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A STUDY ON SANGUINARINE AS AN ANTILEUKEMIC AGENT-INVOLVEMENT OF REACTIVE OXYGEN SPECIES-CERAMIDE-AKT APOPTOTIC SIGNALING PATHWAY

Anees Rahman Cheratta

United Arab Emirates University

College of Medicine and Health Sciences

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Anees Rahman Cheratta

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Under the Supervision of Professor Sehamuddin Galadari

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Declaration of Original Work

I, Anees Rahman Cheratta, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Elucidating the molecular signaling mechanism of anti-leukemic potential of sanguinarine*", hereby, solemnly declare that this dissertation is my own original research work that has been done and prepared by me under the supervision of Professor Sehamuddin Galadari, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this dissertation.

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College of Medicine and Health Sciences

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Title: Associate Professor

Department of Biochemistry

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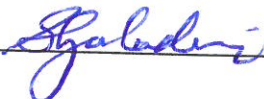University of Ulster

Approval of the Doctorate Dissertation

This Doctorate Dissertation is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Sehamuddin Galadari

   Title: Professor

   Department of Biochemistry

   College of Medicine and Heath Sciences
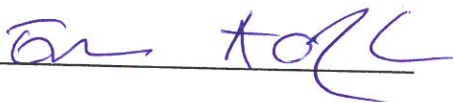
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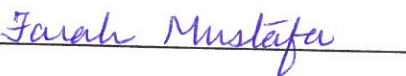
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   Title: Assistant Professor

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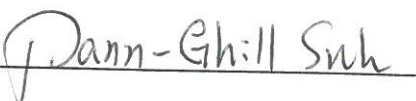
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4) Member (External Examiner):

   Title: Pann-Ghill Suh

   Department of Biological Sciences

   Institution: School of Life Sciences, Ulsan National Institute of Science and Technology (UNIST), South Korea

   Signature _Pann-Ghill Suh_   Date 3/3/16

This Doctorate Dissertation is accepted by:

Dean of the College of Medicine and Health Sciences: Professor Dennis Templeton

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Abstract

Dysregulation of apoptosis is a prime hallmark of leukemia. Therefore, drugs which restore the sensitivity of leukemic cells to apoptotic stimuli are promising candidates in the treatment of leukemia. The main objective of this dissertation was to examine the antileukemic effect of sanguinarine, *in vitro*, and to further examine the signaling mechanisms that may b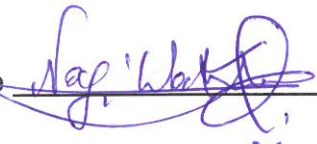e involved. This study demonstrates that in human leukemic cells, sanguinarine activates a caspase-dependent apoptotic cell death pathway that is characterized by reactive oxygen species-dependent ceramide generation, and subsequent inhibition of Akt signaling pathway. In addition, sanguinarine also induces reactive oxygen species-dependent glutathione depletion and activation of extracellular signal-regulated kinase1/2. Moreover, inhibition of reactive oxygen species generation, using reactive oxygen species scavengers and antioxidants, significantly abrogates sanguinarine-induced ceramide generation, Akt dephosphorylation, extracellular signal-regulated kinase1/2 activation, and apoptosis. Sanguinarine-induced ceramide generation is mediated via reactive oxygen species-dependent activation of acid sphingomyelinase in Jurkat cells and inhibition of acid ceramidase and glucosylceramide synthase in both Jurkat and Molt-4 cells. Furthermore, the involvement of ceramide-activated protein phosphatase-1 in sanguinarine-induced Akt dephosphorylation and apoptosis is demonstrated. Altogether, this study underscores the critical role for reactive oxygen species-ceramide-Akt signaling pathway and reactive oxygen species-dependent extracellular signal-regulated kinase1/2 activation in the antileukemic action of sanguinarine. Understanding the molecular signaling mechanism of sanguinarine-induced apoptosis undoubtedly should have a great impact on future sanguinarine-based antileukemic drug development.

Title and Abstract (in Arabic)

دراسة مادة السانجوينارين (sanguinarine) كعامل مضاد لسرطان الدم_ و إرتباطه بإشارات موت الخلايا المبرمج (apoptosis) المرتبط بجزيئات الأكسجين النشطة_السيراميد (ceramide)_الأكيتي (Akt)

الإخلال في موت الخلايا المبرمج (apoptosis) هو السمة المميزة الرئيسية لسرطان الدم. لذلك, فإن الأدوية التي تعيد استجابة خلايا سرطان الدم   لإشارات موت الخلايا المبرمج (apoptosis) تكون مؤهلة للاستخدام كعلاج لسرطان الدم (leukemia) .

الهدف الرئيسي من هذه الأطروحة هو دراسة تأثير مادة السانجوينارين (sanguinarine) كمضاد لسرطان الدم (leukemia)في المختبر, وإلى مواصلة دراسة آليات الإشارات التي يمكن أن تؤثر من خلالها هذه المادة .

هذه الدراسة تبين أن مادة السانجوينارين (sanguinarine) تؤثر على خلايا سرطان الدم البشرية, عن طريق تفعيل موت الخلايا المبرمج بالاعتماد على الكاسبيس (caspase-dependent apoptotic cell death pathway), و التي تتميز بتفعيل جزيئات الأكسجين النشطة المعتمدة على السراميد (reactive oxygen species-dependent ceramide generation), و ينتج عنه تثبيط لإشارات الأكيتي (Akt). بالإضافة إلى ذلك, فإن مادة السانجوينارين (sanguinarine) تقوم بتفعيل جزيئات الأكسجين النشطة المعتمدة على استنزاف الجلوتاثايون (reactive oxygen species-dependent glutathione depletion) و تفعيل اشارات الخلية المنظمة للكاينيز (kinase1/2).و علاوة على ذلك, فإن تثبيط توليد جزيئات الأكسجين النشطة بإستخدام السكافينجرز (scavengers) و مضادات الأكسدة, أدى بشكل ملحوظ إلى إيقاف تولد السراميد المفعل عن طريق مادة السانجوينارين (sanguinarine-induced ceramide generation), إزلة فسفرة الأكيتي (Akt dephosphorylation), تفعيل إشارات الخلية المنظمة من خلال الأكيتي (extracellular signal-regulated kinase1/2 activation), و إلى موت الخلايا المبرمج (apoptosis). إن تولد السيراميد (ceramide) المفعل عن طريق مادة السانجوينارين (Sanguinarine) يتم التحكم بها عن طريق جزيئات الأكسجين النشطة المعتمدة على تنشيط حمض السفينجومايلينيس (acid sphingomyelinase) في خلايا الجيركيت (Jurkat cells), و إلى تثبيط إنتاج حمض السيراميديس (acid ceramidase) و الجلوكوسيراميد (glucosylceramide synthase) في كل من خلايا الجيركيت (Jurkat) و مولت-4 (Molt-4).

زيادة على ذلك, فإننا نظهر علاقة إرتباط بروتين السيراميد فوسفيتيس النشط (activated protein phosphatase-1) في إزالة فسفرة الأكيتي (Akt dephosphorylation) المفعلة عن طريق مادة السانجوينارين (sanguinarine) مع موت الخلايا المبرمج (apoptosis).

بالإجمال, هذه الدراسة تؤكد الدور الحاسم للإشارات الخلوية لجزيئات الأكسجين النشطة المفعلة عن طريق السيراميد-الأكيتي (reactive oxygen species-ceramide-Akt signaling pathway) و جزيئات الأكسجين النشطة المعتمدة على إشارات الخلوية السطحية المنظمة عن طريق تنشيط الكينيز (reactive oxygen species-dependent extracellular signal-regulated kinase1/2 activation) , كعوامل مضادة لسرطان الدم.

في النهاية, إن فهم آلية الإشارات الجزيئية لموت الخلايا المبرمج بفعل مادة السانجوينارين (Sanguinarine),بلا شك سيكون له تأثير أكبر على تطوير أدوية سرطان الدم القائم على هذه المادة في المستقبل .

x

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In retrospect, I realize that this work would not have reached its conclusion without contributions from many people during hour of need because of their sheer goodness of heart. Although, I can never repay anyone, I would like to pen down few words of gratitude.

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I like to express my profound regards and deep sense of gratitude to UAE University for offering me this program and providing all the facilities required. I am grateful for

This is for you, mom.
Thank you is a very small word.
I owe it all to you

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List of Abbreviations

ABC	ATP binding cassette
AIF	Apoptosis inducing factor
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
ANT	Adenine nucleotide translocase
Apaf-1	Apoptotic protease activating factor 1
ASK1	Apoptosis signal-regulating kinase 1
Bad	Bcl-2-associated death promoter
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra-large
Bid	BH3 interacting-domain death agonist
Bim	Bcl-2-like protein 11
CAD	Caspase activated DNase
CalA	Calyculin A
CAPP	Ceramide activated protein phosphatases
CDase	Ceramidase
CDKI	Cyclin-dependent kinase inhibitors
c-FLIP	Cellular FLICE-inhibitory protein
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
CNS	Central nervous system
CREB	cAMP response element-binding protein
Cyt-c	Cytochrome-c

DAPK	Death-associated protein kinase
DCF	2', 7'- Dichlorofluorescein
DCFH	2', 7'-Dichlorodihydrofluorescin
Desip	Desipramine hydrochloride
DHSNG	Dihydrosanguinarine
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
DUSP	Dual specificity phosphatases
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
eIF4EBP	eukaryotic initiation factor 4E binding protein
eIF2B	Eukaryotic initiation factor 2B
EndoG	Endonuclease G
ER	Endoplasmic reticulum
Erg-1	Early growth response gene-1
ERK1/2	Extracellular signal-regulated kinase 1/2
ERM	Ezrin, radixin, and moesin
ETC	Electron transport chain
FAK	Focal adhesion kinase
FasL	Fas ligand
FasR	Fas receptor
FB1	Fumonisin B1
FBS	Fetal bovine serum

FLT3	Fms-like tyrosine kinase 3
FOXO	Forkhead box O
GCS	Glucosylceramide synthase
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grb2	Growth factor receptor-bound protein 2
GSH	Glutathione
GSK3β	Glycogen synthase kinase 3 beta
GSL	Glycosphingolipids
GSSG	Glutathione disulfide
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
HAAD	Heath Authority of Abu Dhabi
HBSS	Hanks' balanced salt solution
HRP	Horseradish peroxidase
I2PP2A	Inhibitor 2 of PP2A
IAP	Inhibitors of apoptosis protein
ICAD	Inhibitor of caspase-activated DNase
IKK	I-kappa B Kinase
JNK	c-Jun N-terminal kinases
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia 1
MDM2	Mouse double minute 2
MitoPY1	Mitochondria peroxy yellow 1
MMP	Mitochondrial membrane permeability

MMP	Matrix metalloprotease
mTOR	Mammalian target of rapamycin
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
Myr	Myriocin
NAC	N-Acetyl-L-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NBD	7-nitro-2-1,3-benzooxadiazolyl amino-dodecanoic acid
NCI	National Cancer Institute
NF-κB	Nuclear factor-kappa B
NOE	N-Oleoyl-ethanolamine
NOX	NADPH oxidase
O_2	Molecular oxygen
$O_2{}^{\bullet-}$	Superoxide anion
OA	Okadaic acid
$^{\bullet}$OH	Hydroxyl radical
OPA	o-phthaladialdehyde
p70S6K	p70 ribosomal S6 kinase
PA	Phosphatidic acid
PAK2	P21 protein (Cdc42/Rac)-activated kinase 2
Par-4	Prostate apoptosis response-4
PARP	Poly (ADP-ribose) polymerase-1
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide dependent protein kinase-1
P-gp	P-glycoprotein

PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PMSF	Phenylmethylsufonyl fluoride
pNA	p-nitroaniline
PP1	Protein phosphatase-1
PP2A	Protein phosphatase-2A
Prx	Peroxiredoxin
PTP	Permeability transition pore
PTPase	Phosphotyrosine-specific phosphatase
PUMA	p53 up-regulated modulator of apoptosis
Rb	Retinoblastoma
ROCK I	Rho-associated coiled-coil-containing protein kinase 1
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
SCT	Stem cell transplantation
SDS	Sodium dodecyl sulfate
SEER	Surveillance, epidemiology and end result
SGOT	Serum glutamic-oxaloacetic transaminase
SGPT	Serum glutamic-pyruvic transaminase
SH2	src homology 2
SH3	src homology 3

Smac/DIABLO	Second mitochondria-derived activator of caspases/direct IAP binding protein with low PI
SMase	Sphingomyelinase
SNG	Sanguinarine
Sod Pyr	Sodium pyruvate
SPT	Serine palmitoyl transferase
Stat3	Signal transducer and activator of transcription 3
TNB	2-nitro-5-thiobenzoic acid
TNFR	TNF receptor
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
TRX	Thioredoxin
TrxR	Thioredoxin reductase
UDP-glucose	uridine 5′-diphosphoglucose
UV	Ultraviolet
VDAC	Voltage-dependent anion-selective channel
VEGF	Vascular endothelial growth factor
Z-VAD-FMK	Z-Val-Ala-Asp-fluoromethylketone

Chapter 1: Introduction

1.1 Overview

According to the National Cancer Institute (NCI), surveillance, epidemiology and end result (SEER) program report, it is estimated that during 2015, approximately 1.6 million new cancer cases will be diagnosed, and more than 580 thousand people will die from cancer in the United States (Howlader et al., 2015). The cancer burden and related deaths is predicted to increase alarmingly by 2020, with approximately 15 million new cases and 12 million deaths globally. This is further expected to witness a catastrophic increase to 21 million new cancer cases and 13 million deaths by 2030 (Kanavos, 2006; "Cancer Facts and Figures 2015," 2015). The burden will soon become a crisis as cancer incidence and death is rapidly and dramatically expanding in countries with less developed economies, where in the past, cancer related burden was significantly less (Kanavos, 2006). Clearly, cancer is an enormous global health burden, and should be effectively addressed with better therapeutic strategies. Currently, the most commonly used anticancer treatment involves chemotherapy, radiotherapy, and surgical resection. In this context, compounds derived from plants (phytochemicals) hold great potential for the development of next generation anticancer agents.

Phytochemicals are non-nutritive components found in plants. They have long been used in the treatment of various ailments such as diabetes mellitus, cardiovascular disease, hypertension, and cancer. Earlier large-scale phytochemical-based clinical trials failed to demonstrate any positive clinical significance, and raised doubts over their use. Notably, lack of mechanism-based study design was considered as the major reason for the failure (K. W. Lee, Bode, & Dong, 2011). As a result, only very few phytochemical-based anticancer agents, such as vinblastine and vincristine, were available for the treatment of cancer (Bailly, 2009).

During the 1980s, thanks to the advent of faster and better technologies, and the availability of large pool of mechanism-based preliminary studies, various plant-based anticancer agents such as etoposide, vinorelbine, topotecan, teniposide, paclitaxel, and docetaxel were approved for the treatment of a number of different malignancies (Bailly, 2009). However, during the late 1990s, with the discovery of receptor specific anticancer agents, such as imatinib and rituximab, the cancer research focus shifted from natural products to synthetic small molecules. As a result, no phytochemical-based anticancer agent was approved during the years 1997-2006 (Bailly, 2009). However, cancer being a heterogeneous disease driven by multiple signaling pathways, receptor specific anticancer agents have not been successful in a number of different cancers (Bailly, 2009). Consequently, interest in phytochemicals has renewed among cancer biologists and the pharmaceutical industry all over the world. Currently, over 60% of the clinically approved anticancer agents are either directly isolated from natural sources, or are modified from natural lead molecules (da Rocha, Lopes, & Schwartsmann, 2001). In addition, a large number of promising candidates are presently in clinical trial or in the development pipeline. Therefore, natural products are moving to be one of the most successful strategy for the discovery of new medicines.

