-Myc and Mcl1 MMP-2,7,9, Rho and Rac), and angiogenesis related factors (e.g., vascular triggered endothelial growth factor (VEGF)(Aggarwal et al., 2006; Siveen et al., 2014; Subramaniam et al., 2013) (Aggarwal, et al., 2006). Alongside with its regular action, STAT3 has been shown to act in the cancer existence and cell alteration. Revolutionary work indicates that sustained STAT3 triggered activation is found on v-Src-transformed NIH-3T3 cell lines (Turkson, et al., 1998) and is sufficient to lead to alteration of normal gonadal cell lines and immortalized fibroblast cells (Bromberg, Horvath, Besser, Lathem, & Darnell, 1998). Such findings specify that dysregulated STAT3 expression can lead to long lasting changes in protein expression, which eventually can lead cancer evolvement . Furthermore, many research demonstrated that increased activation of Janus associated kinase (JAK) or its related receptors -associated to tyrosine kinase (Src) will eventually lead to STAT3 increased phosphorylation (G. Niu et al., 2002; Turkson et al., 1998) cancer (Liu et al., 2014) and triple-negative breast cancer (J. C.

Su et al., 2016). Therefore, focusing to target p-STAT3 by activating of SHP-1 might be one of the strategies to fight off p-STAT3 activating cancers.

Cancer stem cells (CSCs) and thyroid cancer

CSCs develop by genetic, nongenetic alteration, epigenetic variations attainment from adult stem cells type or from their committed progenitor cells(Z. Yu, Pestell, Lisanti, & Pestell, 2012). CSCs owes the capacity of self-regeneration, and are also responsible for keeping cell's cancer potentiality, which is aid CSCs to acquire migration capacity feature, and contribute to resistance to therapeutic options. These cells were extracted from several types of solid tumors like thyroid cancer and can form spherical shape of cells in vitro and to develop same original cancer when xenografted into immunodeficient mice model. CSCs role in thyroid cancer recurrence and resistance is poorly investigated. To understand the biology of thyroid CSCs, we have isolated cancer stem like cells thyrospheres from the PTC cell line BCPAP(Z. Yu et al., 2012).

Model used to study cancer

In vitro models

Cell cultures has been a good model for studying various aspect of cell biology , tissue morphology , mechanism of disease , and therapeutic interaction. Harrison carried out the first type of tissue culture in 1907 through investigating the origin of nerve fibers(R. G. Harrison, Greenman, Mall, & Jackson, 1907).since then, the research using donated cell that is stored in tissue banks and retrieved when needed for testing. There is great benefits when extracting primary cells from donor than when using tissue cultures. Although there is difficulty in extracting the correct cells out of donor samples, the have great advantage of mimicking the in vivo conditions of the disease present. There are two types of cultures that is most commonly used are: cultures of adherent or cells on a flask cells and culture of suspension ;such as cultures of lymphocyte, (Kapałczyńska et al., 2016, 2018).

2D cell lines

The most common type of cells used in research, because of the simplicity and low cost. However, the disadvantage of using it is that it doesn't illustrate cell-cell or cell-extracellular environment, which is an important concept to understand in metastatic cancer. Furthermore, after isolation the cellular morphology gets altered upon isolation and this will ultimately affect the function of the cell and cell interaction. Another drawback of 2D cell cultures, the adherent nature of the cells makes nutrient essential metabolites as well as oxygen inaccessible to the cells themselves thus, altering the results of experiment(Kapałczyńska et al., 2016, 2018). In this particular study, BC-PAP cell lines are adherent in nature and was used along will the thyrosphere stem cells.

3D cell lines

It is considered one of the early three-dimensional types of cell cultures grown on a soft agar suspension in 1970s by Hamburg and Salmon (Hamburger & Salmon, 1977). The main concept of the 3D cell culture is to create a spherical shape of cells that will mimic the morphological features of the cell of various tumors. There are 3 types of 3D cells that had be discovered when analyzing various cell morphologies: 1) tight spheroids,2) compact aggregates and 3) loose aggregates. The main advantage of 3D cell culture is proper cell-cell and cell-environment interaction and maintaining of cellular morphology (Kapałczyńska et al., 2018). In our current study we used thyrosphere as a 3D culture.

In vivo models

Mouse model

Using mouse as a model to study cancer development of great benefit due to the physiological, anatomical, cellular, and molecular similarity between mouse and human. Additionally, there 80 % homology between genome of the mouse and human, thus making it an excellent laboratory manageable model structure as a research method

to investigate the basic cancer pathophysiology as well as drug treatment. Transgenic mice had been a good model in studying disease mechanisms in human but , there is a draw back in such model, because of the heterogeneity of human disease that can't be explained using genetically modified mouse(Kumar, Larson, Wang, McDermott, & Bronshteyn, 2009).These limitation lead the scientist to search for other possible models that would give a reliable answers to variety of evolving cancers (Kumar et al., 2009).

PDX mouse

A lot of cancer related cell linage have been created, which is present for quite some time to fulfil cancer preclinical research. However, those cell lines are artificial and don't reflect the real condition of cancer cells environment, because of the artificial nature of their culture conditions. Patient derived xenograft (PDX) of tumor cells incorporated in to mouse models have attracted scientists attention in the recent decades, aiming to resolve issue of cancer resistance and recurrence (Jackson & Thomas, 2017).

CHAPTER 3: MATERIALS AND METHODS

Material

Reagents

Sanguinarine, VAD ((scientific name: Z-VAD-FMK) (chemical name carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone), NAC(N-Acetyl-L-cysteine)) and SDS (sodium dodecyl sulfate) solution was from Sigma Aldrich company (St. Louis, Missouri, United States). Wide range of antibodies against many proteins associated apoptosis, autophagy and survival pathways was brought from Cell Signaling Technologies Company (Beverly, MA, USA). GAPDH antibody was brought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) (Table-2). Annexin V- and Propidium iodide (PI) staining chemical , Hoechst 33342 Solution, BD Cytofix/Cytoperm plus fixation and permeabilization solution kit, BD MitoScreen (JC-1) Kit, was purchased from BD Biosciences . Laemmli Sample buffer 1X, Resolving Gel Buffer, 20% Acrylamid/Bis Solution , Stacking Gel Solution, Developer kit (Clarity Westeren ECL substrate Cat#170-5061) purchased from BIO-RAD. Flask used for culturing are specific for adherent cells purchased from sigma. BC-PAP cell lines are purchased from American Type Cell Culture. E-plates for Real Time Cell Analysis were purchased from ACEA Bioscience. DMEM (Dulbecco's Modified Eagle Medium) and Transfection, reagent were used: gene plasmid GFP-LC3 (Invitrogen) and Opti-MEM media and lipofectamine 2000 reagent . Growth factors like epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Sigma Aldrich Inc). PTC cell lines BCPAP from DSMZ, Braunschh, Germany and

TPC-1 FROM EMD Millipore, USA.

Table 2. *List of Antibodies used in Western blot.*

List of Antibodies (Rabbit or mouse)	**Band size (kDa)**	**Company(source)**
Anti-p-H2AX	15 kDa	Cell Signaling Technologies Company (Beverly, MA, USA)., Santa Cruz Biotechnology, CA, USA, BIO-RAD comany
Anti-GAPDH	37 kDa	
Anti-Caspase-3	35 kDa	
Anti-Cleaved Caspase-3	19/17 kDa	
Anti-Caspase-9	47 kDa	
Anti-Cleaved Caspase-9	37/35 kDa	
Anti-Survivin	16 kDa	
Anti-MCI1	40 kDa	
Anti-BCL2	28 kDa	
Anti-XIAP	53 kDa	
Anti-Cyclin D1	36 kDa	
Anti-STAT3	86 kDa	
Anti-p-STAT3	79/86 kDa	
Anti-PARP	116/89 kDa	
Anti- Cleaved Caspase-8	43 kDa	
Anti-MMP9	92 kDa	
Anti-MMP2	72 kDa	
Anti-HSP60	60 kDa	

Anti-LC3 I/II	18/16 kDa
Anti-ALDH2	56k Da
Anti-SOX2	35 kDa
Anti-rabbit IgG, HRP-linked Antibody	---
Anti-rabbit IgG, HRP-linked Antibody	---

Equipment utilized

ChemiDoc MP imaging system

Nanordop 1000-Spectrophotometry

xCELLigence Real-Time Cell Analyzer

Digital inverted microscope (ECOS life technology)

Cell culture Incubator from Sigma (37Co 5% Co2, 76% humidity)

Flowcytometry Muse® flow cytometry analyzer.

EVOS FL imaging system

Shaker

Methods

Cell Culture

Human papillary thyroid cancer (PTC) cell lines, BCPAP and TPC-1, were cultured using RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum(5ml) (FBS) as a nutrient for cell, 100U/ml penicillin and 100U/ml streptomycin (50ml), to prevent bacterial contamination, at 37 0C in a humidified atmosphere containing 5% CO2.

Cell Proliferation Assay

The anti-proliferative effect of SNG, on BCPAP and TPC-1, were performed through using Cell Counting Kit 8 kit assay. The main working solution in this kit is highly water-soluble tetrazolium salt WST-8(Formazan). First, culture10^4 cells in each well

in a 96-well plate and then incubate at 37Co / 5% Co2. After 24 hours, cells were treated in with five different doses (0.5 µM, 1 µM, 2µM 4 µM, and 8 µM) of SNG for 24 hours along with control (0 µM). Next day, cell counting Kit-8 solution were added as recommended by manufacturer followed by incubation at 37Co / 5% Co2. Finally, the optical density was read at 450 nm. (Figure-5). The Optical density (OD) of the control is subtracted from the is subtracted from sample OD (to remove background). Percentage of cell viable was calculated as OD of the experiment test sample / OD of the control sample x 100. IC50 (inhibitory concentration) = 3.407 uM is calculated, (Mario. H.vargas.2000. Instututo. Nacional de Enfermedes respiratory. Maxico) Figure 5.

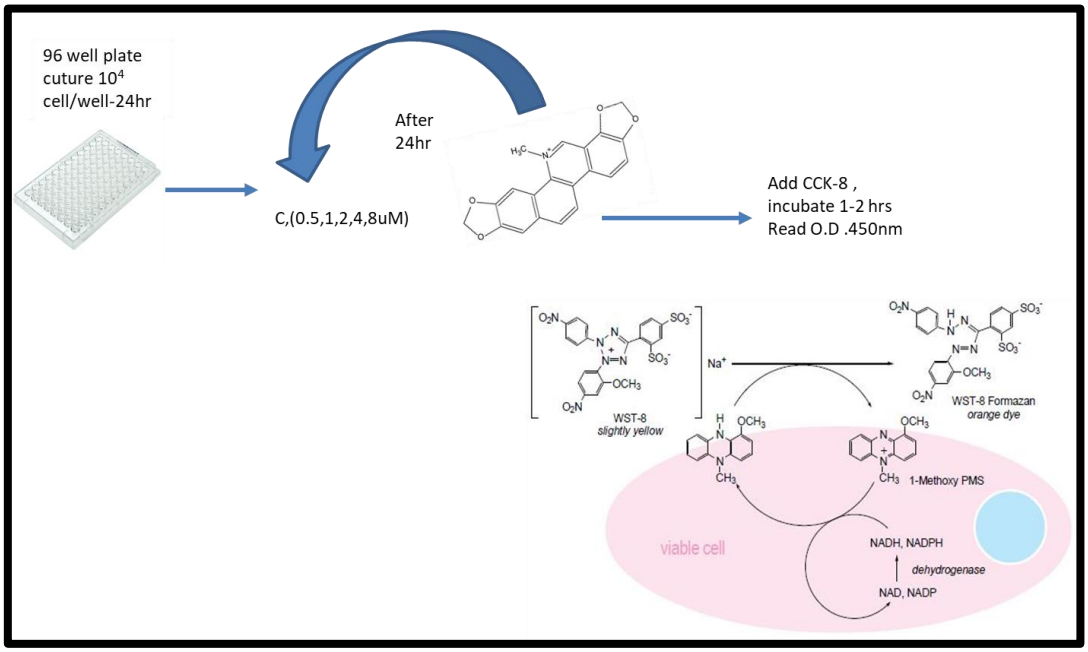


Figure 7. schematic view of the CCK-8 cell proliferation assay.

RTCA-real time cell analyzer

First, load 50ul 10% RPMI media in to the E-plate 16 provided in the kit of Measurement of the real time cell growing of PTC cells that are treated with sanguinarine using the RTCA; xCELLigencecell analyzer BCPAP cells were grown in a single layer (on E-plates) on top of the electrodes and was treated with different doses of SNG. The real time cell analyzer machine and E-plates 16 (RTCA; xCELLigence, Roche, San Diego, CA, USA) were used to determine the cell viability of SNG treated and untreated cultured cells. This method is accomplished in three days (Figure 6).

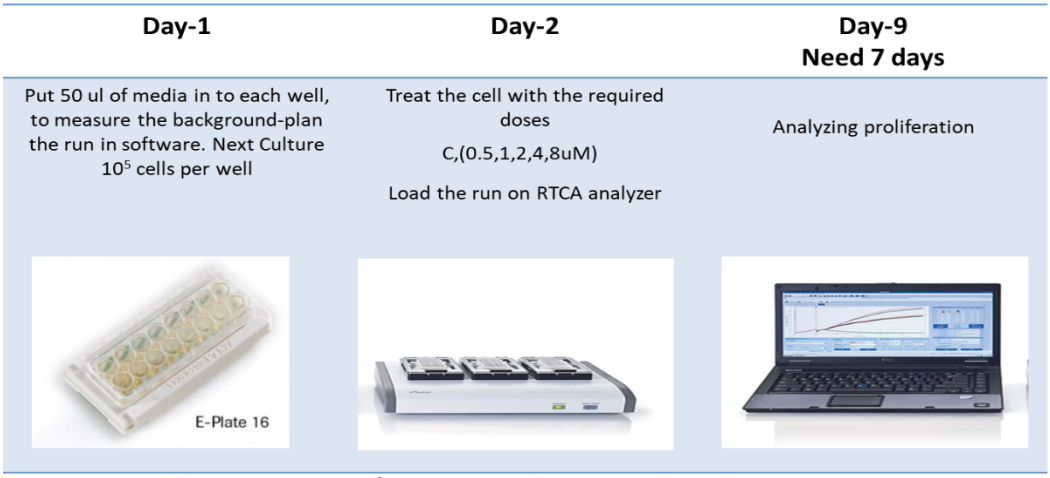


Figure 8. Schematic view of RTCA-Real Time cell analyzer.

Pretreatment of BC-PAP with NAC

First: plate the cells 2X10^6 /Petri dish , and allow it to grow overnight. Next: pretreat the cells with NAC (N-acetyl cystine(0.1M)) , for 1 hour and then followed by SNG

(4uM)treatment. Allow the cells to be incubated with SNG for 4hrs and then prepare protein lysate.

Pretreatment with BC-PAP VAD

First: plate the cells 1X10^6 / 6 well plate , and allow it to grow overnight. Then: pretreat the cells with VAD (40 uM) , for 1 hour and then followed by SNG(4uM) treatment. Allow the cells to be incubated with SNG for 4hrs and then prepare protein lysate.

Protein lysate

The protein lysate is prepared after the accomplishment of treatment time to detect the signaling protein involved in JAK/STAT pathway. Step 1: treated cell are collected in 15 ml falcon and spine at 1500 rpm/5min/4C^{o} and discard supernatant. Step 2: the cell pellet is washed with 10X phosphate buffer and then transferred to 1.5ml Eppendorf tube and spined at 4000rpm/5min/4C^{o}, followed by discarding supernatant. Step 3: A protein test sample is mixed with equal volume of 2X buffer and left on boiling water for 2-5 min. (2x laemmli buffer volume depend on the pellet formed). A 2x laemmli buffer is specifically used for Laemmli SDS-PAGE (Poly Acrylamide Gel Electrophoresis) system, and it contains 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue. The purpose of SDS detergent is to denatures the proteins and subunits and gives each protein an overall negative charge so that each will separate based on size, whereas 2-mercaptoethanol is to reduces the intra and inter-molecular disulfide bonds. The bromophenol blue serves as a dye front that runs ahead of the proteins and also serves to make it easier to see the sample during loading. Step 4: measuring the protein concentration using nanodrop 1000 and protein purity is assessed at absorbance of 280 nm (Note: protein sample should be completely

homogenized, otherwise false low /high results will be read). The purity of proteins is assessed using 260/280nm absorbance, and a purity value < 1.8 considered pure for protein. Step 5: add 2-mercaptoethanol, the volume should be 5% of 2x laemmli buffer added previously. Triplicate reading is taken for protein values and then mean was calculated (Figure 7).

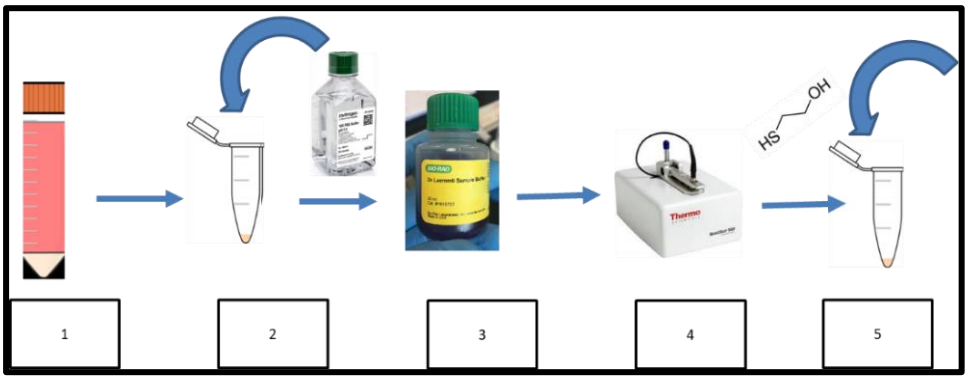


Figure 9. Protein lysate preparation steps.

Muse® Annexin V and dead cell assay

To find out the level of apoptosis due to SNG treatment in PTC cell lines, we plated equal number of cells followed by treatment with var Using Muse™ Annexin V & Dead Cell Kit. Cell were treated with various doses of SNG 0µM, 0.5µM, 1µM, 2µM, 4µM and 8µM SNG for 24 hours. The next day, cell pellet was collected wasted and assess the level of apoptosis as peer the manufacturer. Further, data collection about the number of early and late apoptotic cells was done on Mouse cell analyzer and total apoptosis was calculated by adding percent of cells in early and late apoptosis quadrants of the histograms and plotted in bar graphs. See Figure 8 for the steps of Muse® Annexin V and dead cell assay as well as the graphical presentation of apoptosis profile.

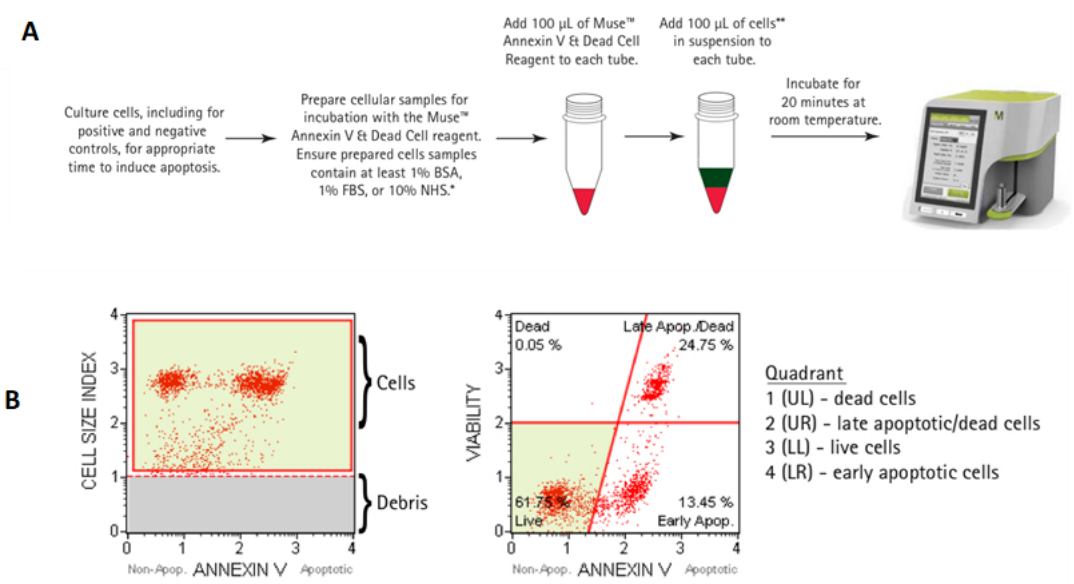


Figure 10. Muse® Annexin V and dead cell assay & graphical presentation of apoptosis profile.(A) assay protocol.(B)apoptosis profile.