Considering their importance, NCI has funded and initiated a major screening program for antitumor activity from natural resources. By conducting a random screening, NCI has determined that more than 1000 different phytochemicals possess potent anticancer activities. Some of the most successful anticancer agents currently being used have been identified as a result of this screening program (K. W. Lee et al., 2011; Surh, 2003). Of the 250,000 plant species available, less than 10% have been tested for biological activities, and among them only a few have gone through extensive high-throughput screening process (Harvey, 2000). This indicates that there is a large untapped source of

phytochemical-based anticancer agents remaining to be discovered. With improvement in the techniques required for natural product isolation and biological testing, and better opportunity to explore previously inaccessible sources of natural products, it is crucial to carry out more rigorous research to identify promising anticancer candidates from natural sources.

Various advantages can be attributed to the study of phytochemicals in cancer therapy: (1) cancer is a heterogeneous disease characterized by extremely complex network of signaling interactions. Therefore, use of a single receptor specific anticancer agent based on lock-and-key model might not be successful in most cases. Phytochemicals usually have complex structures; therefore, they can act on a number of different targets with relatively low specificity. For example, curcumin was shown to induce apoptosis in human non-small cell lung cancer via modulation of multiple signaling pathways, leading to the induction of intrinsic-, extrinsic- and endoplasmic reticulum (ER) stress-mediated apoptosis (S. H. Wu et al., 2010). (2) Phytochemicals can be used as an additive or a synergistic agent together with conventional chemotherapeutic agents (combinatorial therapy), so as to reduce the dose and, therefore, increase the sensitivity and lower the potential for adverse drug reactions. For example, curcumin has been shown to inhibit multidrug resistant protein known as P-glycoprotein (P-gp), thereby promoting the sensitivity of vinblastine in multidrug resistant human cervical carcinoma cell line (Anuchapreeda, Leechanachai, Smith, Ambudkar, & Limtrakul, 2002). (3) Phytochemicals provide the best source for lead moieties, which can be subjected to structural modifications for the development of improved anticancer agents. For example, vinflunine, a modified form of vinblastine, is more efficacious than vinblastine (Kruczynski et al., 2002).

In summary, the immense potential of phytochemicals can serve as an excellent source for the development of novel antitumor agents. Therefore, it is

worthwhile to systematically investigate the anticancer potential of various phytochemicals. Such investigations can provide a scientific basis for designing targeted animal and human studies aimed at developing safe and efficacious anticancer therapy.

1.2 Problem statement

Leukemia is the most common type of childhood cancer in most parts of the world (Gilliland & Tallman, 2002; Harned & Gaynon, 2008; Vandenbriele, Dierickx, Amant, & Delforge, 2010). The estimated leukemia-related death in 2008 was around 250,000 globally ("Global Cancer Facts and Figures," 2011). The overall incidence of leukemia in the developed countries has been increasing since the last few decades ("Global Cancer Facts and Figures," 2011; Shah & Coleman, 2007). According to Heath Authority of Abu Dhabi (HAAD), leukemia is the most common type of cancer among males and the second most common type of cancer among females in Abu Dhabi ("Cancer in Abu Dhabi," 2011). Majority of children and 80% of children with cancer live in the developing countries (Pisani & Hery, 2006). Moreover, mortality trends of childhood cancers in developing countries are mostly underreported (Howard et al., 2008). Acute lymphoblastic leukemia (ALL) is the most common type of leukemia identified in children, accounting for 80% of the total leukemic cases (Morais et al., 2014). Most of the currently available chemotherapy regimens for ALL, or leukemia in general, are very intensive and can lead to severe systemic toxicities (Grigoropoulos, Petter, Van't Veer, Scott, & Follows, 2013). This signifies the importance of identifying novel nature-based therapeutic agents that can target and treat leukemia with least toxicity profile. Sanguinarine (SNG) is a benzophenanthridine alkaloid that has been shown to inhibit proliferation, and induce programmed cell death in various human cancer cell lines (Choi, Kim, Lee, & Choi, 2008; Dong et al., 2013; S. Gu et al., 2015; Hammerová, Uldrijan, Táborská, & Slaninová, 2011; Jang et al., 2009; Kalogris et al., 2014; S. Kim et al., 2008; Rosen

et al., 2015). It has been reported that SNG has a more profound cytotoxic effects against tumor cells when compared to normal cells (Ahmad, Gupta, Husain, Heiskanen, & Mukhtar, 2000; Kaminskyy, Lin, Filyak, & Stoika, 2008; Sun et al., 2010). This preferential selectivity of SNG makes it a 'druggable' moiety in the treatment of cancer. This research was focused on elucidating the molecular signaling mechanism of anti-leukemic potential of SNG.

1.3 Objective

The ultimate goal of this research was identification of the role and mechanism of SNG-induced inhibition of proliferation of human leukemic cells. Such an understanding should provide a basis for designing novel therapeutic approaches to combat leukemia. The overall objectives of this study can be broadly broken down within the following five specific aims:

1. To determine the intracellular mechanisms by which SNG inhibits proliferation and induces death in human leukemic cells.

2. To investigate the role of reactive oxygen species (ROS) generation in SNG-induced death in human leukemic cells.

3. To elucidate the role of the tumor suppressor lipid, ceramide, in SNG-induced cell death.

4. To examine the role of Akt, also known as protein kinase B (PKB), in SNG-induced leukemic cell death.

5. To delineate the involvement of mitogen activated protein kinases (MAPKs) in SNG-induced cell death.

Chapter 2: Review of literature

2.1 Leukemia

2.1.1 Overview of leukemia

Leukemia constitutes a group of cancers that arise from malignant transformation of blood and blood forming organs (Grigoropoulos et al., 2013). According to recent SEER statistics, approximately 1.5% of United States men and women will be diagnosed with leukemia at some point during their lifetime (Howlader et al., 2015). In 2012, there were approximately 310,000 reported cases of leukemia in the United States (Howlader et al., 2015). Over the past 10 years, there has been a gradual, but significant, increase in the incidence of leukemia in the United States, with an estimated rate of approximately 54,000 new cases and 24,000 deaths in 2015 (Howlader et al., 2015). Moreover, according to HAAD surveillance section report, leukemia is the first and second most common type of cancer among Abu Dhabi male and female population, respectively ("Cancer in Abu Dhabi," 2011).

Leukemia can be classified in several ways. Based on the type of white blood cells involved, leukemia may be myelogenous or lymphocytic. Lymphocytic leukemia refers to cancer of the marrow cells which normally produce immune system cells, whereas, myelogenous leukemia is cancer of granulocytes or monocytes. Leukemia is also classified as acute and chronic on the basis of cell maturity. In acute leukemia, cells are immature blast cells, whereas, in chronic leukemia cells are more mature and functionally differentiated. Therefore, generally leukemia is classified as (1) acute lymphocytic leukemia (ALL), (2) chronic lymphocytic leukemia (CLL), (3) acute myelogenous leukemia (AML), and (4) chronic myelogenous leukemia (CML).

Acute lymphoblastic leukemia is a malignant proliferation of lymphoid precursor cells in the bone marrow, blood, and extramedullary sites. It is the most

common type of leukemia identified in children and it accounts for 80% of the total leukemic cases (Morais et al., 2014). Additionally, ALL also accounts for a significant number of adult leukemia cases as well. Basically, a bimodal age distribution of ALL can be seen, with the first peak between 4-10 years, and a second peak at about 50 years (Gilliland & Tallman, 2002; Shah & Coleman, 2007). According to recent SEER report, there were an estimated 75,000 ALL cases in the United States in 2012 (Howlader et al., 2015). Over the past 10 years, the rate of ALL have been gradually rising on an average of 0.6% each year (Howlader et al., 2015). The incidence of leukemia in general is steadily increasing in other developed countries as well ("Global Cancer Facts and Figures," 2011; Shah & Coleman, 2007). Majority of children and 80% of children with cancer live in the developing countries. However, there are no reliable population level data available concerninghe incidence and survival of leukemia patients (Howard et al., 2008; Pisani & Hery, 2006).

2.1.2 Standard treatment of leukemia

Advances in cytogenetic and molecular analyses have significantly improved our understanding of the molecular basis of leukemia. As a result, different therapeutically important subtypes of ALL have been identified, and treatment is more directed towards a genetically different subset of leukemia (Bhojwani, Howard, & Pui, 2009). In addition, results from a number of clinical trials have led to the development of chemotherapeutic regimens composed of multidrug combinations, and intensification of their dose and/or treatment duration (Carroll & Raetz, 2012). Altogether, these factors contributed to considerable improvement in the treatment of ALL, or leukemia in general. Depending on the cytogenetic characteristics, risk of relapse, and age of patients, a number of different treatment protocols have been employed for the management of ALL. Most of the treatment protocols are

traditionally divided into three different phases, namely remission induction, consolidation, and maintenance (Carroll & Raetz, 2012).

Remission induction therapy is usually carried out for a period of 4-6 weeks to enable rapid reduction in vast amount of leukemic cells. The mainstay of induction regimen usually include vincristine, a corticosteroid (prednisone or dexamethasone), L-asparginase, and an anthracycline (Inaba, Greaves, & Mullighan, 2013; Pui, 2010). Patients at high- or very high-risk, in addition may receive cyclophosphamide (Inaba et al., 2013; Pui, 2010). If the biological and clinical features of leukemia are more lucid, additional drugs can be considered for the remission induction of different subsets of ALL. For example, patient with Philadelphia chromosome-positive ALL may benefit from addition of tyrosine kinase inhibitor imatinib to conventional chemotherapeutic regimen (Schultz et al., 2009). Similarly, infants with mixed lineage leukemia rearranged ALL, having increased expression of Fms-like tyrosine kinase 3 (FLT3), may require inclusion of FLT3 inhibitor lestaurtinib (P. Brown, Levis, McIntyre, Griesemer, & Small, 2006). Consolidation is usually carried out for a period of 20-30 weeks with an aim to eradicate any residual leukemic cells. Antimetabolites such as 6-mercaptopurine and methotrexate are the cornerstones of consolidation therapy (Inaba et al., 2013; Pui, 2010). Other commonly used drugs are vincristine and L-asparginase (high-dose). Maintenance therapy typically lasts 2-3 years and comprises of daily dosage of 6-mercaptopurine, weekly methotrexate, and with or without monthly pulses of vincristine and prednisolone. Patients with Philadelphia chromosome-positive ALL may require addition of imatinib to this regimen (Inaba et al., 2013; Pui, 2010).

Control of central nervous system (CNS) relapse remains as the major therapeutic challenge in the successful treatment of leukemia. Standard prophylaxis for CNS relapse involves triple-drug intrathecal chemotherapy with methotrexate, hydrocortisone, and high-dose of cytarabine (Inaba et al., 2013; Pui, 2010). Some of

the leukemic patients are also treated with allogenic stem cell transplantation (SCT). Treatment with SCT along with chemotherapy was demonstrated to be more beneficial than treatment with chemotherapy alone (Aricò et al., 2000).

2.1.3 Complications associated with standard chemotherapy

Although, leukemia therapy has witnessed some improvement at least in some subgroups of ALL, however, treatment results in significant amount of short- and long-term undesired consequences. For examples, glucocorticoids are associated with short term complications such as myopathy, behavioral problem, hyperglycemia and long-term complications such as osteonecrosis. Anthracyclines can cause severe cardiotoxicity. It is demonstrated that children treated with anthracyclines are at higher risk of developing late-onset cardiac arrest (Carroll & Raetz, 2012; D. S. Ziegler, Dalla Pozza, Waters, & Marshall, 2005). Methotrexate is responsible for renal and neurotoxicity, whilst, high-dose of cytarabine is associated with hepatotoxicity and cerebellar dysfunction (Fullmer, O'Brien, Kantarjian, & Jabbour, 2009). Hypersensitivity to L-asparginase is also a common concern (Asselin & Fisher, 2014). Additionally, SCT is associated with high rate of acute transplant related mortality. Therefore, SCT is limited to high-risk patients or patients with very higher prospect of relapse.

2.1.4 Need for further improvement in antileukemic therapy

Significant improvement in treatment of childhood ALL, or leukemia in general, has been achieved during the past few decades. However, infants less than 1 year of age at diagnosis and adults are the exception to this improvement (Kotecha, Gottardo, Kees, & Cole, 2014; Narayanan & Shami, 2012). Moreover, about 20% of children treated with current chemotherapeutic regimes still relapse (D. S. Ziegler et al., 2005). Additionally, some survivors face an uncertain future of treatment related short- and long-term morbidity. To address these challenges, the

current therapeutic regimens are substantially modified to improve survival. However, despite these modifications, no definite advancement in survival was observed in most of these leukemia over the past two decades. Moreover, we are approaching the limit to which currently available chemotherapy can be further intensified with limited toxicity (Kotecha et al., 2014). Currently, more than 100 clinical trials are underway focused on curing relapsed or refractory leukemia, as well as, the reduction of short- and long-term morbidity associated with treatment ("NCI supported clinical trials," 2015). Therefore, there is a compelling rationale to discover and incorporate novel and promising drug candidates for the treatment of ALL and leukemia in general. To achieve this goal, there is a need for increased focus on better understanding the molecular signaling mechanisms of the novel antileukemic agents. Recently, phytochemicals have taken center stage as compounds with potential anticancer properties. Delineating the molecular mechanism of action and their anticancer potential will undoubtedly lead to novel antileukemic agents with improved outcomes in resistant and relapsed ALL patients.

2.2 Apoptosis

2.2.1 Apoptosis as a tumor suppressive mechanism

Apoptosis, also known as type I programmed cell death, plays an important role in the maintenance of tissue homeostasis by eliminating harmful or unwanted cells. Defective apoptosis (increased or decreased) is implicated in various diseases such as diabetes mellitus, Alzheimer's disease, and cancer. Evading apoptosis is one of the major hallmarks of cancer progression and resistance to chemotherapy. This has provided the necessary encouragement for exploiting a variety of therapeutic approaches that restores apoptosis either via inhibiting anti-apoptotic cellular machineries or reactivating pro-apoptotic components.

2.2.2 Morphological and biochemical features of apoptosis

The process of apoptosis is characterized by a series of morphological changes and biochemical events. Some of the important morphological hallmarks of apoptosis are cellular shrinkage and detachment, chromatin condensation, membrane blebbing, and formation of small compact fragments known as apoptotic bodies (Fischer, Jänicke, & Schulze-Osthoff, 2003). Most of these morphological changes are mediated via proteolytic modification of a number of key proteins involved in cytoskeletal regulation and cellular organization. Proteolysis may be mediated by a unique family of cysteine-dependent aspartate-directed proteases known as caspases. However, caspase–independent mechanisms have also been implicated in this. For instance, caspase-mediated cleavage of P21 protein (Cdc42/Rac)-activated kinase 2 (PAK2), gelsolin, and rho-associated coiled-coil-containing protein kinase I (ROCK I) are involved in the apoptotic membrane blebbing. Alternatively, membrane blebbing can be triggered by death-associated protein kinase (DAPK) via caspase-independent mechanism (Fischer et al., 2003; U. Ziegler & Groscurth, 2004). Moreover, caspase-dependent cleavage of acinus contributes to chromatin condensation, while cleavage of focal adhesion kinase (FAK) and paxillin induces cellular shrinkage and detachment (Fischer et al., 2003; U. Ziegler & Groscurth, 2004).

The most important biochemical feature of apoptosis is activation of caspase signaling cascade itself. Most of the biochemical features of apoptosis are facilitated as a result of caspase activation. For example, caspase-3 and -7 mediate the cleavage of DNA repair protein Poly (ADP-ribose) polymerase-1 (PARP), leading to its inactivation (Fischer et al., 2003; Saraste & Pulkki, 2000). Cleavage of PARP is a hallmark of caspase-dependent apoptosis in most cases. Another important protein cleaved by caspases is inhibitor of caspase-activated DNase (ICAD). Caspase-mediated cleavage of ICAD releases caspase activated DNase (CAD) from the hold

of ICAD, and induces DNA fragmentation. This DNA fragmentation can also be mediated by apoptosis inducing factor (AIF) and endonuclease G (EndoG) via caspase-independent mechanism (Fischer et al., 2003; Saraste & Pulkki, 2000). Another key biochemical feature of cells undergoing apoptosis is phosphatidylserine flipping from the inner leaflet to the outer leaflet of plasma membrane. Both caspase-dependent and -independent mechanisms are responsible for this process (Fischer et al., 2003; Saraste & Pulkki, 2000).

2.2.3 Mechanisms of apoptosis

Apoptosis may be manifested via extrinsic death receptor mechanism and/or intrinsic mitochondrial mechanism (Fig. 2.1) (Galadari, Rahman, Pallichankandy, Galadari, & Thayyullathil, 2013; Galadari, Rahman, Pallichankandy, & Thayyullathil, 2015).
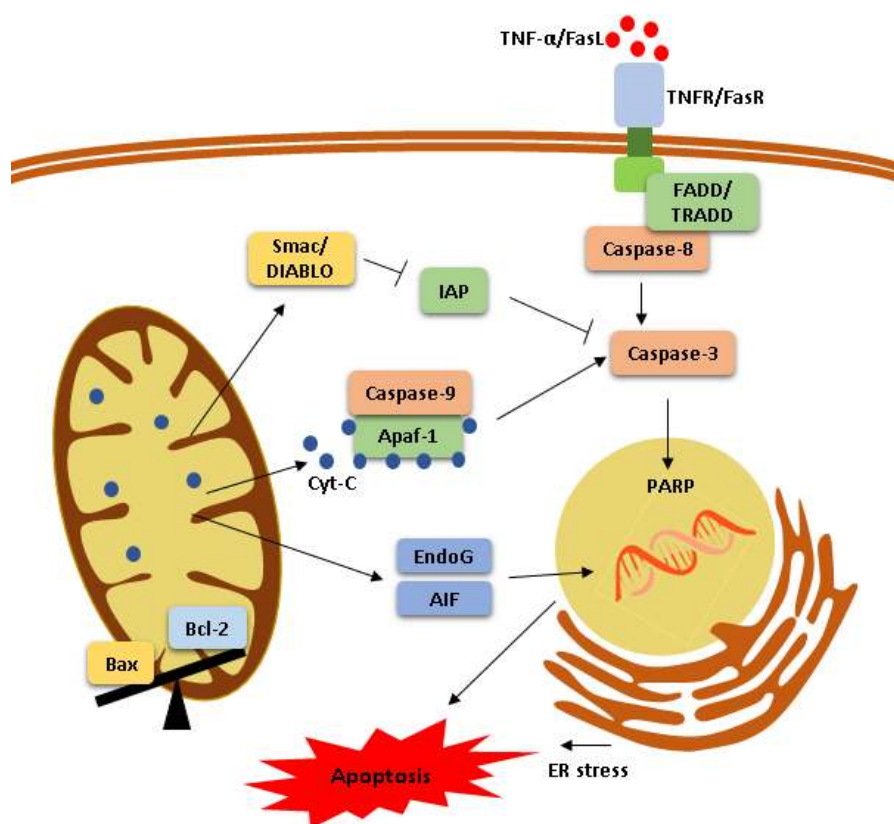
Figure 2.1. Mechanism of apoptosis. Major regulators involved in intrinsic and extrinsic pathway of apoptosis is illustrated.

The extrinsic pathway begins outside the cell with the binding of death ligands, such as tumor necrosis factor-α (TNF-α) and Fas ligand (FasL), to their respective cell-surface death receptors such as TNF receptor (TNFR) and Fas receptor (FasR). The ligand-receptor interaction recruits adaptor protein (FADD for the FasR and TRADD for the TNFR) and procaspase dimer (mostly procaspase-8), leading to death-inducing signaling complex (DISC) formation and subsequent activation of caspase cascade and apoptosis (Fig. 2.1) (Galadari et al., 2013; Galadari et al., 2015).

The intrinsic mitochondrial pathway is initiated within the cell in response to wide range of death stimuli. Regardless of the stimuli, mitochondrial membrane permeability is increased, and pro-apoptotic factors such as cytochrome-c (Cyt-c), AIF, EndoG, and second mitochondria-derived activator of caspases/direct IAP binding protein with low PI (Smac/DIABLO), etc., are released from the mitochondria through the permeability transition pore (PTP). Cyt-c complexes with the apoptotic protease activating factor 1 (Apaf-1), procaspase oligomers (mostly procaspase-9) and ATP, thereby, forming an entity called apoptosome (Fig. 2.1). In turn, the apoptosome once formed leads to the activation of caspase signaling cascade and induction of apoptosis (Galadari et al., 2013; Galadari et al., 2015). Alternatively, AIF and EndoG may translocate to the nucleus where they activate chromatin condensation and large scale DNA fragmentation in a caspase-independent manner. Smac/DIABLO neutralizes the anti-apoptotic functions of inhibitors of apoptosis proteins (IAP), hence, allowing the caspase activation and execution of apoptosis (Fig. 2.1) (Galadari et al., 2013; Galadari et al., 2015; Martinez-Ruiz, Maldonado, Ceballos-Cancino, Grajeda, & Melendez-Zajgla, 2008).

2.2.4 Major regulators of apoptosis

Caspases: These are unique family of proteases that initiate and execute the process of apoptosis. Caspases are generally divided into two classes: the initiator

caspases (caspase-2, -8, -9, -10, and -11) and the effector caspases (caspases-3, -6, and -7). The two major mechanism of caspase activation are recruitment activation and transactivation. In recruitment activation, initiator procaspases such as procaspase-8 and procaspase-9 are recruited to the DISC and apoptosome complexes, respectively. The dimerization or oligomerization of the procaspases mediates proximity-induced autocatalytic cleavage and activation of caspases. In the transactivation mechanism, the effector procaspases, such as procaspase-3 and -7, are cleaved and activated by initiator caspases or other proteases. Following its activation, caspases propagate apoptotic signal via proteolysis of targets involved in the regulation of cell morphology and survival (Galadari et al., 2013; Galadari et al., 2015).

B-cell lymphoma-2 proteins: The intrinsic pathway of apoptosis is regulated by a group of proteins belonging to the B-cell lymphoma-2 (Bcl-2) family. There are two main groups of Bcl-2 proteins, namely the pro-apoptotic proteins (e.g. Bad, Bid, Bax, Bak, Bcl-Xs, Bim, etc.) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-xL, Mcl-1, etc.). The anti-apoptotic proteins reduce apoptosis by blocking the mitochondrial release of Cyt-c, while the pro-apoptotic proteins act by promoting it, and the fate of the cell is determined by the tilt in their ratio towards one or the other (Czabotar, Lessene, Strasser, & Adams, 2014; Youle & Strasser, 2008). Importance of Bcl-2 family proteins in apoptosis regulation is further justified by the number of anticancer lead molecules that target the Bcl-2 family of proteins which are currently under clinical trial (Wong, 2011).

Inhibitors of apoptosis proteins: These are a group of functionally and structurally related proteins that serve as endogenous inhibitors of apoptosis by inhibiting the activity of both initiator and effector caspases. In addition, IAPs are also implicated in cell survival and proliferation (Fulda & Vucic, 2012; Gyrd-Hansen & Meier, 2010). In humans, eight IAP members have been identified, of which cIAP-1, cIAP-2, XIAP,

and survivin are well known. The expression and/or function of IAP proteins are significantly altered in a number of human cancers, and are associated with tumor progression, poor prognosis, and resistance to chemotherapy (Fulda & Vucic, 2012; Gyrd-Hansen & Meier, 2010). For example, increased activity of IAPs are implicated in cancers of lung (Hofmann, Simm, Hammer, Silber, & Bartling, 2002), cervix (Imoto et al., 2002), kidney (Ramp et al., 2004), prostate (Krajewska et al., 2003), breast (Y. Zhang et al., 2011), colon (Xiang, Wen, Wang, Chen, & Liu, 2009), thyroid (L. Q. Gu et al., 2009), pancreas (Vogler et al., 2009), and leukemia (Fulda & Vucic, 2012; Hundsdoerfer, Dietrich, Schmelz, Eckert, & Henze, 2010). IAPs directly inhibit apoptosis and promote resistance to chemotherapy by several mechanisms: (1) XIAP directly antagonizes the apoptotic functions of caspase-3, -7, and -9, (2) XIAP, cIAP-1 and cIAP-2 induce ubiquitination of caspase-3 and -7, leading to their degradation and non-degradative inactivation, (3) cIAP-1, cIAP-2 and XIAP induce nuclear factor-kappa B (NFκB) activation, thereby, promoting tumor cell survival and chemoresistance, (4) survivin complexes with XIAP and protects it from ubiquitination and degradation, and finally (5) survivin inhibits the release of Smac/DIABLO from mitochondria (Ceballos-Cancino, Espinosa, Maldonado, & Melendez-Zajgla, 2007; Dohi, Xia, & Altieri, 2007; Gyrd-Hansen & Meier, 2010). Given their prime importance in tumor progression and chemoresistance, several IAP-based anticancer therapeutic strategies have been exploited via targeting the inhibition of IAP expression and function, amongst them, Smac-derived peptides, Smac-mimetics, IAP antagonists and antisense oligonucleotides have gained most of the attention (Fulda & Vucic, 2012). Owing to their important role in cancer survival, a large number of potential IAP-based anticancer agents are currently undergoing clinical trial (Owens, Gilmore, Streuli, & Foster, 2013).

2.3 Sanguinarine

2.3.1 Overview of sanguinarine

Sanguinarine (13-methyl-[1,3]benzodioxolo[5,6-c]- 1,3-dioxolo[4,5-i]phenanthridinium) ($C_{20}H_{14}NO_4$) (Fig. 2.2) is a quaternary benzophenanthridine alkaloid encountered in many plant species of *Papaveraceae* family.
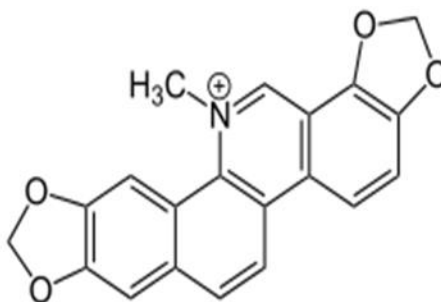
Figure 2.2. Molecular structure of sanguinarine

Sanguinarine is mainly extracted from the roots of bloodroot plant (*Sanguinaria canadensis*) (Deroussent, Ré, Hoellinger, & Cresteil, 2010), seeds of Mexican prickly poppy (*Argemone Mexicana*) (Deroussent et al., 2010), roots and aerial parts of greater celandine (*Chelidonium majus*) (Colombo & Bosisio, 1996; Meng et al., 2009), and fruits and leaves of plume poppy (*Macleaya cordata*) (E. J. Lee, Hagel, & Facchini, 2013; Yao et al., 2010) (Fig. 2.3). It is also found in eastern horned poppies (*Dicranostigma lactucoides*), opium poppy (*Papaver somniferum*), and Kelway's Coral Plume (*Macleaya microcarpa*).
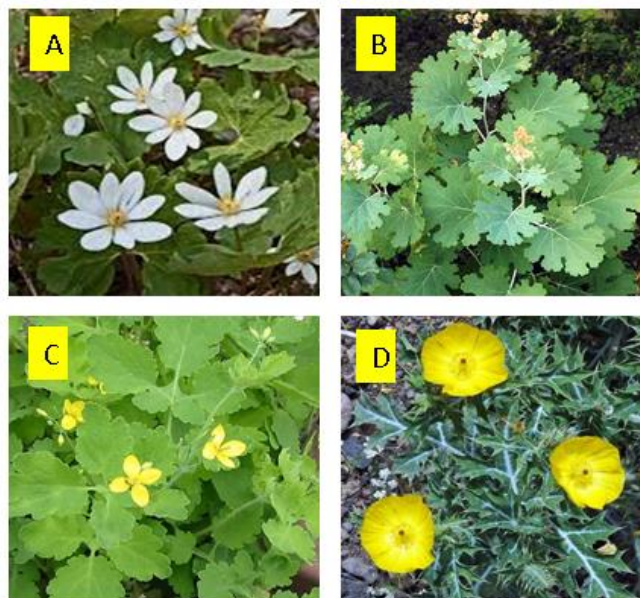
Figure 2.3. Biological sources of sanguinarine. (A) *Sanguinaria Canadensis*, (B) *Macleaya cordata*, (C) *Chelidonium majus*, and (D) *Argemone Mexicana*.

Sanguinarine and extracts of SNG-containing plants have long been used in tooth pastes and other oral hygiene products due to its anti-plaque and anti-inflammatory properties (Hannah, Johnson, & Kuftinec, 1989; Kuftinec, Mueller-Joseph, & Kopczyk, 1990). Furthermore, Sanguiritrin (a mixture of SNG and a structurally similar alkaloid, chelerythrine) has been used as a veterinary preparation for the treatment of mastoiditis in cows (Postova et al., 2006). It is also used in animal husbandry as an additive to animal feeds (Sangrovit) (Zdarilova et al., 2008). A wide range of pharmacological activities have been documented for SNG, including antihypertensive (Mackraj, Govender, & Gathiram, 2008; R. Singh, Mackraj, Naidoo, & Gathiram, 2006), antimicrobial (Hamoud, Reichling, & Wink, 2014; Obiang-Obounou et al., 2011), and anti-inflammatory (Lenfeld et al., 1981; Niu, Fan, Li, Xing, & Huang, 2012) activities. SNG has received significant attention over the past few decades due to its anticancer properties (Choi et al., 2008; Dong et al., 2013; S. Gu et al., 2015; Hammerová et al., 2011; Jang et al., 2009; Kalogris et al., 2014; S. Kim et al., 2008; Rosen et al., 2015).

2.3.2 Pharmacokinetics and toxicological parameters of sanguinarine

Only limited information is available on the pharmacokinetics and toxicological parameters of SNG. Gastrointestinal absorption of SNG is poor, which may be due to its quaternary nitrogen atom (Becci, Schwartz, Barnes, & Southard, 1987; Hong, Moon, & Shim, 2006). In an *in vivo* experiment conducted in rats, it was reported that only 2% of sanguiritrin was absorbed from the gastric tract following its daily oral administration (10 mg/kg animal body weight) for 109 days. The remaining alkaloid was unabsorbed and excreted in the feces (Postova et al., 2006). Following absorption, SNG was found to be distributed in plasma, liver, and kidney of pigs and rats (Kosina et al., 2004; Postova et al., 2006). Psotova et al. and Vecera et al. identified that dihydrosanguinarine (DHSNG), a less toxic benzophenanthridine, as the principal metabolite of SNG (Psotová et al., 2006; Vecera et al., 2007).

Kosina et al. also conducted an *in vivo* safety assessment of sanguiritrin (5 mg/kg body weight) in pigs following its oral administration for 90 days. In this study, there were no signs of toxicity and impairment in the health status of the animals. Histological examination of gastrointestinal tract, cardiac myocardium, renal cortex, and hepatic parenchyma did not reveal any pathological changes (Kosina et al., 2004). Analogous results were also observed by Psotova et al., after oral administration of a higher dose of sanguiritrin (10 mg/kg body weight) in rats for 109 days (Postova et al., 2006). Studies conducted using Sangrovit also demonstrated the safety of SNG. Indeed, administration of Sangrovit for 90 days at doses higher than the daily recommended dosage were well tolerated in rats and they were free from systemic toxicity (Stiborova et al., 2008; Zdarilova et al., 2008). In addition, studies with oral DHSNG (58 mg/kg body weight) for 90 days also showed no evidence of systemic toxicity (Vrublova et al., 2008). Contrary to the above, toxic effect of SNG have been reported in a few studies. Das et al. demonstrated that intraperitoneal administration of SNG (10 and 15 mg/kg body weight) induced a

significant number of chromosome aberrations and sister chromatid exchanges in bone marrow cells (Das, Mukherjee, & Chakrabarti, 2004). In addition, single intraperitoneal administration of SNG (10 mg/kg body weight) in rats substantially increased the activity of serum glutamic-pyruvic transaminase (SGPT) and serum glutamic-oxaloacetic transaminase (SGOT), demonstrating its potential hepatotoxicity (Dalvi, 1985). These studies used SNG at a concentration of 10 mg or higher/kg body weight. Kosina et al. and Psotova et al. attributed these toxicity to the high dose administration via intraperitoneal route (Kosina et al., 2004; Mackraj et al., 2008; Postova et al., 2006).