Transfection experiment

1. At day one BCPAP cells (1X10^6 cells) are cultured in 6 well plate and labeled properly.
2. Day two, inspection of cell confluency (must be >90 % of cells confluent), after that remove the media in the wells, and follow steps bellow:
    a. Add one ml of Opti-MEM in to each well
    b. Two Master mix are made (A tube: 4 ul of gene plasmid GFP-LC3(100 pmol)+ 250 ul Opti-MEM, and B tube: 250 ul Opti-MEM mixed with 5ml of lipofectamine 2000 reagent).
    c. Both master mixes are incubated at room temperature for 10 minutes separately and after that mix (both A & B) together and incubate again

for 30 minutes.

d. Add 500 ul of the mix prepared to each well , and incubate the plate in incubator for 6 hrs , then remove media and add 2 ml Opti-MEM and allow transfection to occur overnight.

e. After 24hr of transfection, change the media to 10% RPMI add SNG (0 uM , 4uM, GFP-LC3,GFP-LC3+4uM) to the respective wells for 24 hr

f. successful Transfection is assessed under Digital inverted microscope (ECOS life technology)

Cologenic Assay

Plating cells on 6-well plate ($2X10^4$ and $1X10^4$ BCPAP cells), after that allow the cells to be seeded for 24hr. After that various SNG doses (0,0.5,1,4,8uM) were applied to each well. Soon after cell treatment for 2 hrs., the treatment media (5%RBMI) is change to (10% RBMI) and cells were grown overnight. Cellular growth was stained with crystal violet 0.2% (0.1g/50ml Distilled water). Each well is subjected to 1ml of crystal violet stain and incubate it for 30min, followed by washing in running tap water. The stained cellular aggregates appear in a form of colony (Figure 9).

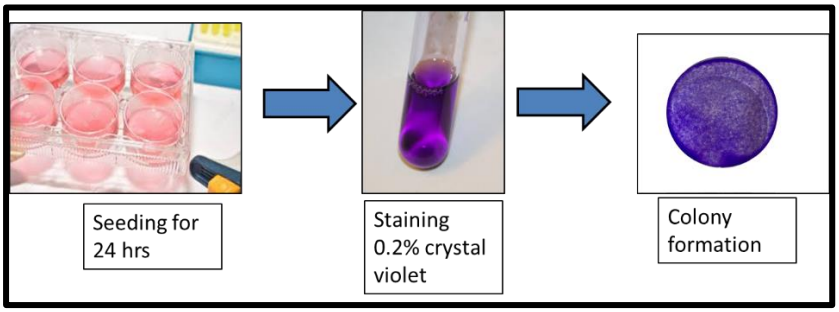


Figure 11. Cologenic Assay protocol.

Scratch Assay

One million cells were plated in a 6-well plate and allowed to grow overnight. Using 100ul pipette tip, a solid scratch line is applied on each well of the 6 well plate. Cells were treated with SNG doses (0,1,2,4,8 uM) for 2hrs , and then the cells were allowed to grow in 10% overnight. Migration rate is assessed ,which represent the gap fill of the scratch made previously(Figure 10).

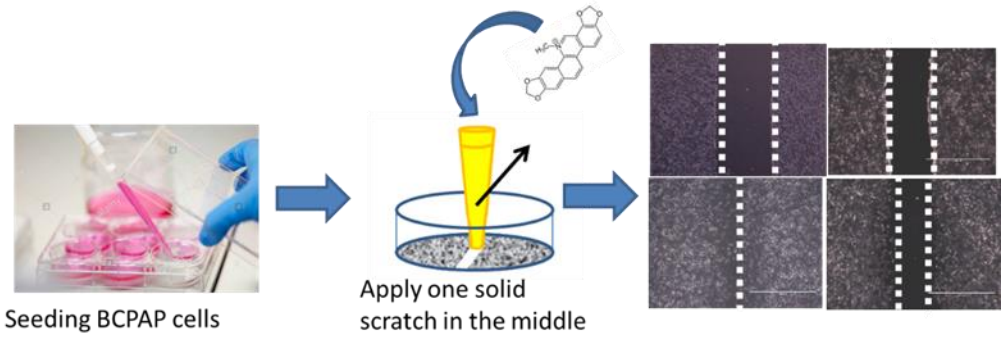


Figure 12. Scratch Assay protocol.

Thyrosphere cell culture

Thyrosphere cells first were generated inside the lab in the form of spheres based on published method generated by Caria et al (Caria et al., 2017). At first, 2X106 PTC cells were cultured in 5% DMEM media. After that, the cells are subjected to enzyme treatment to make detach cells in a single cell suspension, using trypsin. A second culturing step is followed using DMEM-F12 medium supplemented with and growth

factors like epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Sigma Aldrich Inc) Figure 11.

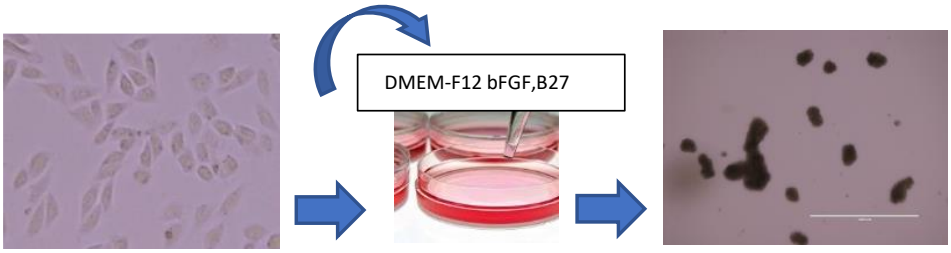


Figure 13. Thyrospheres Forming Assay and Stemness Profile.

SNG suppresses PTC cells through upregulation of apoptosis markers

A group of experiments were used to investigate the effect of SNG on the proliferation of PTC cells. First, cell viability assay was performed to assess the antiproliferative effect on PTC cells. Both, BCPAP (Figure 12) and TPC-1 cells (Figure 13) showed a significant dose dependent inhibition of cell viability (p-value***$p<0.001$). Both BCPAP and TPC-1 cells were treated with 0µM, 0.5µM, 1µM, 2µM, 4µM and 8µM SNG for 24 hours and the next day cells were assessed for the viability using CCK8 assay. The inhibitor concentration 50 (IC-50) of both BCPAP and TPC-1 is 3.40 µM and 4 µM, respectively.

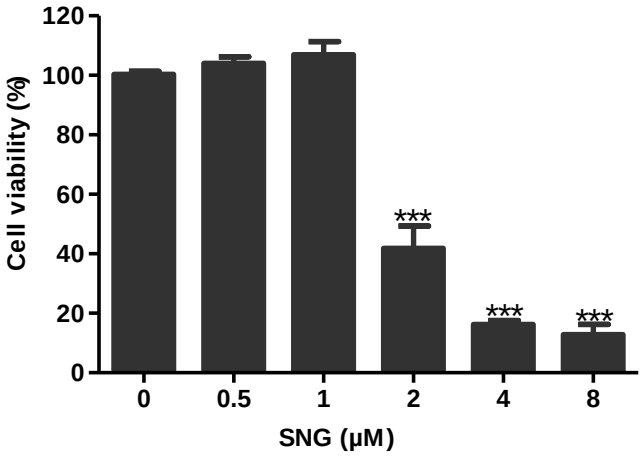


Figure 14. BCPAP cell viability after 24hr treatment.

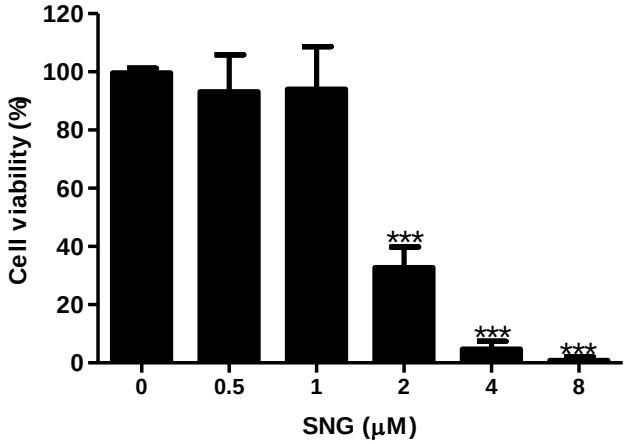


Figure 15. TPC-1 cell viability after 24hr treatment.

Real time cell analysis revealed similar results to the CCK8 cell viability assay, were the cell viability was assessed in real time. Both cell lines were cultured on an electrode containing plate, and after 24, cells were treated with 0µM, 0.5µM, 1µM, 2µM, 4µM and 8µM SNG. Both BCPAP cells (Figure 14) and TPC-1(Figure15) showed real time cellular inhibition after 25 hours, since experiment started.

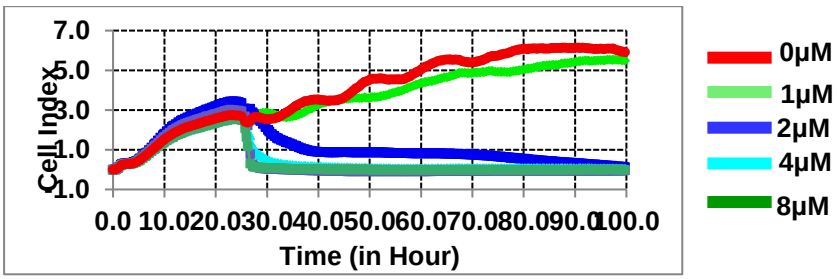


Figure 16. Real time cell analysis using xCELLigenc cell analyzer for BCPAP cells.

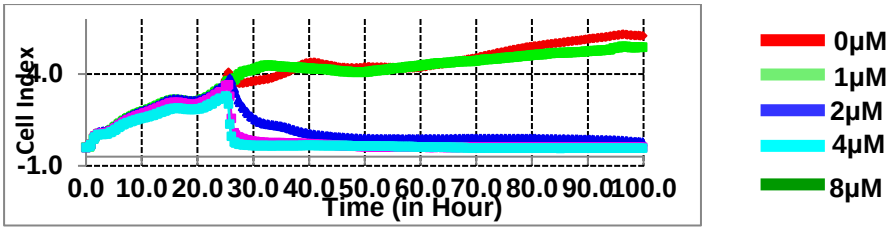


Figure 17. Real time cell analysis using xCELLigenc cell analyzer for TPC-1 cells.