2.3.3 Anticancer effects of sanguinarine

Although SNG was isolated in the late 1940s, extensive studies on the mechanism underlying SNG-mediated tumor suppression have only been carried out during the past two decades (Kalogris et al., 2014; Sarkar, 1948). During this time, numerous *in vivo* and *in vitro* studies have been published signifying the anticancer properties of SNG. Some of the important anticancer functions of SNG are induction of apoptosis (S. Gu et al., 2015; Rosen et al., 2015), autophagy (Pallichankandy, Rahman, Thayyullathil, & Galadari, 2015), oncosis (Ding et al., 2002; Weerasinghe, Hallock, & Liepins, 2001), cell cycle arrest (Adhami et al., 2004; Reagan-Shaw, Breur, & Ahmad, 2006), and chemosensitization (W. Y. Choi et al., 2009; Gatti et al., 2014; S. Kim et al., 2008). SNG has also shown to inhibit invasion and metastasis (Kalogris et al., 2014; S. Y. Park, Jin, Kim, Lee, & Park, 2014; Sun et al., 2012). These effects of SNG are illustrated in Fig. 2.4.
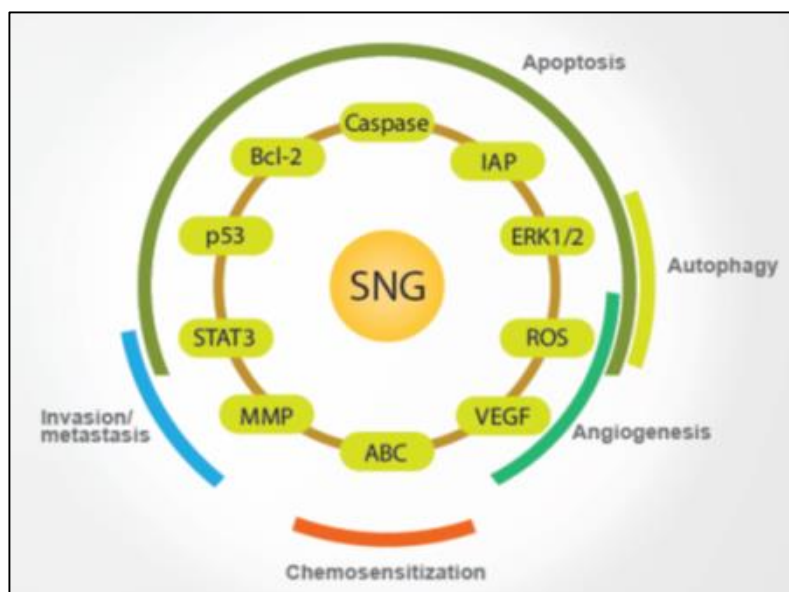
Figure 2.4. Anticancer effects of sanguinarine and its main targets. Various tumor suppressive functions of SNG such as induction of apoptosis, autophagy, and chemosensitization, as well as, inhibition of angiogenesis, invasion and metastasis are illustrated with major molecular targets that are involved in each process.

Most of the anticancer activities of SNG have been attributed to its ability to induce apoptosis. SNG has been shown to inhibit cancer cell growth through induction of apoptosis in a number of different cancers such as skin (Hammerová et al., 2011; Rosen et al., 2015), lung (S. Gu et al., 2015; Jang et al., 2009), breast (Choi et al., 2008; Dong et al., 2013; Kalogris et al., 2014; S. Kim et al., 2008), bladder (Han, Park, et al., 2013; J. S. Lee, Jung, Jeong, Yoon, & Kim, 2012), colon (Han, Kim, Yoo, & Choi, 2013; J. S. Lee et al., 2012), cervix (Xu et al., 2012), prostate (Adhami et al., 2004; Debiton, Madelmont, Legault, & Barthomeuf, 2003; Sun et al., 2010), bone (H. Park et al., 2010), blood (Han, Yoo, & Choi, 2008; Hussain et al., 2007), brain (Cecen, Altun, Ercetin, Aktas, & Olgun, 2014; Han et al., 2007), and pancreas (Ahsan, Reagan-Shaw, Breur, & Ahmad, 2007).

2.3.4 Molecular targets of sanguinarine

The anticancer properties of SNG are manifested through its action on various targets. The most established targets are summarized below.

Caspases: SNG has shown to activate caspase-3, -8, and -9 in T24, EJ, and 5637 human bladder cancer cells (Han, Park, et al., 2013), HCT-116 colorectal cancer cells (Han, Kim, et al., 2013), SK-MEL-2 melanoma cells (Burgeiro, Bento, Gajate, Oliveira, & Mollinedo, 2013), HaCaT immortalized human keratinocytes (Adhami, Aziz, Mukhtar, & Ahmad, 2003), and HL-60 and CEM leukemia cells (Jang et al., 2009; Kaminskyy, Kulachkovskyy, & Stoika, 2008). SNG can also impart anticancer effects via activation of caspase-7 in BxPC-3 and MIA PaCa-2 pancreatic cancer cell lines (C. K. Singh et al., 2015), HaCaT immortalized human keratinocytes (Adhami et al., 2003), and SK-MEL-2 melanoma cells (Burgeiro et al., 2013). Caspase-4 and -10 have also been shown to be activated in SK-MEL-2 melanoma cells (Burgeiro et al., 2013).

B-cell lymphoma-2 proteins: SNG has been shown to up-regulate pro-apoptotic Bcl-2 members such as Bcl-2-associated X protein (Bax) and BH3 interacting-domain death agonist (Bid), leading to apoptosis induction in AsPC-1 and BxPC-3 pancreatic cancer cells (Ahsan et al., 2007), primary effusion lymphoma cells (Hussain et al., 2007), and DU145 prostate cancer cells (Malíková, Zdarilová, Hlobilková, & Ulrichová, 2006). Role of SNG in the regulation of anti-apoptotic Bcl-2 members such as Bcl-2, B-cell lymphoma-extra-large (Bcl-xL), and myeloid cell leukemia 1 (Mcl-1) have also been reported. For example, SNG has been shown to decrease the expression of Bcl-2 in HCT-116 colorectal cancer cells (Han, Kim, et al., 2013), AsPC-1 and BxPC-3 pancreatic cancer cells (Ahsan et al., 2007), MDA-231 breast cancer cells (S. Kim et al., 2008), HeLa and SiHa human cervical cancer cells (Xu et al., 2012), and HT-29 human colon cancer cells (J. S. Lee et al., 2012). Furthermore, SNG has also been shown to down-regulate the expression of Bcl-xL

in a variety of cancer cells (Ahsan et al., 2007; Hammerová et al., 2011; Reagan-Shaw et al., 2006). Another important anti-apoptotic Bcl-2 member down-regulated by SNG is Mcl-1. Hammerova et al. observed that SNG down-regulates the expression of Mcl-1 in A-375 and SK-MEL-2 melanoma cells (Hammerová et al., 2011).

Inhibitors of apoptosis protein: Numerous studies have reported the role of SNG in the down-regulation of IAP family of proteins. For instance, SNG-induced inactivation of XIAP has been reported in T24, EJ, and 5637 bladder cancer cells (Han, Park, et al., 2013), MDA-MB-231 breast cancer cells (S. Kim et al., 2008), HCT-116 colorectal cancer cells (Han, Kim, et al., 2013), A-375 and SK-MEL-2 melanoma cells (Hammerová et al., 2011), and in different primary effusion lymphoma cells (Hussain et al., 2007). Sue et al. demonstrated that SNG induces survivin degradation through the ubiquitin proteasome-dependent pathway in DU145 prostate cancer cells (Sun et al., 2010). In addition to XIAP and survivin, SNG has been shown to down-regulate cIAP-2 in MDA-MB-231 breast cancer cells (S. Kim et al., 2008), cIAP-1 in HCT-116 colorectal cancer cells (Han, Kim, et al., 2013), and both cIAP-1 and cIAP-2 in different primary effusion lymphoma cells (Hussain et al., 2007).

Reactive oxygen species: Numerous studies suggest that ROS generation by SNG initiates many of its antiproliferative and tumor suppressive functions. For instance, SNG has been shown to induce ROS-dependent activation of Early growth response gene-1 (Erg-1) and subsequent induction of apoptosis in human bladder (T24, EJ, and 5637) and colorectal cancer (HCT-116) cell lines (Han, Kim, et al., 2013; Han, Park, et al., 2013). Treatment with ROS scavenger significantly abrogates the Erg-1 activation, and subsequent apoptosis induction, demonstrating that ROS-mediated Erg-1 activation is a vital step in apoptosis. ROS production

accompanying the anticancer effects of SNG has also been demonstrated in various cancer cell lines (Dong et al., 2013; Hussain et al., 2007; Kaminskyy, Kulachkovskyy, et al., 2008; S. Kim et al., 2008; Matkar, Wrischnik, & Hellmann-Blumberg, 2008a, 2008b).

Other targets: In addition to the above mentioned pathways, SNG has been shown to modulate several other important pathways. Some of the other important SNG targets include: (1) inhibition of potent pro-angiogenic vascular endothelial growth factor (VEGF) (Basini, Santini, Bussolati, & Grasselli, 2007; Eun & Koh, 2004), (2) down-regulation of signal transducer and activator of transcription 3 (Stat3)-mediated pro-tumorigenic targets (Kalogris et al., 2014; Sun et al., 2012), (3) overcoming ATP binding cassette (ABC) transporters-mediated tumor resistance (Ding et al., 2002; Eid, El-Readi, Eldin, Fatani, & Wink, 2013; Gatti et al., 2014; Weerasinghe, Hallock, Tang, Trump, & Liepins, 2006), (4) induction of extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation (activation), leading to the activation of autophagic cell death in U87MG and U118MG malignant glioma cells (Pallichankandy et al., 2015), and (5) down-regulation of mRNA levels of matrix metalloproteases (MMP-2 and -9), leading to decrease in their activities (Y. H. Choi et al., 2009).

2.4 Reactive oxygen species

2.4.1 Introduction to reactive oxygen species

Reactive oxygen species are heterogeneous group of highly reactive ions and molecules derived from molecular oxygen (O_2) (Schieber & Chandel, 2014). Some of the most important ROS with physiological significance are free radicals such as hydroxyl radical ($\cdot OH$), superoxide anion ($O_2 \cdot^-$), as well as non-radical molecules like hydrogen peroxide (H_2O_2) (Auten & Davis, 2009; Schieber & Chandel, 2014). When introduced for the first time, ROS were thought to be very

toxic and only associated with various pathological conditions (Brieger, Schiavone, Miller, & Krause, 2012). Since then, tremendous amount of published research has linked ROS to various physiological process as well (D'Autréaux & Toledano, 2007; Weidinger & Kozlov, 2015). In this context, the biological reality of ROS is more complex and paradoxical (D'Autréaux & Toledano, 2007; Fruehauf & Meyskens, 2007; Halliwell, 2013). ROS is involved in numerous biological processes that are important for the integrity and fitness of living organisms; yet, ROS is also implicated in various disease states such as cancer, diabetes mellitus, atherosclerosis, and cardiovascular diseases, to name few (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012).

2.4.2 Reactive oxygen species generation

Reactive oxygen species, as byproducts of oxygen metabolism, are constantly produced and removed via a variety of complex biosynthetic and degradative pathways. $O_2^{\bullet-}$ is generally considered as the primary ROS which can be rapidly inactivated by superoxide dismutase (SOD), yielding H_2O_2. In the presence of Fe^{2+} or Cu^{2+} ions, H_2O_2 can be further converted to $^{\bullet}OH$ in a process called the Fenton reaction (Fig. 2.5) (Glasauer & Chandel, 2014; Schieber & Chandel, 2014).
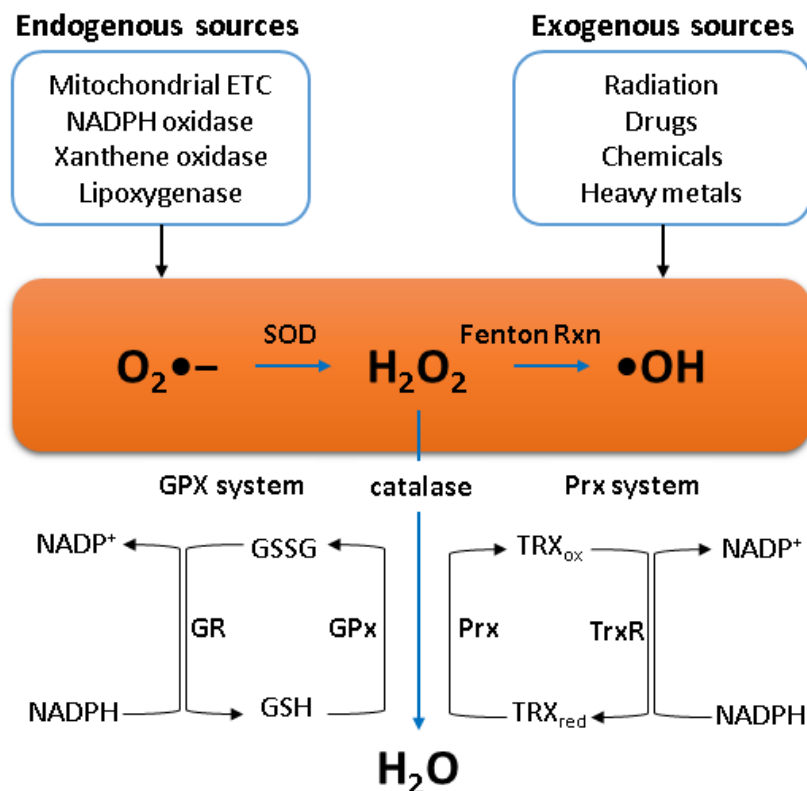
Figure 2.5. Reactive oxygen species formation and clearance. Major sources of ROS formation and antioxidant system involved in ROS conversion and clearance are illustrated.

Different types of ROS have different diffusion capabilities and reactivity towards their targets. For instance, •OH is extremely reactive and does not diffuse outside their site of formation, while $O_2\bullet-$ is highly active and has limited diffusion capacity, especially through certain channels. In contrast, H_2O_2 readily diffuses through membranes, making it an ideal candidate for intracellular signaling (Wojtovich & Foster, 2014). ROS generation can be triggered by several exogenous and endogenous factors. Mitochondria-catalyzed electron transport chain (ETC) is the major endogenous source of ROS in most mammalian tissues (Sena & Chandel, 2012). In ETC, mainly at complex I and III, $O_2\bullet-$ is formed in the mitochondrial matrix

by the single electron reduction of O_2 (Saybaşili, Yüksel, Haklar, & Yalçin, 2001; Staniek, Gille, Kozlov, & Nohl, 2002). Simultaneously, complex III can also release $O_2^{\bullet-}$ into the mitochondrial intermembrane space (Muller, Liu, & Van Remmen, 2004). Some of the other important endogenous sources of ROS are enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) (Vignais, 2002), xanthene oxidase (Hodges, Young, Paul, & Ingold, 2000), and lipoxygenase (L. Zuo, Christofi, Wright, Bao, & Clanton, 2004). In addition, ROS generation can be facilitated by a multitude of exogenous factors such as radiation, drugs and chemicals, heavy metals, etc. (Cadenas, 1989).

2.4.3 Conversion and clearance of reactive oxygen species

The balance between production and scavenging of ROS is of critical importance as ROS have been linked with number of biological processes and pathophysiological conditions. Cellular redox balance is typically maintained by a powerful battery of antioxidant system that is present in different subcellular compartments (Hanschmann, Godoy, Berndt, Hudemann, & Lillig, 2013; Perkins, Nelson, Parsonage, Poole, & Karplus, 2015). These antioxidant species can be divided into (1) enzymatic: SOD, catalase, peroxiredoxin (Prx; also known as thioredoxin peroxidase) system, glutathione peroxidase (GPx) system, etc. and (2) non-enzymatic: L-ascorbic acid, α-lipoic acid, α-tocopherol, etc.

As mentioned earlier, SOD catalyzes the conversion of $O_2^{\bullet-}$ into H_2O_2, which can be further reduced to water by Prx system, GPx system, and catalase (Fig. 2.5). The Prx system consists of two electron donors and two types of antioxidant enzymes: Prx itself, thioredoxin reductase (TrxR), thioredoxin (TRX), and NADPH. During the metabolism of H_2O_2, Prx gets oxidized and inactivated. TRX acts as an immediate electron donor and reduces Prx back to active form. Consequently, TRX is reduced by TrxR which receives its electrons from NADPH (Fig. 2.5). In the GPx system, GPx catalyses the reduction of H_2O_2 leading to the oxidation of glutathione

(GSH), which can be reduced back by glutathione reductase (GR), using NADPH (Fig. 2.5) (Brigelius-Flohé & Maiorino, 2013).

2.4.4 Apoptotic functions of reactive oxygen species

Cancer cells exhibit greater ROS level than normal cells. Nevertheless, these ROS levels are counteracted by increased activity of antioxidant enzymes in cancer cells. This common effect suggests that the therapeutic strategies, which either increase ROS generation or decrease antioxidant defense, may push cancer cells beyond the breaking point, leading to the activation of different cell death pathways, thus limiting the cancer progression. Accordingly, large variety of anticancer agents effectively kill cancer cells and overcome drug resistance via modulating ROS generation and antioxidant defense.

Induction of apoptosis is one of the key tumor suppressive functions of ROS. Cellular FLICE-inhibitory protein (c-FLIP) competes with the procaspase for adaptor protein binding site, leading to the inhibition of DISC formation, and subsequent activation of apoptosis (Safa, 2012). ROS have been shown to negatively regulate c-FLIP post-transcriptionally, leading to the facilitation of extrinsic pathway of apoptosis (L. Wang et al., 2008). Two different mechanisms have been proposed for ROS-dependent down-regulation of c-FLIP: (1) Through negatively regulating nitric oxide that prevents S-nitrosation and ubiquitin-proteasomal degradation of c-FLIP (Chanvorachote et al., 2005; L. Wang et al., 2008) and (2) ubiquitination of c-FLIP (phospho-Thr166) at Lys167, leading to its subsequent degradation by proteasomes (Wilkie-Grantham, Matsuzawa, & Reed, 2013).

Being the major source of ROS generation, most of the apoptosis-inducing actions of ROS is undoubtedly in the mitochondria. During the PTP formation, a number of proteins associate to form a non-selective pore at the mitochondrial membrane. The three major components of PTP are voltage-dependent anion-selective channel (VDAC; also known as porin), adenine nucleotide translocase

(ANT), and cyclophilin D (Marchi et al., 2012). Madesh and Hajnoczky demonstrated that $O_2^{\bullet-}$, but not H_2O_2, triggers rapid and massive Cyt-c release, leading to the induction of apoptosis via VDAC-dependent permeabilization of mitochondria (Madesh & Hajnóczky, 2001). Additionally, McStay et al. demonstrated that oxidation of ANT at Cys160 and Cys257 promotes the opening of the PTP (McStay, Clarke, & Halestrap, 2002). In mitochondria Cyt-c exists as a complex with cardiolipin, therefore, the complex must be breached in order to facilitate its release. In the presence of H_2O_2, cardiolipin gets oxidized, reducing its affinity for Cyt-c, leading to its release into the cytosol (Bayir & Kagan, 2008; Kagan et al., 2005). As mentioned earlier, following the release from mitochondria, Cyt-c must interact with Apaf-1 and procaspase-9, to form apoptosome. Zuo et al. demonstrated that oxidative modification of Cys403 of procaspase-9 can mediate its interaction with Apaf-1 via disulfide bond formation, and can thus promote its auto-cleavage and activation (L. Zhang et al., 2015; Y. Zuo et al., 2009). An alternative mechanism for oxidation-dependent caspase-9 activation has also been proposed. Katoh et al. demonstrated that H_2O_2 induces disulfide bridge-mediated dimer formation of caspase-9 in the mitochondria, leading to its auto-proteolytic cleavage and subsequent activation in Apaf-1-independent manner (Muller et al., 2004; L. Zhang et al., 2015).

The process of apoptosis can be positively or negatively regulated by a large variety of tumor suppressive and proliferative proteins and lipids. Many of the current anticancer therapies have been shown to exert their cytotoxicity via ROS-dependent regulation of these proteins and lipids. For instance, ROS have been shown to induce apoptosis signal-regulating kinase 1 (ASK1) dimer formation, leading to its activation (Gotoh & Cooper, 1998). Activation of ASK1 induces phosphorylation and activation of MAPKs such as c-Jun N-terminal kinases (JNK) and p38, leading to the induction of the intrinsic pathway of apoptosis (Hatai et al.,

2000). Another key mediator of the biological effects of ROS is the tumor suppressor lipid ceramide. A strong interplay between ceramide generation and oxidative stress has been demonstrated. ROS generation were reported to generate ceramide in lipid rafts; specialized micro-domains of plasma membrane that contain high concentrations of lipids (Galadari et al., 2013). For example, ROS-dependent accumulation of ceramide in raft micro-domains was observed following ultraviolet (UV)-irradiation (Charruyer et al., 2005) and TNF-related apoptosis inducing ligand (TRAIL) treatment (Dumitru & Gulbins, 2006). Similar results were also observed in radiosensitive human head and neck squamous carcinoma cell line (SCC61) following gamma-irradiation (Bionda et al., 2007).

2.5 Ceramide

2.5.1 Introduction to sphingolipids

Sphingolipids comprise a complex family of membrane lipids which are ubiquitously expressed in eukaryotic cells. Currently, sphingolipids are also implicated in mediating a wide variety of intracellular functions (Galadari et al., 2015; Hannun & Obeid, 2008). Ceramide is the most crucial sphingolipid molecule which mediates a variety of antiproliferative cellular responses. Diverse array of stressors such as genotoxic damage, inflammatory mediators, heat shock, oxidative stress, and anticancer drugs can induce ceramide generation, which in turn orchestrates a myriad of tumor suppressive signaling pathways such as apoptosis, autophagy, senescence, and necroptosis (Albi et al., 2006; Galadari et al., 2015; Hannun & Obeid, 2008; Kimura, Markowski, Edsall, Spiegel, & Gelmann, 2003; MacKichan & DeFranco, 1999; Pfeilschifter & Huwiler, 1998; Yabu, Imamura, Yamashita, & Okazaki, 2008).

2.5.2 Ceramide biosynthetic pathway

The biosynthetic pathway of sphingolipids consists of a complex network of synthetic and degradative reactions. Some of the major pathways of intracellular ceramide accumulation are (1) *de novo* pathway of ceramide synthesis, (2) sphingomyelinase (SMase)-mediated hydrolysis of sphingomyelin, and (3) inhibition of ceramidase (CDase)- or glucosylceramide synthase (GCS)-mediated conversion of ceramide into sphingosine and glucosylceramide, respectively, and (4) catabolism of complex glycosphingolipids (GSL) by different glycosidases (Galadari et al., 2013; Galadari et al., 2015). Ceramide biosynthetic pathway and inhibitors of various ceramide biosynthetic enzymes are depicted in Fig. 2.6.
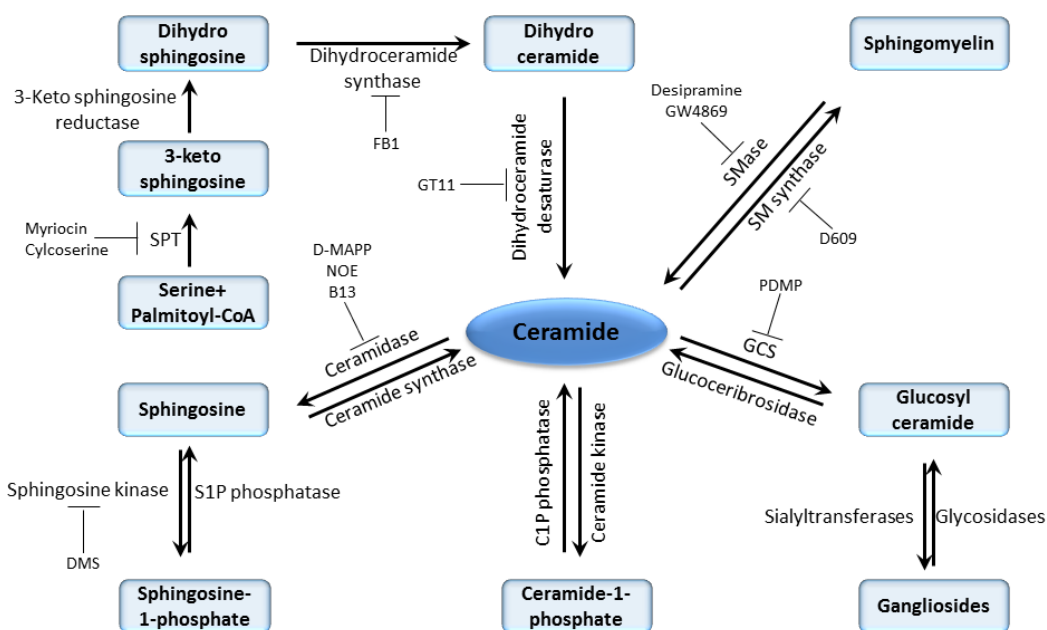
Figure 2.6. Schematic representation of ceramide biosynthetic pathway that illustrates enzymes that catalyze each step and their inhibitors. Adapted from: Galadari S, Rahman A, Pallichankandy S, Thayyullathil F. Tumor suppressive functions of ceramide: evidence and mechanisms. Apoptosis. 2015; 20(5):689-711.

The *de novo* ceramide biosynthesis begins in the ER with condensation of palmitoyl-CoA and the amino acid serine, catalyzed by the action of an enzyme

called serine palmitoyl transferase (SPT), which proceeds through a series of reducing, acylating, and oxidizing reactions until ceramide is formed (Fig. 2.6) (Galadari et al., 2013; Galadari et al., 2015). Ceramide may be hydrolyzed by CDase to generate sphingosine, which may be further phosphorylated to sphingosine-1-phosphate (S1P) by sphingosine kinase. Ceramide synthase and S1P phosphatase can reverse the formation of sphingosine from ceramide and S1P from sphingosine, respectively (Fig. 2.6) (Galadari et al., 2013; Galadari et al., 2015). Once ceramide is formed in the ER, ceramide transfer protein transport ceramide to the Golgi, where it can be converted into sphingomyelin and glucosylceramide by the action of sphingomyelin synthase and GCS, respectively. Glucosylceramide can be further converted to the complex GSL by different sialyl transferases (Galadari et al., 2013; Galadari et al., 2015).

2.5.3 Role of ceramide in apoptosis

One of the key tumor suppressive functions of ceramide is its ability to induce apoptotic cell death in variety of tumor models. Ceramide exercises its apoptosis-inducing activity via directly or indirectly modulating a diverse range of molecular targets as depicted in Fig. 2.7.
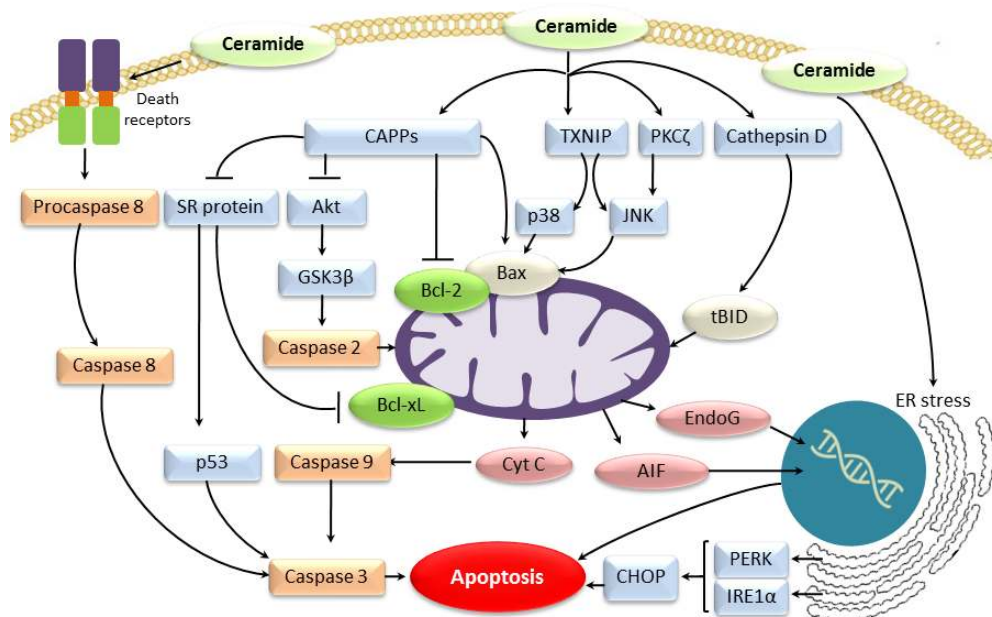
Figure 2.7. Pathways of ceramide-driven apoptotic response. Major molecular targets that are involved in the ceramide-induced extrinsic, intrinsic and ER-stress mediated apoptotic responses are shown. Adapted from: Galadari S, Rahman A, Pallichankandy S, Thayyullathil F. Tumor suppressive functions of ceramide: evidence and mechanisms. Apoptosis. 2015; 20(5):689-711.

Some of the important pro-apoptotic targets of ceramide are (1) ceramide-activated protein phosphatases (CAPP): protein phosphatase-2A (PP2A), protein phosphatase-1 (PP1), and protein phosphatase-2C (PP2C), (2) protein kinase C (PKC), (3) MAPKs: JNK and p38, (4) mitochondrial pro-apoptotic proteins: Cyt-c, AIF, EndoG, and Smac/DIABLO, (5) caspases, (6) cathepsin D, (7) p53, (8) Bcl-2 family proteins, and (9) ER stress proteins.

A significant body of previous reports have shown the role of ceramide in the activation of extrinsic and intrinsic pathway of apoptosis. For instance, activation of TRAIL receptors was shown to induce ROS-dependent acid sphingomyelinase (aSMase) activation, leading to subsequent ceramide generation and formation of ceramide-enriched membrane domains. These domains serve as signaling platforms, where clustering of various death receptors mediates proximity-induced caspase activation and subsequent induction of extrinsic pathway of apoptosis

(Dumitru & Gulbins, 2006; Galadari et al., 2015). In addition, ceramide has also been shown to down-regulate caspase-8 inhibitor, c-FLIP protein (Asakuma, Sumitomo, Asano, & Hayakawa, 2003; Galadari et al., 2015; Yoon et al., 2002). As mentioned earlier, ceramide has been implicated in the induction of the intrinsic pathway of apoptosis. Various mechanism have been proposed for this: (1) activation of Bax leading to the release of Cyt-c (Birbes et al., 2005; Galadari et al., 2015; Kashkar, Wiegmann, Yazdanpanah, Haubert, & Krönke, 2005), (2) directly forming channels in the mitochondrial membrane (Galadari et al., 2013; Siskind, Kolesnick, & Colombini, 2006), (3) inhibition of Akt activation and thereby preventing the Akt-induced inactivation of pro-apoptotic Bcl-2 proteins (Galadari et al., 2015; Navarro, Valverde, Rohn, Benito, & Lorenzo, 2000), and (4) translocation of PKCδ to the mitochondria, which in turn, leads to the release of Cyt-c and caspase-9 activation (Galadari et al., 2015; Sumitomo et al., 2002).

2.5.4 Ceramide activated protein phosphatases

Ceramide has been shown to mediate many of its biological effects via activation of serine/threonine protein phosphatases (PP1 and PP2A) collectively referred to as CAPPs. Some of the important substrates of CAPPs include Akt, Bcl-2, c-Jun, retinoblastoma (Rb) protein, PKCα, DAPK, and ERM (ezrin, radixin, and moesin) proteins (Galadari et al., 2015). Numerous studies have shown that CAPPs dephosphorylate Akt and oppose its anti-apoptotic actions (Galadari et al., 2015; Salinas, López-Valdaliso, Martín, Alvarez, & Cuadrado, 2000; Schubert, Scheid, & Duronio, 2000; Zhu et al., 2011). For instance, Schubert et al. have demonstrated that treatment of TF-1 human erythroleukemia and PC-12 rat pheochromocytoma cells with exogenous ceramide mediates CAPP-dependent dephosphorylation of Akt at Ser473 (Schubert et al., 2000).