SNG mediates apoptosis in both BCPAP and TPC-1 cells, when Muse Annexin apoptosis kit used, as illustrated in material and methods. Both cell lines showed an increase in the Annexin V staining expression in dose dependent manner with BCPAP cells (Figure 16) and TPC-1(Figure 17).

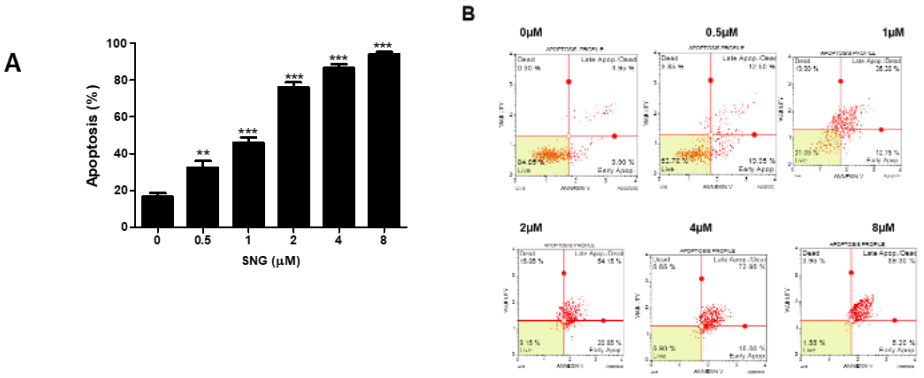


Figure 18. Apoptosis Annexin V staining in BCPAP cells using Muse Annexin apoptosis kit.(A): BCPAP cells percentage of Apoptosis, p-value***p<0.001.(B): BCPAP apoptosis profile results from muse flow cytometry machine.

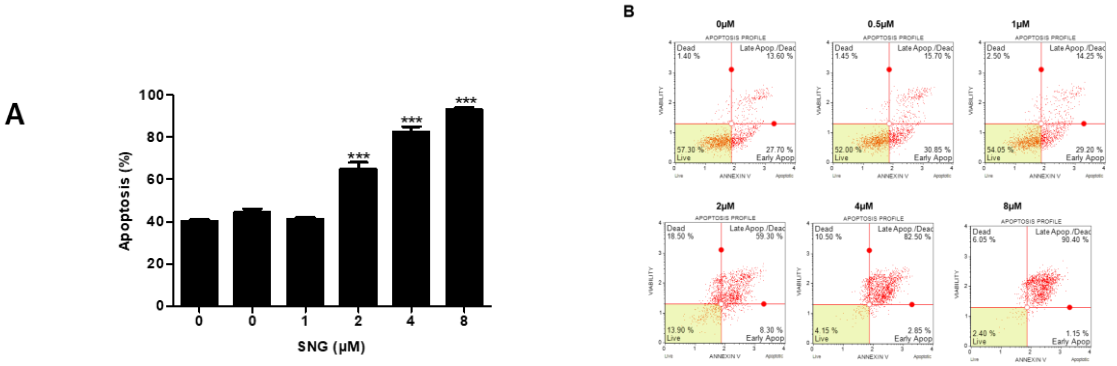


Figure 19. Apoptosis Annexin V staining in TPC-1 cells using Muse Annexin apoptosis kit. (A): TPC-1 cells percentage of Apoptosis, p-value***p<0.001.(B): TPC-1 apoptosis profile results from muse flow cytometry machine.

Besides, SNG was found to phosphorylate Gamma H2AX, which is a novel biomarker for DNA double-strand breaks, which initiate the cell to go through cellular damage and eventually apoptosis. Furthermore, SNG was found to generate double-stranded breaks (DSBs) in both cell lines BCPAP (Figure-18) and TPC-1(Figure-19). In both cell lines, 2uM of SNG stimulate DSB with maximum level of expression at 4uM, as illustrated in Figure 18 &19. GAPDH proteins is used to assess the equal loading and it is expressed in all cells as a housekeeping protein.cologenic assay confirm what had been seen in CCK8 assay and flow cytometry,were colony formation in inhibited in a dose dependant manner.however at 4µM and 8µM cells were found floating dead (Figure-20).

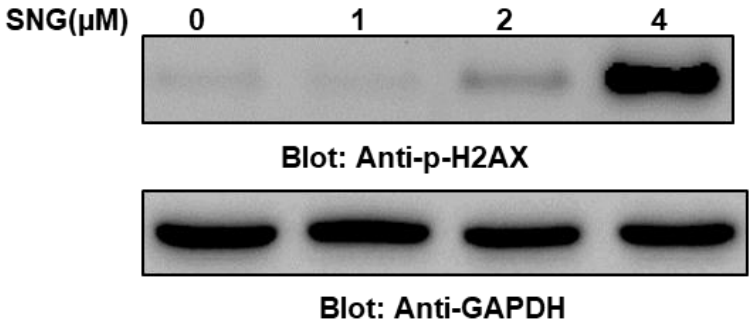


Figure 20. SNG stimulates phosphorylation of H2AX in BCPAP cells.

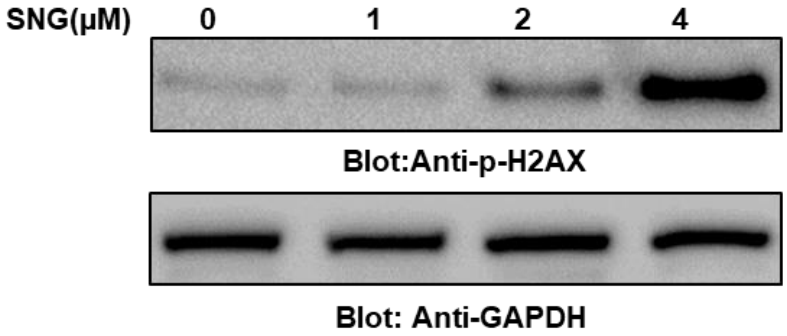


Figure 21. SNG stimulate phosphorylation of H2AX in TPC-1 cells.

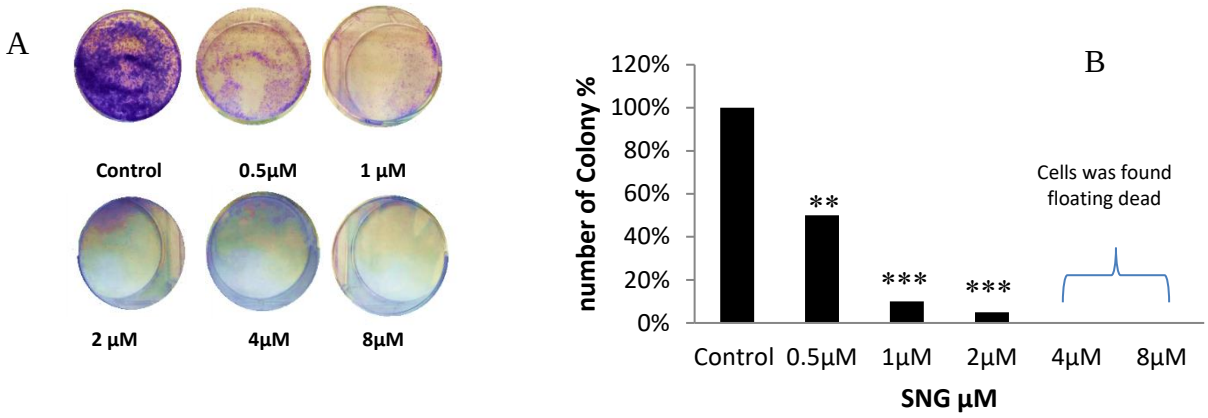


Figure 22. SNG causes dose dependent decrease in colony formation in BCPAP cell lines.(A) Cologenic assay in 6-well plate,(B) Number of colonies after SNG treatment.

SNG mediate the activation of caspase cascade in PTC cell lines

SNG mediated its effect on PTC cells though activation of caspases; caspase 3, caspase 9, caspase 8 and PARP (Poly (ADP-ribose) polymerase) (Figure 20). In both cell lines cells were treated with 0 µM ,1 µM ,2 µM and 4 µM for 4 hours and immunoblotted for several caspase proteins. At dose 4 µM SNG activate caspase cascades, however at 2 µM there is slight activation Figure 20.

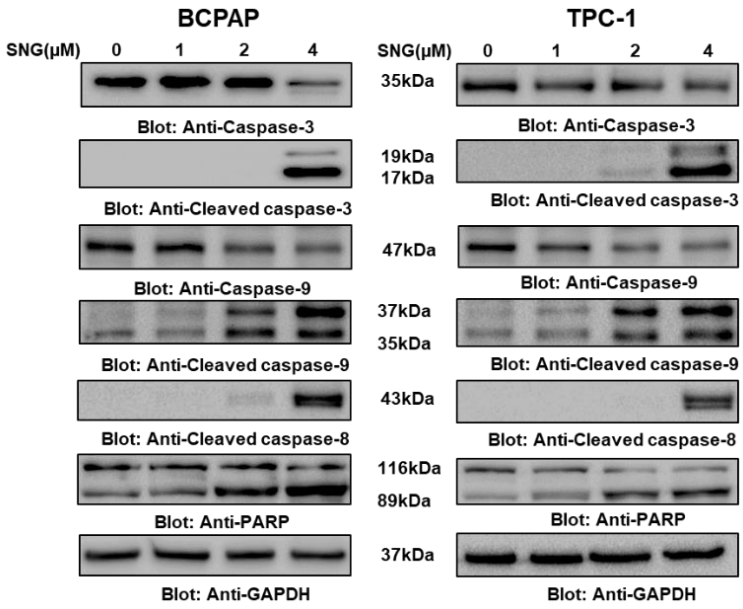


Figure 23. SNG mediate caspase cascade activation in both BCPAP and TPC-1 cells.

In similar conditions stated earlier, PTC cells were pretreated with z-VAD-FMK; a universal inhibitor of caspase activity, and it prevented SNG-mediated caspase activation. Morphological assessment of BCPAP cells clearly showed that z-VAD-FMK was able to revers morphological changes only partially (Figure 21-A(z-VAD-FMK+SNG4μM)). Western blot analysis revealed reversed action of SNG in apoptosis markers (caspase-3,PARP) but only partially reversed DSBs cellular markers(p-H2AX)(Figure 20-B).