2.6 Akt

2.6.1 Overview of Akt

Akt is a serine-threonine protein kinase that plays a key role in multiple tumorigenic cellular processes such as cell proliferation, cell cycle progression, inhibition of apoptosis, and induction of cell motility, angiogenesis and metastasis (Brazil, Park, & Hemmings, 2002; Qiao, Sheng, & Pardee, 2008). The three highly homologous isoforms of mammalian Akt which been identified so far are Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ) (Scheid & Woodgett, 2003). These isoforms share similar structural organization, but appear to have variability in cellular distribution and carry distinct biological processes (Laine, Künstle, Obata, & Noguchi, 2002). Akt1 is ubiquitously expressed in a wide variety of tissues and is implicated in cell growth and survival, while Akt2 is highly expressed in adipose tissues, liver, and skeletal muscles, and is implicated in glucose homeostasis. Though virtually seen in all tissues, the expression of Akt3 is more predominant in testes and brain (Hers, Vincent, & Tavaré, 2011).

2.6.2 Akt in cancer

Over past few decades, significant advances have been made in understanding the biological processes that are regulated by Akt. Akt has emerged as a central player in cellular survival and proliferation. Considering its proliferative functions, Akt has one of the highest number of mutation associated with cancer (Fresno Vara et al., 2004). For instance, somatic missense activating mutation of Akt1 (E17K) was found in breast, colorectal, and ovarian cancers (Carpten et al., 2007). Moreover, gene amplifications in different Akt isoforms have been demonstrated in pancreatic and head and neck cancers (Engelman, Luo, & Cantley, 2006; Yuan & Cantley, 2008). In addition to tumorigenesis, constitutive activation of Akt is also associated with poor prognosis and resistance to chemotherapy

(Morishita et al., 2012; Yoshioka et al., 2008). Therefore, therapeutic strategies that target Akt may ultimately prove more efficacious for the management of cancer. Simultaneously, many such compounds (perifosine, MK2206, GDC-0068, GSK2110183, and ARQ 092) are in clinical trial or in the development pipeline (Dienstmann, Rodon, Serra, & Tabernero, 2014).

2.6.3 Regulation of Akt

The signaling events required for the Akt activation are well characterized. Akt is one of the key downstream mediator of the phosphoinositide 3-kinase (PI3K) cell growth and survival pathway. The PI3K pathway begins at the plasma membrane with the binding of growth factors and cytokines to their respective cell-surface receptors. This binding results in the recruitment and activation of PI3K (Galadari et al., 2013). Once activated, PI3K phosphorylates the membrane lipid phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3) (Galadari et al., 2013). Akt and phosphoinositide dependent protein kinase-1 (PDK1) are recruited to the PIP3 and Akt is activated by PDK-dependent phosphorylation at Thr308 of catalytic domain (Chong, Li, & Maiese, 2005; Galadari et al., 2013). However, complete activation of Akt requires phosphorylation at Ser473 of regulatory domain (Galadari et al., 2013). The mechanism of Ser473 phosphorylation is not completely elucidated as yet. Several likely candidates have been proposed, including Akt itself, PDK1, PKC, and mammalian target of rapamycin (mTOR) complex 2 (Hassan, Akcakanat, Holder, & Meric-Bernstam, 2013; Martelli, Evangelisti, Chiarini, & McCubrey, 2010). Following its activation, Akt translocates to the cytosol and various subcellular compartments such as the nucleus, ER, and mitochondria where it catalyzes the phosphorylation of a number of downstream targets leading to tumorigenesis (Bononi et al., 2011) (Fig. 2.8).
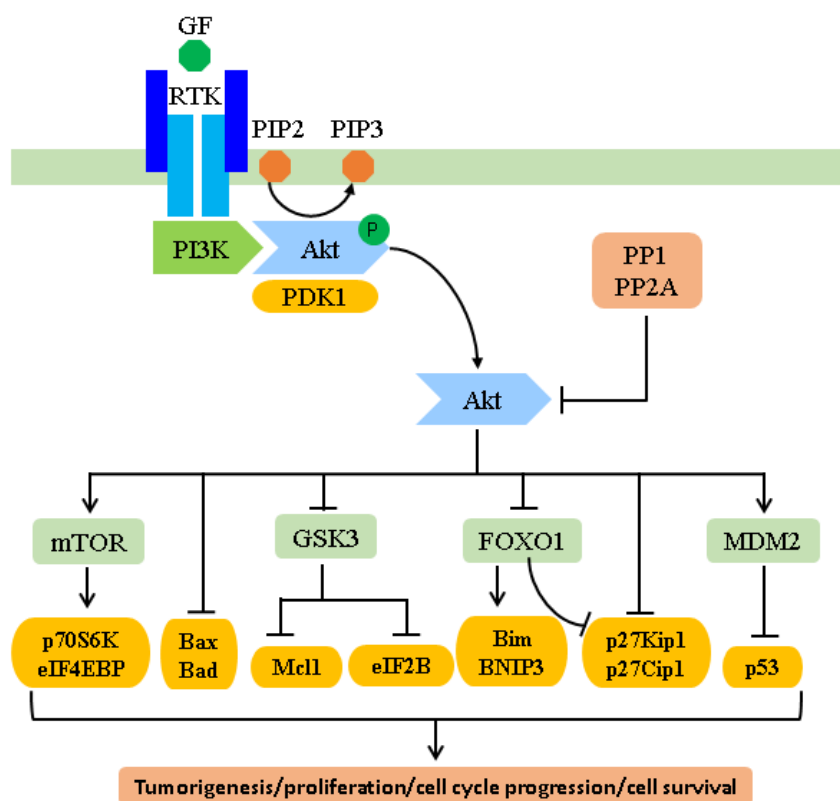
Figure 2.8. Pathways of Akt-driven pro-tumorigenic response. Major molecular targets that are involved in the Akt-induced proliferation, cell cycle progression, and survival responses are shown.

Following its activation, Akt may be inactivated by its dephosphorylation which in turn switches off its varied anti-apoptotic functions. As mentioned earlier, CAPPs such as PP1 and PP2A can dephosphorylate Akt and oppose its anti-apoptotic actions (Galadari et al., 2015; Schubert et al., 2000). Apart from directly activating CAPPs, ceramide has been shown to reduce the association between inhibitor 2 of PP2A (I2PP2A; also known as SET), resulting in increased PP2A activity (Chalfant, Szulc, Roddy, Bielawska, & Hannun, 2004; Mukhopadhyay et al., 2009). Although, it was hypothesized that CAPPs equally dephosphorylates both the Thr308 and Ser473 residues, possibility of differential affinity towards each site has not been addressed.

2.6.4 Downstream targets of Akt

Some of the important downstream targets of Akt are listed below (Fig. 2.8):

Glycogen synthase kinase 3 beta: Akt has been shown to phosphorylate glycogen synthase kinase 3 beta (GSK3β) at Ser9, leading to its inactivation, and subsequent attenuation of its inhibitory effect on anti-apoptotic Bcl-2 member, Mcl-1 (Chalfant et al., 2004). Another important primary target for GSK3β is β-catenin, of which phosphorylation by GSK3β at Ser33, Ser37, and Thr41 leads to its degradation by the 26S proteasome complex (Takahashi-Yanaga & Sasaguri, 2008). Akt-mediated inactivation of GSK3β blocks its ability to phosphorylate and degrade β-catenin, leading to its accumulation in the nucleus, where it induces the expression of tumorigenic Wnt target proteins such as c-myc, cyclin D1, and VEGF (Takahashi-Yanaga & Sasaguri, 2008; X. Zhang, Gaspard, & Chung, 2001). In addition, GSK3β is also an endogenous inhibitor of eukaryotic initiation factor 2B (eIF2B), an essential participant in the initiation of protein translation. So, inactivation of GSK3β by Akt promotes protein synthesis, a requisite in tumor progression (Galadari et al., 2013; White-Gilbertson, Kurtz, & Voelkel-Johnson, 2009).

Forkhead box O transcription factors: Akt has been shown to directly phosphorylate forkhead box O (FOXO) transcription factors leading to their displacement from target genes, nuclear exclusion and subsequent proteasomal degradation (X. Zhang, Tang, Hadden, & Rishi, 2011). Akt-mediated phosphorylation of FOXO1 (Thr24, Ser256, and Ser319), FOXO3 (Thr32, Ser253, and Ser315), FOXO4 (Thr28, Ser193, and Ser258), and FOXO6 (Thr26 and Ser184), may promote their interaction with 14-3-3 chaperone molecules. The adapter protein 14-3-3 displaces FOXO from target genes, and escorts their re-localization from the nucleus to the cytoplasm, thereby inhibiting their transcriptional functions (X. Zhang et al., 2011). Through this mechanism, Akt blocks FOXO-mediated transcription of tumor suppressive genes such as (1) apoptosis inducing

death receptor ligands such as FasL and TRAIL, (2) pro-death Bcl-2 family protein such as Bcl-2-like protein 11 (Bim), p52 up-regulated modulator of apoptosis (PUMA), and Noxa (Latin word for damage), and (3) cyclin-dependent kinase inhibitors (CDKI) such as p27Kip1 and p21Cip1 (X. Zhang et al., 2011).

p53: Akt phosphorylates mouse double minute 2 (MDM2) at Ser166 and Ser186, which results in its enhanced nuclear localization, where it negatively regulates p53 function by potentiating its ubiquitination (Mayo & Donner, 2001; B. P. Zhou, Liao, Xia, Zou, et al., 2001). Some of the important transcriptional targets of p53 are: (1) pro-apoptotic Bcl-2 members such as PUMA and Noxa, (2) CDKI such as p21Cip1, and (3) pro-autophagic genes such as Atg4, Atg7, and Atg10 (Bieging, Mello, & Attardi, 2014).

Cyclin dependent kinase inhibitors: It was shown that Akt could directly phosphorylate p27Kip1 at Thr157 and subsequently abolish its nuclear localization and CDKI function (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002). As with p27Kip1, Akt phosphorylates p21Cip1 at Thr145, leading to its cytosolic localization and subsequent promotion of cell growth (B. P. Zhou, Liao, Xia, Spohn, et al., 2001).

Mammalian target of rapamycin: Akt directly phosphorylates mTOR at Ser2448, leading to mTORC1 activation, which promotes tumorigenesis via activation of p70 ribosomal S6 kinase (p70S6K) and the inhibition of eukaryotic initiation factor 4E binding protein (eIF4EBP) (Galadari et al., 2013).

B-cell lymphoma-2 proteins: Mitochondrial membrane insertion of Bax is essential for the release of pro-apoptotic factors from the mitochondria (Czabotar et al., 2014; Youle & Strasser, 2008). Akt phosphorylates Bax at Ser184, thereby inhibiting its conformational change necessary for the oligomerization and mitochondrial membrane insertion (Gardai et al., 2004; Stiles, 2009; Xin & Deng, 2005). Another important Bcl-2 protein which is directly phosphorylated by Akt is Bcl-2-associated

death promoter (Bad). Akt directly phosphorylates Bad at Ser136, resulting in its dissociation from Bcl-2 or Bcl-xL, thereby regaining the anti-apoptotic functions of Bcl-2 and Bcl-xL (Datta et al., 1997; Masters, Yang, Datta, Greenberg, & Fu, 2001).

Other targets: In addition to the above mentioned functions, Akt has been shown to phosphorylate: (1) procaspase-9 at Ser196, leading to a decrease in its proteolytic activity (Cardone et al., 1998), (2) I-kappa B Kinase (IKK) at Thr23, thereby inducing the transcriptional activation of NFκB, which is widely perceived as an anti-apoptotic transcriptional factor (Romashkova & Makarov, 1999), (3) ASK1 at Ser83, thereby inhibiting ASK1-mediated JNK activation and subsequent inhibition of apoptosis (A. H. Kim, Khursigara, Sun, Franke, & Chao, 2001), and (4) cAMP response element-binding protein (CREB) transcription factor at Ser133, thereby possibly promoting transcription of CREB-regulated survival genes such as Bcl-2 and Mcl-1 (Du & Montminy, 1998).

2.7 Extracellular signal-regulated kinase 1/2

2.7.1 Introduction to extracellular signal-regulated kinase 1/2

Mitogen activated protein kinases are highly conserved family of serine/threonine protein kinases which regulate wide variety of cellular process such as cell growth and differentiation, gene expression, cell motility and invasion, cell survival, apoptosis, and autophagy. Three key members of MAPK family are ERK (ERK1 and ERK2), JNK (JNK1, JNK2, and JNK3), and p38 (p38α, p38β, p38γ, and p38δ) (Lu & Xu, 2006). Human ERK1 and ERK2 share 84% structural identity with ERK1 having an additional 17 amino acid residue insertion at its N-terminal extension. Most, if not all, the stimulants of the ERK pathway can activate both ERK1 and ERK2 independently, and both ERKs possess matching substrate specificity (Roskoski, 2012). Considering their overlapping functions, they are generally referred to as ERK1/2 (Lloyd, 2006).

2.7.2 Regulation of extracellular signal-regulated kinase 1/2

The canonical ERK1/2 signaling pathway is initiated by the binding of growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) to their respective tyrosine kinase receptors, leading to their autophosphorylation and activation (Bivona & Philips, 2003; Ramos, 2008). Receptor activation recruits the adaptor protein growth factor receptor-bound protein 2 (Grb2) through src homology 2 (SH2) domain, which further recruits and binds to guanine nucleotide exchange factor SOS through its src homology 3 (SH3) domain (Shaul & Seger, 2007). Following its recruitment, SOS activates membrane-localized Ras GTPase which subsequently mediates a relay of phosphorylation cascade, finally leading to the ERK1/2 activation. Initially, Ras recruits and activates Raf kinase (also known as MAPK kinase kinase), which subsequently phosphorylates two serine residues on the activation loop of MEK (also known as MAPK kinase), leading to its activation (Ramos, 2008; Shaul & Seger, 2007). Activated MEK phosphorylates tyrosine and threonine residues on the activation loop of ERK1/2 leading to its activation.

Following its activation, ERK1/2 may be inactivated by its dephosphorylation at either or both threonine and tyrosine residues on the activation loop. This action may be mediated by (1) dual specificity phosphatases (DUSP) (also known as MAPK phosphatase) which dephosphorylates both threonine and tyrosine residues, (2) serine/threonine phosphatase such as CAPP (PP2A), and (3) tyrosine phosphatase such as phosphotyrosine-specific phosphatase (PTPase) (Roskoski, 2012). In addition, ERK1/2 activation may also be attenuated by the inhibition of its upstream kinases. ERK1/2 can phosphorylate its upstream regulators such as SOS, Raf, and MEK, thereby, inhibiting further ERK1/2 activation via a negative feedback mechanism (Ramos, 2008).

2.7.3 Opposing functions of extracellular signal-regulated kinase 1/2

Following its activation, ERK1/2 can translocate mainly into the nucleus and cytoplasm, but also into other organelles where they can phosphorylate and regulate various signaling molecules such as transcription factors, kinases, and cytoskeletal proteins (Roberts & Der, 2007). More than 160 substrates with diverse and sometimes opposing functions have been identified as ERK1/2 targets (Roberts & Der, 2007). ERK1/2 signaling is particularly interesting as it exhibits both tumor promoting and tumor suppressive activity (Cagnol & Chambard, 2010). This raises the question as to how these varied and opposing functions are regulated by ERK1/2. Several mechanisms have been proposed for the specificity determination by ERK1/2. These are (1) strength and duration of its phosphorylation, (2) interaction with various scaffold proteins and other intracellular signaling molecules/pathways, and (3) subcellular localization of ERK1/2 (Shaul & Seger, 2007).

2.7.4 Tumor suppressive functions of extracellular signal-regulated kinase 1/2

Initial reports on the tumor suppressive functions of ERK1/2 signaling pathway came in the 1990s where Blagosklonny et al. demonstrated that depletion of Raf protects MCF-7 breast cancer cells from taxol-induced apoptosis (Cagnol & Chambard, 2010). Since then, numerous articles have been published linking the Ras∕Raf∕ERK1/2 pathway in the induction of cell death mediated by UV-gamma irradiation (Y. J. Lee et al., 2003; Tang et al., 2002), chemotherapeutic agents (Martin, Poggi, Chambard, Boulukos, & Pognonec, 2006; X. Wang, Martindale, & Holbrook, 2000), phytochemicals (Stefanelli et al., 2002; Yeh et al., 2004), and other anticancer therapies (Cagnol & Chambard, 2010). Some of the important pro-apoptotic mechanism of ERK1/2 include:

1. Activation of extrinsic apoptotic pathway: ERK1/2 have been shown to induce death ligands (TNF-α and FasL) (Cagnol & Chambard, 2010; Ulisse et al., 2000), death receptors (FasR, death receptor 4, and death receptor 5) (Drosopoulos et al., 2005; Shenoy, Wu, & Pervaiz, 2009; Tanaka et al., 2002; Tewari, Sharma, Koul, & Sen, 2008), death inducing adaptor protein (FADD) (Tewari et al., 2008), and extrinsic apoptosis initiator caspase (caspase-8) (Cagnol, Van Obberghen-Schilling, & Chambard, 2006) in different cancer models.

2. Activation of intrinsic apoptotic pathway: ERK1/2 have also been shown to decrease the mitochondrial membrane potential, which could mediate the release of Cyt-c from the mitochondria to the cytosol, which may further facilitating the intrinsic pathway of apoptosis (G. S. Kim et al., 2003; Nowak, Clifton, Godwin, & Bakajsova, 2006; C. L. Zhang et al., 2004; X. Zhang et al., 2003). In addition, down-regulation of anti-apoptotic Bcl-2 family members (Bcl-2 and Bcl-xL) (DeHaan, Yazlovitskaya, & Persons, 2001; Li et al., 2007) and up-regulation of pro-apoptotic Bcl-2 members (Bax and PUMA) (L. Brown & Benchimol, 2006; Cagnol & Chambard, 2010; Gomez-Sarosi, Strasberg-Rieber, & Rieber, 2009; Panaretakis et al., 2008; Z. Wu, Wu, Tashiro, Onodera, & Ikejima, 2005) have also been shown to be facilitated by ERK1/2.

3. Phosphorylation and stabilization of p53: ERK1/2 bind to and phosphorylate p53 at Ser15, which subsequently promotes p53 stabilization and accumulation via inhibiting its association with MDM2, a negative regulator of p53 (Cagnol & Chambard, 2010).

Chapter 3: Materials and methods

3.1 Resources and Materials

3.1.1 Cell lines and culture media

Human acute T lymphocytic leukemia cells (Jurkat and Molt-4) were purchased from ATCC (VA, USA). Tissue culture media such as RPMI 1640 GlutaMAX medium, phenol red free RPMI, as well as, biochemical such as phosphate-buffered saline (PBS), fetal bovine serum (FBS), trypsin-EDTA, and penicillin-streptomycin were bought from Gibco BRL (NY, USA).

3.1.2 Reagents, chemicals, and inhibitors

Sanguinarine chloride hydrate, methylthiazolyldiphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), proteinase K, RNase A, ethidium bromide, L-GSH reduced, N-Acetyl-L-cysteine (NAC), L-Ascorbic acid, α-Lipoic acid, bovine liver catalase, bovine erythrocytes SOD, mitochondria peroxy yellow 1 (MitoPY1), o-phthaladialdehyde (OPA), myriocin (Myr), fumonisin B1 (FB1), desipramine hydrochloride (Desip), uridine 5′-diphosphoglucose disodium salt (UDP-glucose), phosphatidic acid (PA), octylphenoxy poly (ethyleneoxy) ethanol (IGEPAL CA-630), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), phenylmethylsufonyl fluoride (PMSF), Hanks' Balanced Salt solution (HBSS), and 1,9-Pyrazoloanthrone (SP600125), caspase-3 colorimetric assay kit and GSH assay kit were were purchased from Sigma-Aldrich (MO, USA). Pan-caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK), DL-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), C_6-ceramide, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126), and 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190) were bought from Enzo

(Lausen, Switzerland). 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), C_6-NBD-ceramide and Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) H_2O_2 assay kit were obtained from Molecular Probes (OR, USA). Calyculin A (CalA) and Okadaic Acid (OA) were from Merck Millipore (MA, USA). N-Oleoyl-ethanolamine (NOE) was from Matreya (PA, USA), N,N′- Bis[4-(4,5-dihydro-1H-imidazol-2-yl)phenyl]-3,3′-p-phenylene-bis-acrylamide dihydrochloride (GW4869) was from Santa Cruz Biotechnology (TX, USA), H_2O_2 was from Merck (NY, USA), Sodium pyruvate (Sod Pyr) was from GIBCO BRL (NY, USA), and C_6-NBD-sphingomyelin was from Avanti Polar Lipids (AL, USA). FICT Annexin V apoptosis detection kit was purchased from BD pharmingen (NJ, USA), and catalase assay kits were obtained from Cayman (MI, USA).

3.1.3 Antibodies

Anti-caspase-8, anti-caspase-9, anti-caspase-3, anti-PARP, anti-Akt pS473, anti-Akt, anti-PI3K p110β, anti-PI3K p85, anti-PDK1 pS241, anti-PDK1, anti-4EBP1 pT37/46, anti-4EBP1, anti-mTOR pS2448, anti-mTOR, anti-GSK3β pS9, and anti-ERK1/2 pT202/Y204 were purchased from Cell Signaling Technology (MA, USA). Anti-actin, anti-cIAP-1, anti-cIAP-2, anti-survivin, anti-GSK3β, and anti-ERK1/2, were from Santa Cruz Biotechnology (TX, USA). Anti-XIAP was from BD Biosciences (NJ, USA).

3.2 Experimental procedures and methods

3.2.1 Cell culture and sanguinarine treatment

Jurkat and Molt-4 cells (ATCC, VA, USA) were grown in RPMI 1640 GlutaMAX medium supplemented with 10% vol/vol heat-inactivated FBS, 50 IU/mL penicillin, and 50 µg/mL streptomycin in an incubator containing humidified atmosphere of 95% air and 5% CO_2 at 37°C. Sanguinarine was dissolved in DMSO at a concentration of 10 mM and was stored in a dark colored bottle at -20°C. The

stock was diluted to the required concentration with DMSO as and when needed. Prior to SNG treatment, cells were grown to about 80% confluence, and then exposed, at the desired concentration, to SNG for the required time period. Cells that were grown in a medium containing an equivalent amount of DMSO without SNG served as control.

3.2.2 MTT viability assay

Cell viability was measured by MTT assay. The assay involves conversion of the water soluble yellow colored MTT to an insoluble purple colored formazan. Only viable cells having active mitochondrial dehydrogenases are able to catalyze this conversion. To perform the assay, leukemic cells (20,000 cells/well) grown in 96-well flat bottom plates were exposed to the required concentration of SNG. At the desired times, 25 µL of MTT (5 mg/mL) in PBS were added to each well. The plates were then incubated for a further 4 h at 37°C. After the incubation, the plates were centrifuged at 600 g for 5 min and the media were removed from all the wells. The remaining formazan crystals were then solubilized in a 200 µL of DMSO, and the absorbance at 570 nM was measured using Victor-X3 multilabel reader (PerkinElmer, MA, USA). The cytotoxicity was expressed as percentage over control.

3.2.3 DNA fragmentation analysis

Cleavage of chromosomal DNA into oligonucleosomal fragments (180-200 bp) by endonuclease called CAD is a distinctive feature of apoptosis in many cell types. The fragmented DNA can be detected by agarose gel electrophoresis followed by ethidium bromide staining. Leukemic cells (3.5×10^6 cells/plate), treated with various concentrations of SNG, were collected by centrifugation (600 g for 5 min) and the pellets were washed with PBS, and re-suspended in 200 µL of lysis buffer (50 mM Tris-HCl, pH 7.5, 3% nonionic detergent IGEPAL CA-630, and 20 mM

EDTA) for 10 min. The samples were centrifuged (1000 g for 5 min) and 10 µL of 20% SDS were added to the supernatant. RNA was digested using RNase A (40 µg/mL at 56°C for 2 h), followed by Proteinase K (40 µg/mL at 56°C for 2 h) to remove the proteins. The DNA was then precipitated with 0.1 vol of 3 M sodium acetate and 2.5 vol of ice-cold absolute ethanol. After centrifugation (13,500 g for 20 min), the DNA pellets were washed with 70% ethanol and air-dried. The dried pellets were re-suspended in 20 µL TE buffer (10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA) and incubated at 65°C for 5 min. The re-suspended DNA was subjected to electrophoresis (constant voltage of 70 V for 2–3 h) on 1.8% agarose gel containing 1 µg/mL ethidium bromide and photographed under UV transillumination.

3.2.4 Fluorescein isothiocyanate (FITC) - Annexin V propidium iodide FACS analysis for apoptosis quantification

During early apoptosis phosphatidylserine, which is normally found exclusively in the inner leaflet of the plasma membrane, translocates to the cell surface. This can be detected by using FITC (fluorochrome)-conjugated Annexin V, a phosphatidylserine binding protein. During the late phase of apoptosis, cells lose its membrane integrity which allow the staining of DNA with a fluorescent dye, propidium iodide (PI). By conducting FACS analysis using FITC-Annexin V and PI together, both early (annexin V-positive, PI-negative) and late (annexin V-positive and PI-positive) apoptotic cells can be identified and quantified. Annexin V-PI apoptosis measurement was performed according to the manufacturer's protocol with slight modification. Briefly, SNG-treated cells (1×10^6 cells/plate) were washed twice with ice cold PBS and re-suspended in 1 mL of 1X binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, and 2.5 mM $CaCl_2$). To the 100 µL of re-suspended solution, 3 µL of FITC-Annexin-V and 3 µL of PI were added. After 15 min incubation at room temperature, 400 µL of binding buffer was added to each

tube and apoptosis was quantified by BD FACSCanto II and FACSDiva software (MA, USA).

3.2.5 Enzymatic caspase-3 assay

The assay is based on the hydrolysis of Ac-DEVD-pNA by caspase-3, resulting in the release of yellowish colored p-nitroaniline (pNA) moiety which can be measured at 405 nM. The assay was performed according to the kit manufacturer's protocol with slight modification. Briefly, SNG treated cells (3.5×10^6 cells/plate) were washed twice with ice cold PBS and re-suspended in 200 µL of lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, and 5 mM DTT). The cell lysates were centrifuged (20,000 g for 15 min), total protein content was determined (Bio-Rad protein assay), 100 µg of protein was incubated (37°C for 90 min) with 10 µL of Ac-DEVD-pNA (20 mM), and the volume was made up to 100 µL using assay buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT). After the incubation, the absorbance was read using Victor-X3 multilabel plate reader.

3.2.6 SDS-PAGE and Western blot analysis

SDS-PAGE and Western blotting are used to detect specific protein in a given sample. In this technique, proteins are separated based on their molecular weight using SDS-PAGE, and then transferred on to a membrane. The membrane is then stained with labelled antibodies specific to the target protein, which can be then visualized as bands. By analyzing the mobility and intensity of the bands, expression details of target proteins in the sample could be identified. Leukemic cells (3.5×10^6 cells/plate) treated with SNG were collected by centrifugation (600 g for 5 min). Pellets were washed twice with ice cold PBS and then re-suspended in 200 µL of RIPA lysis buffer (50 mM Tris HCl, pH 7.4, 1% IGEPAL CA-630, 40 mM NaF, 10 mM NaCl, 10 mM Na_3VO_4, 1 mM PMSF, 10 mM DTT, and EDTA-free Rosh protease inhibitor tablets per 20 mL buffer). The cell lysates were centrifuged

(20,000 g for 15 min) and total protein content were determined (Bio-Rad protein assay), and the sample was prepared by mixing with 6X loading buffer and boiling at 100°C for 3 min. Equivalent amount of proteins were resolved on a 7-15% SDS-PAGE gel using Bio-Rad electrophoresis apparatus (Bio-Rad Laboratories, CA, USA). Following the electrophoresis, the proteins were transferred on to nitrocellulose membrane (Whatman, Little Chalfont, UK) by the wet transfer method using Bio-Rad electrotransfer apparatus (Bio-Rad Laboratories, CA, USA). The membranes were then blocked with 5% nonfat milk (in Tris buffer saline containing 0.1% Tween 20), incubated with the desired primary antibodies (at room temperature for 2 h), followed by corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (at room temperature for 1 h). The bands were then visualized using the enhanced chemiluminescence reagent (Thermo Scientific, MA, USA) according to the manufacturer's protocols and recorded on Kodak BioMAX MR Film (Carestream Health, NY, USA).\

3.2.7 Intracellular measurement of reactive oxygen species

2',7'-dichlorodihydrofluorescein diacetate is a cell-permeable fluorogenic probe retained intracellularly by cellular esterase-mediated deacetylation to 2', 7'-dichlorodihydrofluorescin (DCFH). Upon oxidation by ROS, the non-fluorescent DCFH rapidly gets oxidized to a highly fluorescent 2', 7'-dichlorofluorescein (DCF). The fluorescence intensity can be observed under fluorescence microscopy and measured using spectrophotometry. Leukemic cells were pre-incubated with 25 µM H_2DCFDA for 30 min, washed twice with HBSS buffer, treated with SNG for 30 min, and washed again with HBSS buffer. Cells were then photographed under DP71 fluorescent microscope (Olympus, Tokyo, Japan) and fluorescence intensity was quantitatively measured using Victor-X3 multilabel plate reader at 485 nM for excitation and at 535 nM for emission.

3.2.8 Amplex red assay for hydrogen peroxide

Amplex Red, in the presence of HRP, reacts with H_2O_2 in a 1:1 stoichiometry to produce highly fluorescent resorufin which can be measured using spectrophotometry. The assay was performed according to the kit manufacturer's protocol with slight modification. Briefly, leukemic cells (20,000 cells/well) grown in 96 well black plates were incubated with 100 µL of reaction mixture (50 µM Amplex Red in DMSO, 100 µL of 0.1 U/mL HRP in Krebs–Ringer phosphate buffer, and required dose of SNG). The cells were incubated for 30 min in the dark, and fluorescence intensity was quantitatively measured using Victor-X3 multilabel reader at 540 nM for excitation and 590 nM for emission.

3.2.9 Detection of mitochondrial hydrogen peroxide

Mitochondria peroxy yellow 1 is a fluorescent probe that specifically localizes to the mitochondria and selectively respond to H_2O_2 by fluorescence enhancement. Mitochondrial-derived H_2O_2 was detected as described previously (Dickinson, Lin, & Chang, 2013) with slight modification. Briefly, leukemic cells (0.5×10^6 cells/well in 6 well plates) were exposed to 10 µM of MitoPY1 for 1 h. After the incubation, cells were centrifuged (600 g for 5 min), washed with PBS, and the medium was replaced with fresh medium containing SNG and inhibitors. Following a 30 min incubation, cells were washed with PBS and supplied with phenol red free RPMI. Changes in fluorescence was photographed using a DP71 fluorescent microscope (Olympus, Tokyo, Japan).

3.2.10 Catalase activity assay

Catalase reacts with methanol in the presence of H_2O_2 to form formaldehyde. The formaldehyde produced reacts with purpald (4-Amino-3-hydrazino-5-mercapto- 1,2,4-triazole), and the adduct formed from the reaction is oxidized by potassium periodate under alkaline condition to give a purple colored

compound which can be measured using spectrophotometry. The assay was performed according to the kit manufacturer protocol with slight modification. Briefly, after SNG treatment, the cells (3.5×10^6 cells/plate) were collected by centrifugation (600 g for 5 min), washed with ice cold PBS, and homogenized in 200 µL of lysis buffer (50 mM K_2HPO_4, pH 7.0, and 1 mM EDTA). Cell lysates were centrifuged (20,000 g for 15 min), 20 µL of supernatants were incubated with 100 µL of assay buffer (100 mM K_2HPO_4, pH 7.0), 30 µL of methanol, and 20 µL of H_2O_2 (8.82 M) in a 96 well microtiter plate. The plate is then shaken for about 20 min, 30 µL of potassium hydroxide (10 M), and 30 µL of purpald were added. The plate is shaken for further 20 min, and 10 µL of potassium periodate was added to each well. After 5 min incubation, absorbance was read at 540 nM using Victor-X3 multilabel plate reader.