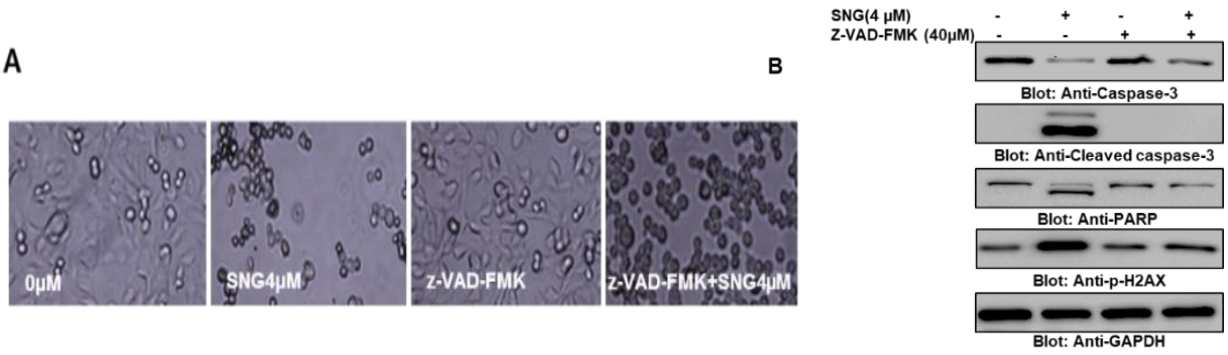


Figure 24. Z-VAD-FMK reverse SNG effect on BCPAP cells.(A): morphological assessment of BCPAP cells, Figure(B): western blot analysis of BCPAP cells in combination with Z-VAD-FMK.

SNG- suppresses p-STAT3 , IL-6 secretion as well as IL-6 induced STAT3 activation in PTC cells

JAK/Stat3 pathway is constructively activated in PTC cells, were pSTAT3 protein is downregulated primarily. PTC cells were cultured and treated with different doses of SNG 0 μM ,1 μM ,2 μM and 4 μM for 4 hours. After that, protein lysate was immunoblotted for several antibodies such as: STAT3, cyclin D1,XIAP,Bcl2,MCI1 and survivin. SNG downregulated STAT3 in a dose dependent manner in PTC cells, with maximum inhibition at 4 μM. In addition, there is no change in Bcl2 proteins,

however survivin, Cyclin D1 showed marked decrease at 4 µM (Figure 22).

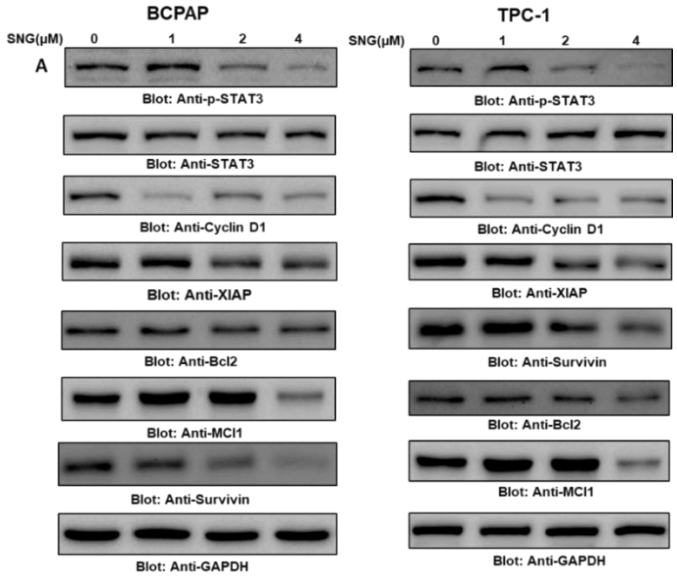


Figure 25. SNG suppresses the p-STAT3 proteins and its associated proteins.

Additionally, BCPAP cells were serum starved for 24 h, next pretreated with 2 µM SNG for 1h and then stimulated with IL 6 (100 µg/ml) for 30 min as described in materials and method section. After cell lysis, equivalent amounts of proteins were separated by SDS–PAGE, transferred to PVDF membrane and immunoblotted with antibodies of p-STAT3 (Try705) and STAT3. SNG was found to block the IL-6 induced STAT3 activation as seen in Figure 23.

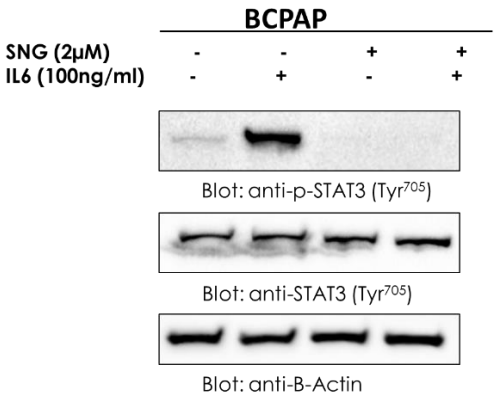


Figure 26. SNG mediated inhibition of IL-6 induced STAT3 activation in BCPAP cells.

SNG- suppresses the matrix metalloproteinases (MMP) of BCPAP.

MMP property of BCPAP was assessed using scratch assay, were $1X10^6$ cell were cultured in 6 well plate for 24 hours as described in material and methods. Subsequently a scratch was made, and cells were treated with SNG 0 µM ,1 µM ,2 µM , 4 µM and 8 µM for 4 hours. Morphological assessment of gab fill was assessed as seen in Figure 24 (A&B). As illustrated in figure A, the gab fill is inversely proportional to the increase in SNG doses. Although SNG 4 µM and 8 µM showed an abolished gab fill, at 4 µM cells were in tacked, compared to 8 µM were cells were floating dead. Western blot analysis was performed by treating cultured BCPAP cells and protein lysate is prepared and immunoblotted for MMP-9 and MMP-2. MMP-9 was downregulated at 4 µM, whereas MMP-2 showed decrease in expression starting from 2 µM(Figure 24-C).

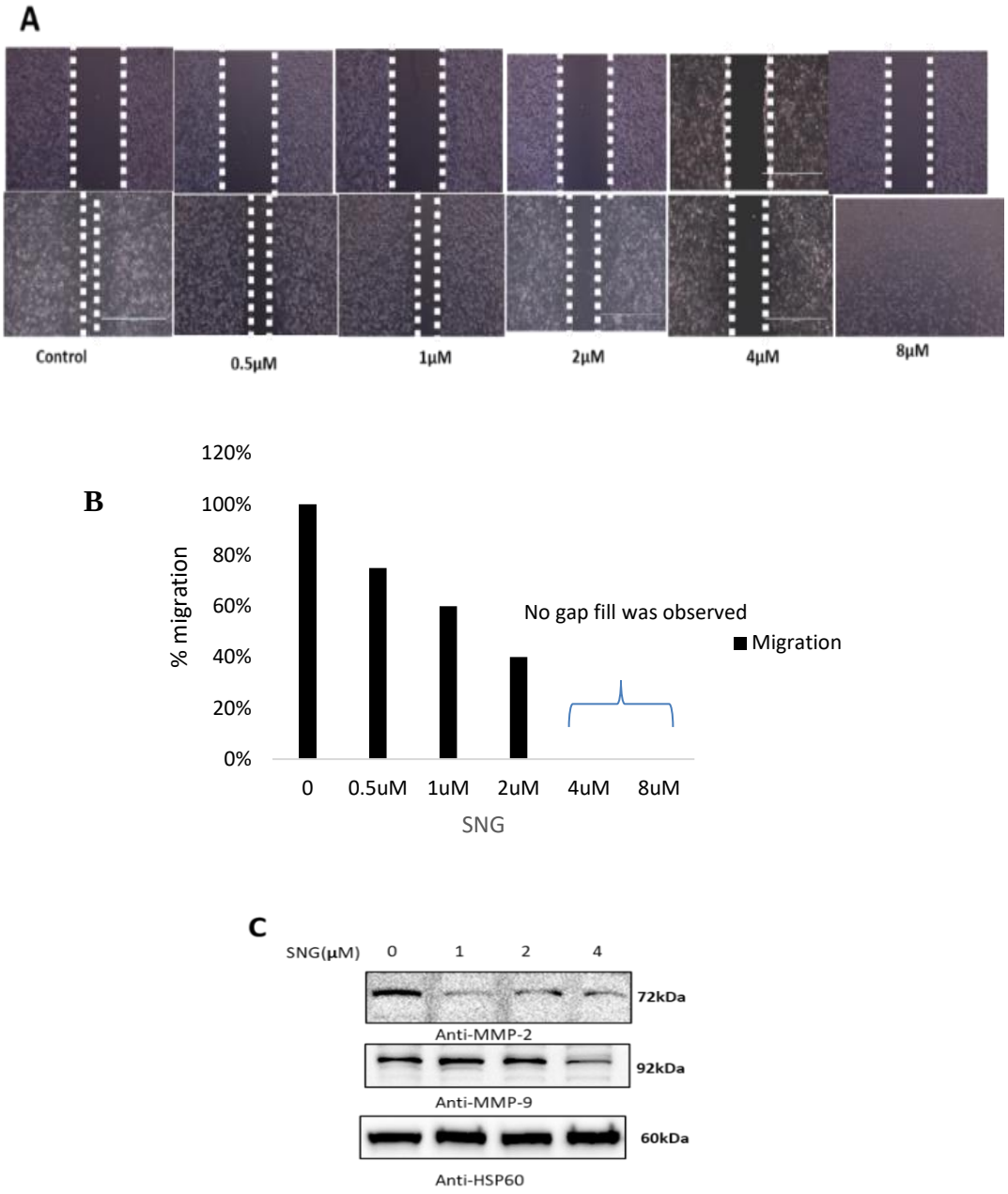
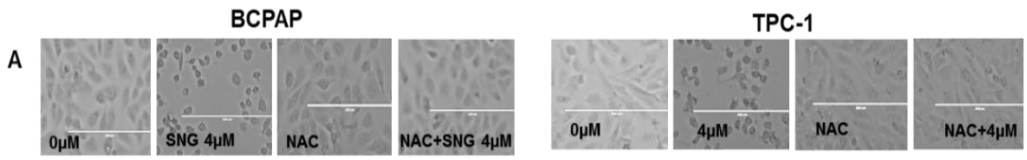


Figure 27. Assessment of MMP properties of PTC. (A) Scratch assay in a dose-dependent manner 0 μM ,1 μM ,2 μM , 4 μM and 8 μM. (B) Measurement of gab fill in figure-A.(C) Western blot analysis of MMP-2 and MMP-9.

SNG mediated anticancer action involves generation of ROS in PTC cell

SNG mediate it antitumor action through generation of ROS (reactive oxygen species). When PTC cells were pre-treated with NAC (N-Acetyl-L-cysteine), it was found that NAC reverse SNG-induced morphological changes in PTC cells (Figure-24-A). At 4 µM, PTC cell lost the spindle like shape and the cell membrane is curled and over all shape is turned to circular shape. On the other hand, NAC+4 µM the cells resembled that of the control (0 µM) and NAC treatment. Furthermore, cellular viability was assessed using CCK8, were BCPAP and TPC-1 cells were pretreated with 10 mM NAC subsequently treated with 4µM SNG as indicated for 24 hours, as described in materials and methods (Figure 25-B). In similar conditions for CCK8 assay, PTC cells were treated for 4 hours, then cells were lysed and proteins were separated on SDS-PAGE (Figure 25-C)



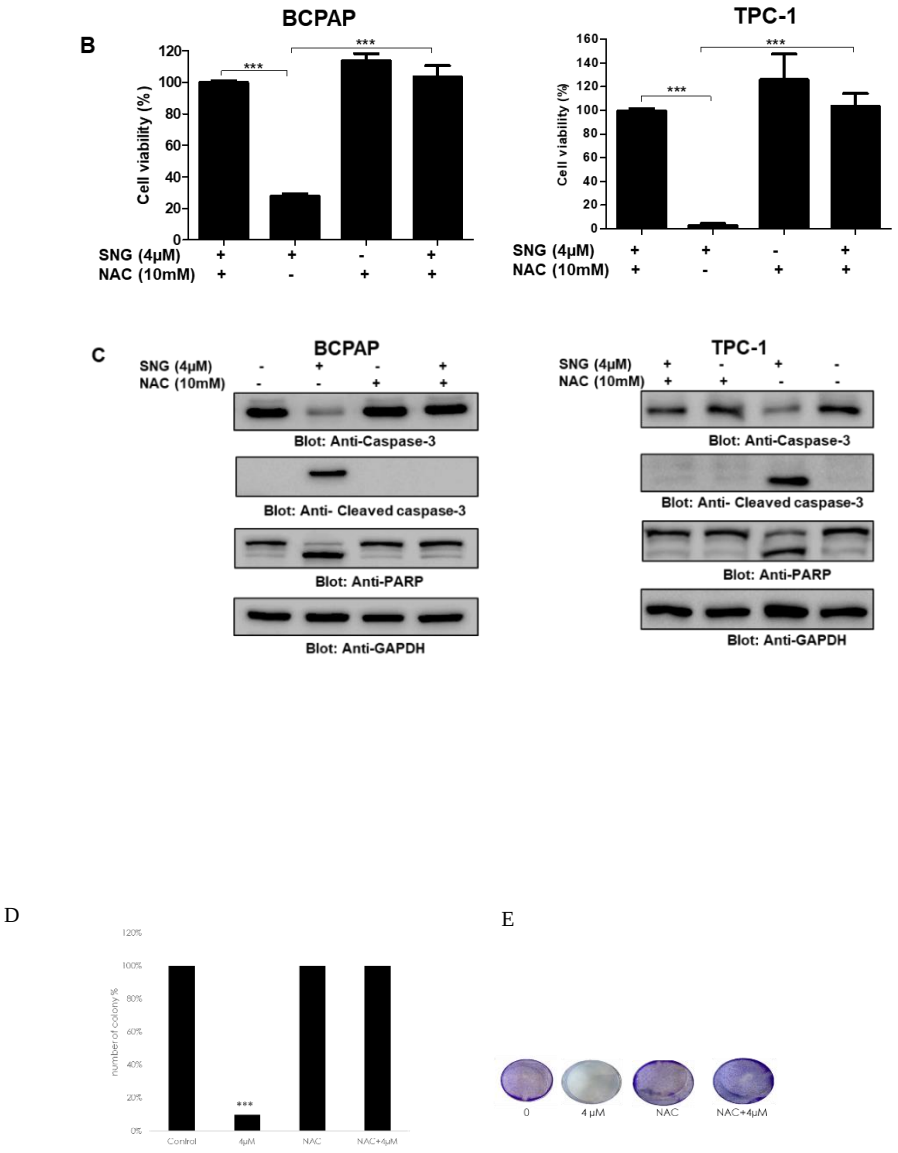


Figure 28. SNG mediated anticancer action involves generation of ROS in PTC cell.(A) NAC reverse SNG-induced morphological changes in PTC cells ;BCPAP and TPC-1.(B) BCPAP and TPC-1 cells were pretreated with 10 mM NAC subsequently treated with 4μM SNG as indicated for 24 hours. (C) western blot analysis, PTC cells were pre-treated with NAC ,after that treated with 4μM, and separated on SDS-PAGE.(D) NAC reversed SNG ROS dependant killing and enhanced colony formation ,Number of colonies after SNG treatment in cologenic assay.(E) Cologenic assay in 6-well plate.

Western blot analysis supports the viability assay, were NAC completely reverse the effect of SNG in both cell lines. This can be clearly noticed by the upregulation of cleaved caspase-3 and PARP proteins, and when cells are pre-treated the effect is completely reversed (Figure 25-C).

SNG mediates its anticancer action through induction of autophagy

Beside upregulation of apoptotic markers, SNG was found to induce autophagy side by side with apoptosis for inhibiting PTC cell proliferation. Figure 26 A & B showed increased expression of LC3-II in both cell lines, BCPAP and TPC-1. Additionally, NAC revered the effect of SNG and reduced expression of LC3 proteins. At Dose 4µM LC3 expression peaked with equal GAPDH housekeeping proteins in both cell lines.

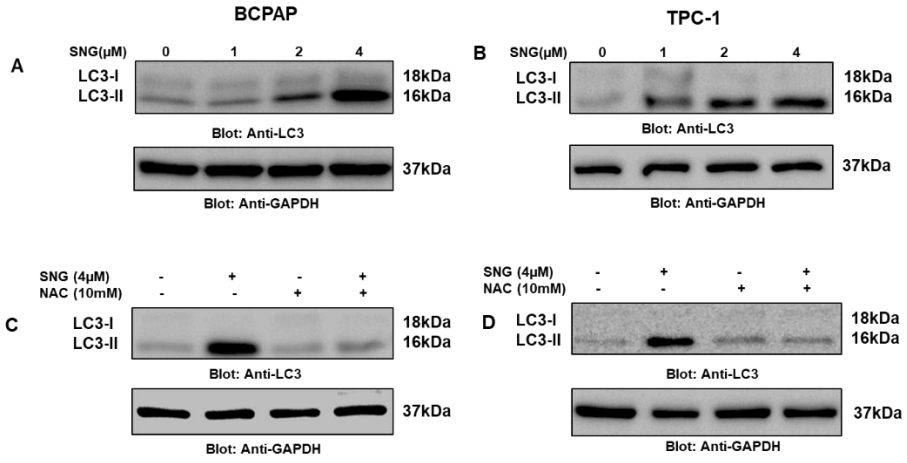


Figure 29. SNG induce activation of autophagy in PTC cells. (A)BCPAP cells were treated for 4 hours and lysed to be separated on SDS-PAGE. (B) TPC-1 cells were treated for 4 hours and lysed to be separated on SDS-PAGE. (C) western blot analysis,

PTC cells were pre-treated with NAC, after that treated with 4µM, and separated on SDS-PAGE (D) western blot analysis, PTC cells were pre-treated with NAC, after that treated with 4µM, and separated on SDS-PAGE.

To assess the LC3 protein expression in BCPAP cells, BCPAP cells were stably transfected with lipofectamine 2000 (GFP-LC3) with 4µM SNG. After treatment, SNG found to induce the autophagy manifested as LC3 puncta were assessed by floursent microscopy.

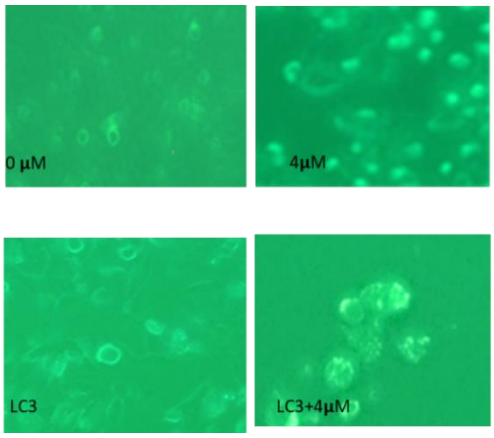


Figure 30. Induction of Autophagy in BCPAP cell lines using GFP-LC3 plasmid.

Drug combination enhanced SNG antiproliferative effect on TPC-1 cells

Drug combination lead to an enhanced antiproliferative effect in cancer therapy, and SNG was found to have an enhance anticancer effect when combined with cisplatin. TPC-1 cells were treated with 2µM SNG and 10 µM of cisplatin alone and with combination for 24h. after that cells were lysed and separated using SDS-PAGE. As it is illustrated in figure-16, SNG and cisplatin enhanced the downregulation of p-STAT3 protein than when each drug was used alone. This synergistic effect lead to activation of apoptosis with increased expression of caspase-3, PARP, and DSB(Figure-27A&B). Other inhibitor of apoptosis protein (IAP) proteins such as, XIAP (X- inhibitor of apoptosis protein (IAP) and survivin, are downregulated to enhance apoptosis to occur (Figure 28A&B). SNG and cisplatin combination was found to induce ROS to mediate apoptosis in TPC-1. This was clearly seen when cells were pre-treated with NAC and then supplied with SNG and cisplatin. NAC was able to reverse the synergistic effect of both SNG and cisplatin (Figure 28 C). With similar conditions stated earlier, TPC-1 cell viability was assessed with CCK8 assay. The results in Figure 28 D revealed reduced viability when SNG & cisplatin added to TPC-1 cells and NAC was able to restore their viability.

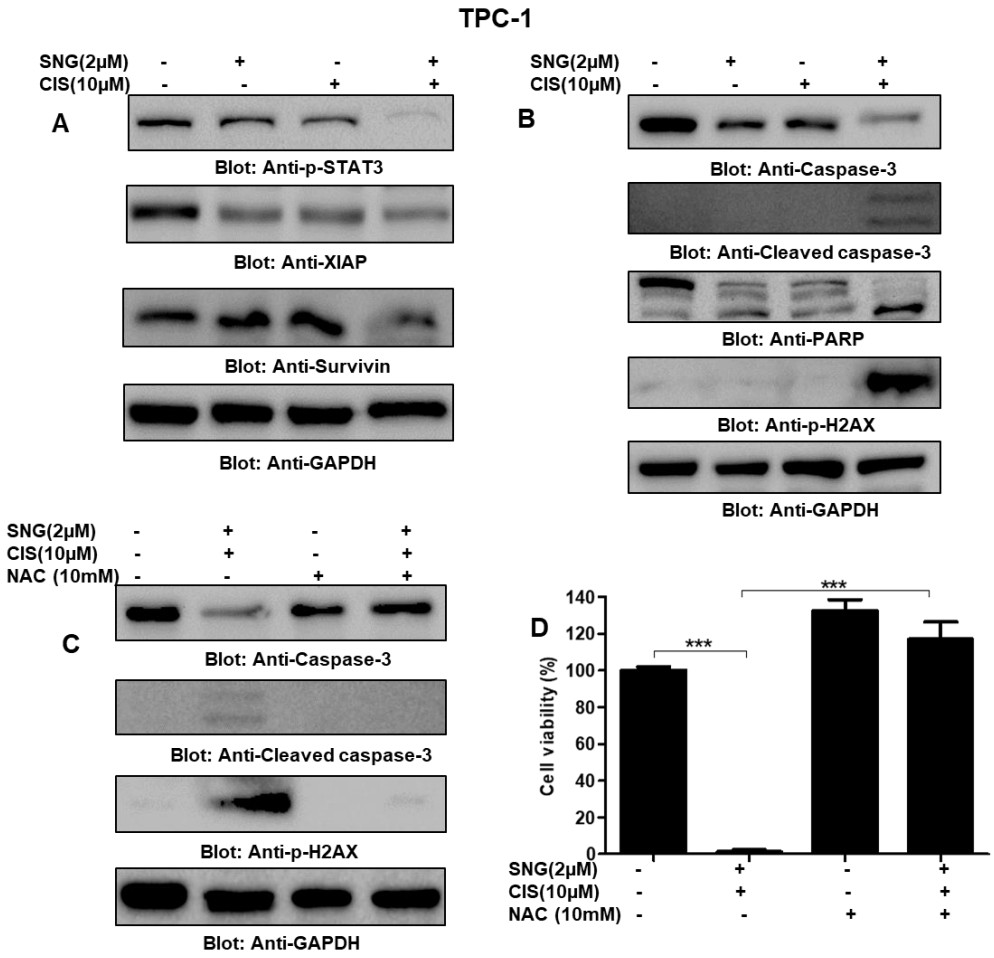


Figure 31. SNG enhanced its anticancer activity when combined with drug cisplatin, through inactivation of STAT3 protein. (A) TPC-1 cells were treated with 2μM SNG and 10 μM of cisplatin alone and combined. Cell lysate was immunoblotted for p-STAT3,XIAP and survivin.(B) TPC-1 cells were immunoblotted for caspase-3, PARP and pH2AX. (C) TPC-1 cells were treated with 10 mM NAC, 2μM SNG and 10 μM of cisplatin alone and in combination. Cells were immunoblotted with caspase-3, PARP and pH2AX.(D) TPC-1 cells were treated with 10 mM NAC, 2μM SNG and 10 μM of cisplatin alone and in combination and as indicated for 24 hours. CCK8 was used to determine cell viability(p-value***$p<0.001$).

SNG-treatment inhibited thyrosphere formation of PTC cells

BCPAP and TPC-1 cells spheres were grown and treated in ultralow attachment plates

with 0 µM, 1µM, 2µM and 4µM of SNG for 3 days, followed by cell lysis and western blotting with antibodies ALDH2, SOX-2, and HSP60. Figure 29-A showed the decrease in the size of the thyrosphere in SNG dose dependent manner, were 2µM showed small fragmented thyroshpere compared to 0 µM (control). With the same conditions stated earlier, western blot analysis was performed, and lysate was immunoblotted for ALDH2, SOX-2, and HSP60. TPC-1 & BCPAP showed inhibitory effect in the stemness marker SOX2 at 4 µWhereas on TPC-1 the inhibitory effect was seen earlier at 2µM with ALDH2 and at 4µM ALDH2 for BCPAP cells (Figure 29-B&C).

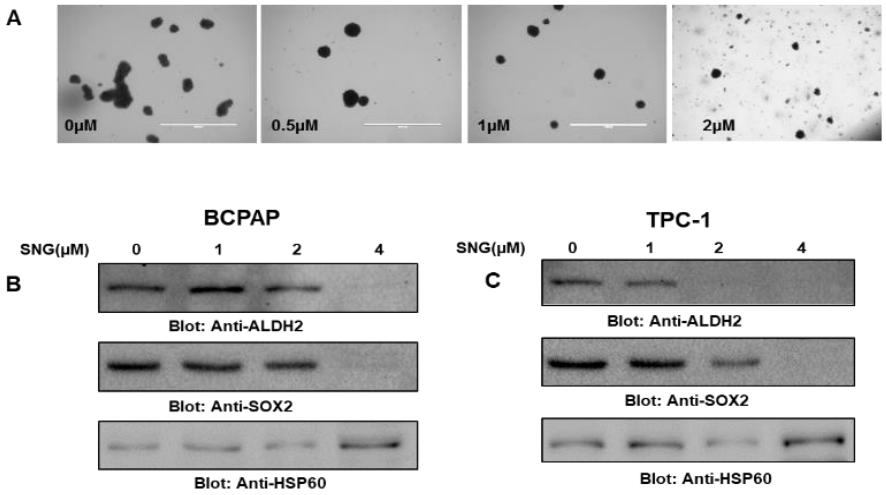


Figure32.SNG-treatment inhibited thyrosphere formation of PTC cells. (A)morphological assessment of PTC thryosphere size with different SNG doses .(B)western blot analysis of PTC thyrosphere stemness markers.

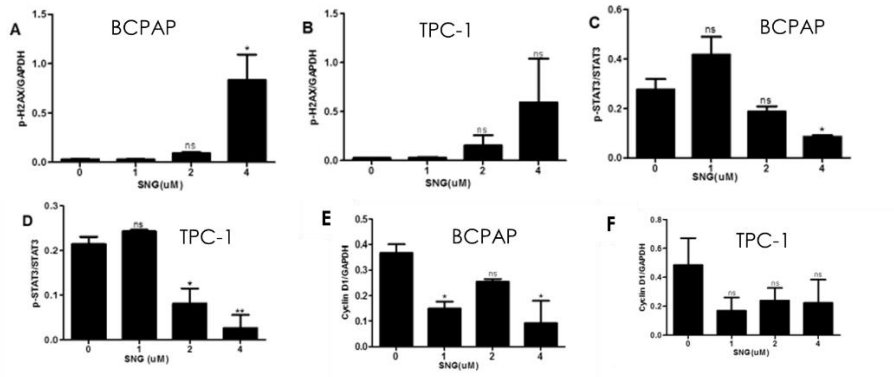


Figure 33. Densitometric analysis of p-H2AX in BCPAP (A) and TPC-1 (B), p-STAT3 in BCPAP (C) and TPC-1 (D) respectively. Cells treated with increasing doses of SNG for 4 hours as indicated in the main text. After cell lysis, equal amounts of proteins were separated by SDS–PAGE, transferred to PVDF membrane and immunoblotted with antibodies against p-H2AX, p-STAT3, STAT3 and GAPDH. Data expressed as relative density normalized with GAPDH and represented as mean ± SD (*$p<0.05$).

CHAPTER 5: DISSCUION

Plant alkaloids have shown to be a promising option for the treatment of cancer patient in many types of cancer(Isah, 2016). Understanding the drug inhibition mechanism is key factor for novel drug development. In this study, we demonstrated consistent decrease in cell viability in both cell lines (TPC-1 and BCPAP). IC50, as a measure of drug efficacy, is an important key to determine in order to downregulate the biological processes in cancer cells and it was found to be higher in TPC-1 cells (4 µM) than in

BCPAP (3.40 µM). Although BCPAP cells reach IC50 at slightly lower concentration, still viability blot is similar in both cell lines. Morphological assessment of BCPAP and TPC-1 cells was nearly comparable, except at 8 µM BCPAP, it was found floating dead, but in case of TPC-1 was shown to be slightly adherent on the Petri dish. This can explain why the IC50 is higher in TPC-1, which allowed some cells to be still viable at higher concentration. A recent study showed that the SNG was found to have lower IC50 in Pre-ALL cell lines including 697, REH, RS4;11, and SupB15 cells, which was between 0.5-0.1 µM (Kuttikrishnan et al., 2019) and higher IC50 of 10 µM recorded in human tumor cell line with high MKP-1 levels (Vogt et al., 2005) and in Hep3B (hepatocellular carcinoma cell) with 4.49 µM(Su et al., 2019). TPC-1 cell lines showed exactly similar IC50 with HepG2 hepatocellular cancer cell lines(Su et al., 2019). Our findings clearly supported by other research finding in various cell lines treated with SNG and this increase the quality of our finding.

On the other hand, RTCA showed similar growth inhibition pattern in both cell lines and data are expressed as a measure of cell index. As it is clearly shown in Figure 14 & 15 both cell lines behave in similar way, but it was noticed that there is a sharp decrease in TPC-1 cell viability compared to BCPAP at 30^{th} hour of treatment at 2 µM. Real-time impedance assay showed all doses were able to decrease cell proliferation, except 0 µM & 1 µM and this assay valuable to study the behavior of cancer cells for long periods to mimic real cancer patient treatment.

SNG was found to induce apoptosis and it is inevitably lead to cell death. Annexin V Muse assay showed gradual increased in apoptosis profile in BCPAP at 0.5 µM, but TPC-1 showed significant increase at 2 µM , and this prove why there is an increase in IC50 of TPC-1 earlier. The 8 µM dose was found to be toxic for PTC cells, and all cells are accumelated in the upper right quadrant of apoptosis profile (Figure 15B and

Figure 16B). Caspases are proteolytic protein that play an important role in apoptotic signaling pathway for rapid apoptosis. Western blot analysis showed increase in Cleaved Caspase-3 activition, which interact with caspase-8,9 in order to activate extrinsic (death ligand) (Figure19). This finding was incorconcance with the slight decrease of anti-XIAP, survivin proteins ,which are antiapoptotic proteins (Figure-21). These proteins prevent apoptosis from occuring, so their downregulation is consistant with SNG antiproferative effect (M. Sun et al., 2012; M. Sun et al., 2010) (Meng Sun et al., 2010). As a precursor for cell death, DNA breakage through the upregulation of p-H2AX protein in both cell lines contribute to cells death. This finding correlated with the acivation of PARP protien in order to aid DNA repair (figure-18,19 &20). Therefore, SNG can target PTC cells through induction of apoptosis is essential for future theraptuic options such, anti-antiapoptotic protiens. BCL-2 as anti-antiapoptotic protien play an essential role in maintaining the integrity of outer mitochonderial membrane , so targetting cancer cells with anti-antiapoptotic protiens will enhance it's killing (Carrington et al., 2017). In order to examine the specific effect of SNG on PTC apoptosis, caspase inhibitor was able to reverse apoptosis only partially (Figure-21 A), suggesting other mechansims might be involved in cell inhibition (ex:autophgy). Other studies concluded similar outcomes such as: Pre-ALL) cell lines (Kuttikrishnan et al., 2019), hepatic cancer cell lines (Su et al., 2019), K562 human erythroleukemia cells (Weerasinghe, Hallock, Tang, & Liepins, 2001) and Primary Effusion Lymphoma Cells (Azhar R. Hussain et al., 2007).

Accumulating evidence has suggested the crosstalk between Apoptosis and autophagy signaling in cancer cells. In our study, autophagic cells death was accombinead side by side with apoptosis cell death , as clearly shown in Figure -26. LC3 II (Microtubule – associated protein light chain 3 (LC3)) increased expression in

both cell lines ,which intiate generation of autophagosomes to strart the process of autophagy (Parzych & Klionsky, 2014). At 4 µM the highest expression of LC3-II fraction in both cell lines, which means high autophagosom was generated to intiate cellular death. GFP-LC3 plasmid transfection experiment, support the westeren blot analysis were puncate expression in inceased in SNG +GFP-LC3 treatment than when GFP-LC3 or SNG alone (Figure 28). This finding is essential as less study have investigate the interconnections between autophgy (Type-II programmed cell death) and apoptosis (type I programmed cell death). Few studies support our finding were SNG induced autophagy in malignant glioma (Pallichankandy, Rahman, Thayyullathil, & Galadari, 2015), which was a noval pioneer finding. SNG found to increase GFP-LC3 punctate formation and it was Pallichankandy's major finding and this support our finding in the transfection experiment.

Our findings support critical role of ROS in SNG mediated PTC cell death as NAC reversed molecular and cellular alterations related to apoptosis and autophagy. As clearly stated in results section (Figures 25&26) , NAC reversed the expression of many cellular markers, as well as, colony formation of BCPAP(Xu et al., 2012) (Figure 21) and this confirms that SNG initiates its antiproliferative action on PTC through ROS-depenant manner. Several studies have shown similar finding in bladder cancer (Han et al., 2013), melanoma cells (Burgeiro, Bento, Gajate, Oliveira, & Mollinedo, 2013) and primary effucion lymphoma cells (A. R. Hussain et al., 2007).

In many types of cancer constitutive activation of JAK/STAT3 pathway was observed in many solid and hematological malignancies thus became targeted for threaputic options. Our study reveled a noval finding which is a decrease expression of phosphrelated STAT3 protien with equal total STAT3 protien and our finding is in concordance finding published in literature such as in prostate cancer cells (Meng

Sun et al., 2012), multiple myloma cell line (Akhtar et al., 2019) and ovarian cancer cell line (Achkar et al., 2019). In addition, cytokines have been linked to cancer activation (Fisher, Appenheimer, & Evans, 2014), and in our study, IL-6 was the main JAK/STAT3 pathwya activator were SNG prevent IL6 mediated-STAT3 activation.

Malignant cancer cells can migrate and metastasize to various body sites. Up-to-date factors governing cancer metastasis and their cancer spread and establishment at secondary locations is still poorly unstated (Jiang et al., 2015). Our assessment of Matrix metalloproteases markers (MMP-9 and MMP2) and scratch assay showed markedly decrease expression, supporting the decreased ability of PTC cell invasion and migration (Figure 24). Published data showed that SNG was able to decrease MMP potential of TPA-induced breast cancer cells (Park, Jin, Kim, Lee, & Park, 2014) and MDA-MB-231 human breast carcinoma cells (Choi et al., 2009)

Drug co-treatment approaches have been given attention and provided evidence for better therapeutic outcomes. Our findings revealed that SNG induces sensitivity of PTC cells to anticancer drug cisplatin via targeting apoptosis signaling proteins. Initially At 2 µM there was no significant downregulation of any protein markers in western blot, but when cisplatin was added a remarkable change in protein expression was detected (Figure 28). An enhanced Phosphorylation of H2AX, cleaved PARP, caspase activation and down regulation of survivin and STAT3 in combined treatment compared when both drugs are challenged alone. In combination with the results stated earlier, SNG provide a good co-treatment option for cancer cells. several studies have used drug combination (SNG+cisplatin), and an enhanced effect was concluded in ovarian cancer (A2780) cells (Gatti et al., 2014; Sarkhosh-Inanlou, Molaparast, Mohammadzadeh, & Shafiei-Irannejad, 2020) and in drug-resistant on–small-cell lung cancer (Gatti et al., 2014) and colorectal cancer cells (Gong et al., 2018).

Cancer stem-like cells are the small population of tumor cells well known for their important role in carcinogenesis, cancer resistance, recurrence and self-renewal (Al-Suhaimi & Al-Khater, 2019; Hardin et al., 2017; Z. Yu et al., 2012). This feature of cancer (Cancer stem-like cells) is due to many cancer reccurance , relapse and aggressiveness. SNG mediated inhibition of thyroid cancer stem-like cells (thyrospheres) and stemness marker provide a clear picture for SNG anti-cancer potential. Published data have showed that SNG induced stemness markers decreased expression in pancreatic cancer stem cell (Ma et al., 2017).

The overall Data generated in this research suggests that SNG compounds have a potential application in the prevention and treatment of cancer. Further research on animal model will help broaden the knowledge of how such plant alkaloid will behave *in vivo*.

CHAPTER 6: CONCLUSION

The present study intended to investigate the antiproliferative action of SNG on PTC cells. To our knowledge, this is first study to investigate detailed molecular mechanisms that are involved in the of anti-cancer pathways that are medicated by SNG in PTC cell line.

At first, SNG was found reduce cell viability as seen in flow results of CCK8 assay as well as RTCA. Furthermore, AnnexinV/PI staining analyzed by flow cytometry showed that SNG induce apoptosis in a dose dependent manner, with maximum effect at 4 µM as clearly seen in western blotting data. Both cell viability and RTCA assay are compatible with each, were SNG dose dependently inhibit PTC cell proliferation. Caspase inhibitor, Z-VAD-FMK, proved that caspase cascade activation is specific,

because when cells pretreated with Z-VAD-FMK , no marked caspase activation was seen in western blotting. SNG was found to phosphorylate Gamma H2AX in PTC cells and this is key marker DNA damage in order to initiate apoptosis. Additionally, SNG was found to mediate anti-cancer action through generation of reactive oxygen species (ROS). NAC (N-Acetyl-L-cysteine), was found to revers the effect of SNG on PTC morphologically, through reversing apoptosis cellular changes, as well as on molecular level, by reversing apoptosis markers in western blot analysis.

Our study findings supported the proposed hypothesis stated earlier in introduction, were SNG was found to downregulate JAK/STAT3 pathway in PTC cell lines. The pSTAT3 protein was downregulated in PTC cell lines, dose dependently, with equal housekeeping stat3 protein. Furthermore, SNG was found to inhibit IL-6 induced stat3 activation ,which support that SNG disrupt IL-6-STAT3 cycle and prevent survival of PTC cells. Scratch assay, on the other hand, showed that SNG can inhibit the migration and invasion in in vitro settings. This Functional assay show that SNG lowered cell-to-cell interaction, thus reduce chance of invasion of surrounding through downregulation of molecular markers MMP-9 and MMP-2.

Beside apoptosis, SNG was found to induce autophagy as clearly seen in the upregulation of their associated proteins (LC3) as clearly shown in the results section. Our research finding corelated with the novel finding illustrated by Pallichankandy and his team were autophagy and apoptosis work side by side to downregulated proliferation of malignant glioma cancer cells.

Addtionally, drug commbination was found to enhance antiproliferative effect in many cancer in literture. Cisplatin, a conventional chemotheraputic drug, had been used in many cancer treatments .Studied in literature showed its potiential synergistic effect in treating differentiated thyroid cancers. As a conventional therapy, cisplatin

has it own side effects ,so when combining SNG with cisplatin ,it can minimiz the side affect of conventional therapy. Initially, At concentration 2 µM, SNG showed no marked activation of apoptosis markers (caspase3, Cleaved caspcase3 ,PARP, XIAP) , but when Cisplain was added marked activation was noted. This inidicate that SNG sensetize PTC cells for killing. Furthuremore, we found that ROS mediated SNG-sensitization of PTC cells to cisplatin and clearly seen when NAC treatment reversed SNG and cisplatin-induced activation of apoptotic markers.This finding suggested that NAC treatment reversed SNG and cisplatin-induced activation of apoptotic markers, which means that ROS played an important role in the combinational apoptotic effect of SNG and cisplatin in PTC cell. CCK8 and cell viability correlated with molecular apoptotic markers in western blotting.

last but not least, as clearly stated in the introduction papillary thyroid cancers have high recurrance rate and aggresiveness. Agrowing focous in research on the importance of cancer stem cells (CSCs) in thyroid tumorigenesis, relapse and unresponsiveness. In our research, stemness markers (ALDH-2 & SOX2), were attenuated at 2-4 µM. on the other hand thyrosphere size and shape was lost at 2 µM , as clearly showen in results section. This finding is extermly essential for recurrant cancer patient , since plants alkhaloids can attenuate CSCs that causes relaps with minimal side effect.

At last, how can all these findings act in in vivo setting? This question can be answered with further research to explore how SNG act in real cancer Environment.

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