3.2.11 Determination of total glutathione

The assay is based on the continuous reaction of GSH and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to produce yellow colored 2-nitro-5-thiobenzoic acid (TNB) and glutathione disulfide (GSSG). The GSSG formed can be recycled back to GSH by GR in the presence of NADPH and the reaction is repeated to produce more yellow color which can be measured using spectrophotometry at 412 nM. The assay was performed according to the kit manufacturer's protocol with slight modification. Briefly, after the SNG treatment, cells (3.5×10^6 cells/plate) were collected by centrifugation (600 g for 5 min), washed with ice cold PBS, deproteinized with 200 µL of 5% 5-Sulfosalicylic acid, and centrifuged (20,000 g for 15 min) in order to remove the precipitated protein. Working mixture was prepared by adding 228 µL of the GR enzyme solution (6 units/mL) and 228 µL of DTNB stock solution (1.5 mg/mL) to 8 mL of assay buffer (100 mM K_2HPO_4 buffer, pH 7.0, and 1 mM EDTA). The supernatant (20 µL) was incubated with 150 µL of working mixture,

and 50 µL of NADPH (0.16 mg/mL). Absorbance was read using Victor-X3 multilabel plate reader.

3.2.12 Intracellular ceramide measurement

In this assay, ceramide was converted to sphingosine using CDase enzyme. The sphingosine formed is derivatized with OPA, which can be quantified using HPLC as described previously (Thayyullathil, Chathoth, Kizhakkayil, et al., 2011). Briefly, after the SNG treatment, cells (3.5×10^6 cells/plate) were collected by centrifugation (600 g for 5 min), washed in PBS, and lysed using ceramide assay lysis buffer (50 mM Tris, pH-7.4, and 0.4% IGEPAL CA 630) by freeze and thaw method. Lysate were heated at 70°C for 5 min in a water bath to precipitate the proteins, and centrifuged (20,000 g for 15 min) at 4°C. The reaction was started by incubating 10 µL of supernatant with 20 ng of recombinant human neutral ceramidase (nCDase) enzyme (10 µL) for 1 h at 37°C. During this incubation, any ceramide in the sample will be converted in to sphingosine. The reaction was stopped by adding 55 µL of stopping buffer (1:9; 0.07 M potassium hydrogen phosphate buffer: methanol). After stopping the reaction, the sphingosine formed was derivatized with OPA reagent (12.5 mg OPA dissolved in 250 µL ethanol and 12.5 µL β-mercaptoethanol. The volume was made up to 12.5 mL with 3% w/v boric acid). The mixture was allowed to stand in the dark for 30 min and an aliquot of 25 µL was analyzed by HPLC using Waters 1525 binary pump system, Waters XTerra C18 column (5 µM, 3 mM × 250 mM), and Waters 2475 fluorescence detector (Walters, MA, USA) at an excitation and emission wavelength of 340 nm and 455 nm, respectively. The mobile phase used was 20% methanol and 80% 1:9 stopping buffer at a flow rate of 0.5 mL/min.

3.2.13 Assay for sphingomyelinase, ceramidase and glucosylceramide synthase activity

The assay is based on the conversion of fluorescent 7-nitro-2-1,3-benzooxadiazolyl amino-dodecanoic acid (NBD)-conjugated substrate by corresponding enzyme into NBD-conjugated product. The enzyme activity was measured as described previously (Thayyullathil, Chathoth, Hago, et al., 2011). Briefly, NBD-conjugated product was separated from remaining substrate using TLC on silica gel. The spot corresponding to the NBD-conjugated product were scraped, incubated with ethanol at 37°C for 5 min to extract the compounds from silica, and fluorescence intensity was quantitatively measured using Victor-X3 multilabel reader at 485 nM excitation and at 535 nM emission.

Assays for aSMase and neutral sphingomyelinase (nSMase) activities were performed as previously described (Loidl, Claus, Deigner, & Hermetter, 2002; Taguchi et al., 2004; Zeidan et al., 2006) with slight modification. Briefly, after SNG treatment, cells (3.5×10^6 cells/plate) were collected by centrifugation (600 g for 5 min), washed in PBS, and lysed using 200 µL of aSMase assay lysis buffer (250 mM CH_3COONa, pH 5.0, and 0.2% Triton X-100) and nSMase assay lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, 10 µg/mL pepstatin A, 0.15 U/mL aprotinin, and 50 µg/mL leupeptin) for corresponding enzyme activities. The cell lysates were centrifuged (20,000 g for 15 min), and total protein content were determined (Bio-Rad protein assay). Lysates normalized to equal total proteins (50 µg) were incubated with 50 µL of corresponding assay buffer. The assay buffer for different enzyme activities are: aSMase assay reaction buffer (0.1 M CH_3COONa, pH 5, 10 µM C_6-NBD-sphingomyelin, and 0.1% Triton X-100) and nSMase assay reaction buffer (0.1 M Tris-HCl, pH 7.5, 10 µM C_6-NBD-sphingomyelin, 10 mM $MgCl_2$, 0.1% Triton X-100, and 5 mM DTT). Final volume of 0.1 mL was made using corresponding lysis buffer. The reaction mixture was

incubated at 37°C for 1 h. Reaction was stopped by addition of 90 µL of distilled water and 200 µL of chloroform/methanol (2:1, vol/vol). After properly mixing, the tubes were centrifuged at 1000 g for 5 min to separate the two phases. The lower organic phase was collected, dried in a speed vacuum concentrator (Savant Instruments, Hyderabad, India), and dissolved in 25 µL of chloroform/methanol (2:1, vol/vol). The sample (20 µL) were applied to the TLC plate and developed using chloroform/methanol/12 mM $MgCl_2$ in distilled water (65:25:4, vol/vol) as solvent and the fluorescent intensity corresponding to the SMase-mediated conversion of C_6-NBD-sphingomyelin to C_6-NBD-ceramide was quantified.

Assay for GCS activity was performed using a similar method as described previously (Kizhakkayil et al., 2012; Taguchi et al., 2004) with slight modification. Briefly, after the treatment and harvesting, cells were lysed in 200 µL of GCS assay lysis buffer (20 mM Tris–HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 1 mM PMSF, and 2.5 µg/mL leupeptin) using a homogenizer. Lysates were centrifuged (1000 g for 5 min at 4°C), supernatant normalized to equal total proteins (50 µg) were incubated (37°C for 2 h) with 25 µL of GCS assay reaction buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 µg/mL C_6-NBD-ceramide, 100 µg/mL phosphatidylcholine, 500 µM UDP-glucose. Final volume of 0.1 mL using corresponding lysis buffer). The reaction was stopped by the addition of 90 µL of distilled water and 200 µL of chloroform/methanol (2:1, vol/vol). After properly mixing, the tubes were centrifuged at 10,000 g for 1 min to separate the two phases. The lower organic phase was collected, dried, and developed in similar way to the SMase activity assay and the fluorescent intensity corresponding to the GCS-mediated transfer of glucose residue from UDP-glucose to C_6-NBD-ceramide was quantified.

In the case of acid ceramidase (aCDase) and nCDase activity assay, after the treatment and harvesting, the cells were lysed in 200 µL of aCDase assay lysis

buffer (50 mM CH$_3$COONa, 5 mM MgCl$_2$, 1 mM EDTA, pH 4.5, and 0.5% Triton X-100) or nCDase assay lysis buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 0.5% Triton X-100, 5 mM Cacl$_2$, and 2mM PMSF). The cell lysates were centrifuged (20,000 g for 15 min), lysates normalized to equal total proteins (50 µg) were incubated with 50 µL of aCDase assay reaction buffer (100 µM D-erythro-C12-NBD-ceramide in 250 mM CH$_3$COONa, pH 5.0, and 0.2% IGEPAL-CA 6300) or nCDase assay reaction buffer (100 µM D-erythro-C12-NBD-ceramide in 50 mM Tris buffers, pH 7, and 0.3% Triton X-100). Final volume of 0.1 mL was made using corresponding lysis buffer. The reaction mixtures were incubated at 37°C for 1 h. The reaction was stopped by adding 100 µL of chloroform/methanol (1:1, vol/vol). The sample was dried, dissolved in 25 µL of chloroform/methanol (2:1, vol/vol), applied to a TLC plate, and developed using chloroform, methanol, and ammonia (75:15:0.9, vol/vol). Fluorescent intensity corresponding to the conversion of C12-NBD-ceramide to NBD-dodecanoic acid was quantified.

3.2.14 Statistical analysis

Statistical analysis was performed using Graph pad Prism 5.0 software. Data are shown as mean ± standard deviation. Significance was analyzed by one-way anova using Bonferroni post hoctest. A difference was considered significant when $p<0.05$. $p<0.05$ (*); $p<0.01$ (**); $p<0.001$ (***).

Chapter 4: Results and findings

4.1 Sanguinarine induces growth arrest and apoptosis in human leukemic cells

The primary objective of this study was to determine the intracellular mechanisms by which SNG inhibits proliferation and induces cell death in human leukemic cells. To systematically address the inhibitory effect of SNG on human leukemic cells, viability was evaluated by MTT assay following the SNG treatment. As shown in Fig. 4.1.A and B, SNG treatment resulted in a significant dose- and time-dependent growth inhibition in both Jurkat and Molt-4 cells.

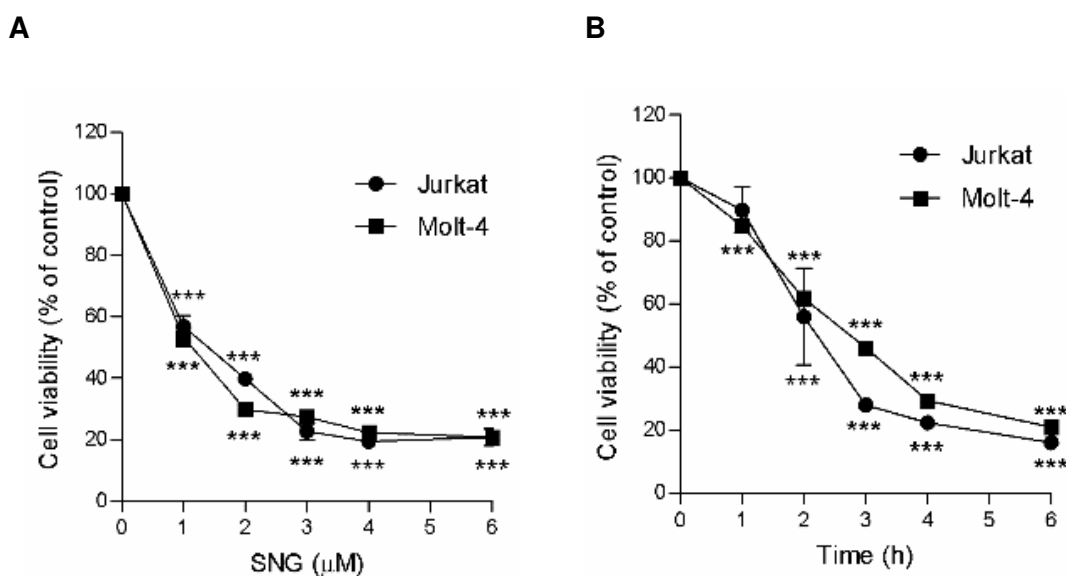**A**   **B**

Figure 4.1. Sanguinarine induces loss of viability in human leukemic cells. (A) Leukemic cells were treated with the indicated concentration of SNG for 4 h, (B) Jurkat and Molt-4 cells were treated with 3 µM and 1.5 µM SNG, respectively, for the indicated time period. Cell viability was assessed. Data shown are means ± SD (n=3) (***p<0.001 vs control).

Several lines of evidence indicate that SNG induces apoptotic cell death in a variety of cancer models (Adhami et al., 2004; Choi et al., 2008; Jang et al., 2009; J. S. Lee et al., 2012; Rosen et al., 2015). To determine whether SNG-induced loss of viability was as a result of apoptosis, two of the defining characteristics of apoptosis

such as plasma membrane blebbing and DNA fragmentation were evaluated following SNG treatment. As shown in Fig 4.2.A, in response to SNG treatment, some of the cells showed membrane blebbing as observed under an inverted microscope. In line with this, treatment with SNG also resulted in the appearance of "DNA ladder" fragmentation pattern (Fig. 4.2.B), indicating that SNG induces apoptosis in human leukemic cells. Cell surface exposure of phosphatidylserine is a classic feature of cells undergoing apoptosis, which can be detected by using Annexin V-FITC. During the late phase of apoptosis, cell loses its membrane integrity which allows the entry of PI, a DNA-binding fluorescent dye. By conducting FACS analysis using FITC-Annexin V and PI together, both early and late phase of apoptotic cells can be identified and quantified. To quantitatively analyze SNG-induced apoptotic cell death, Annexin V-FITC/PI staining followed by flow cytometry analysis was performed. As shown in Fig. 4.2.C, significant increase in apoptosis was observed in cells treated with increasing concentrations of SNG.
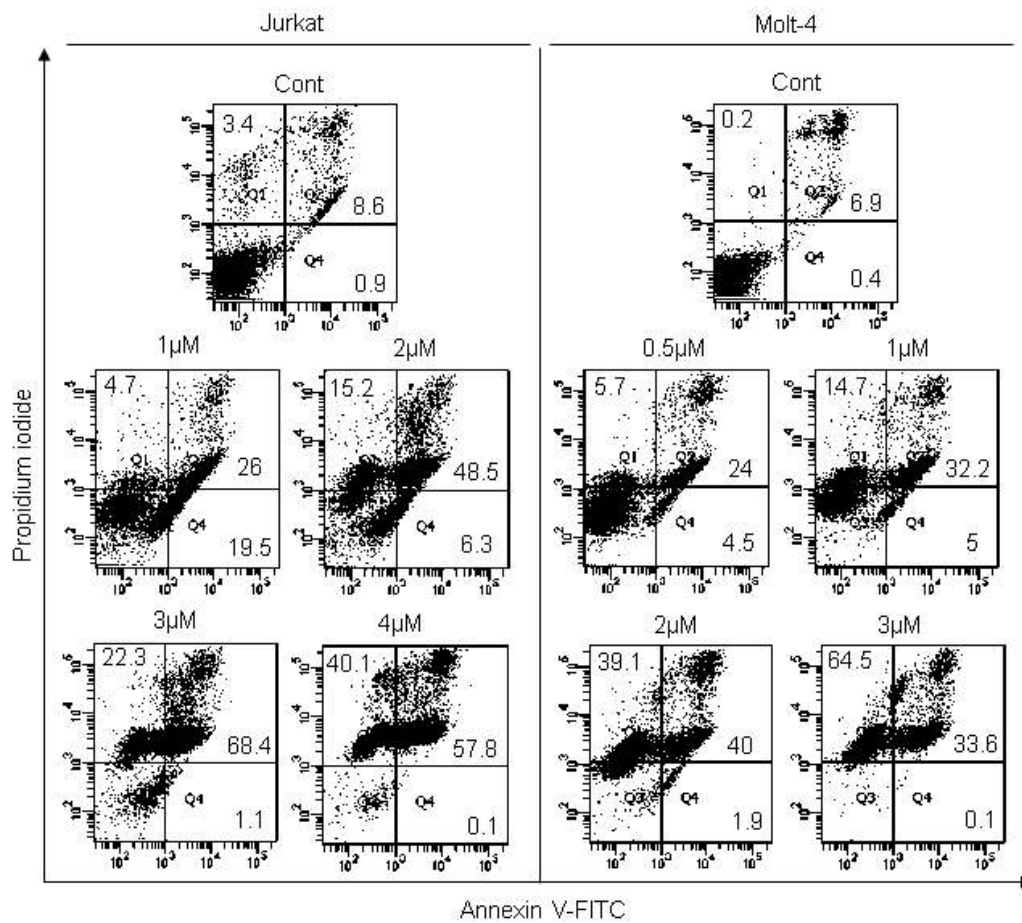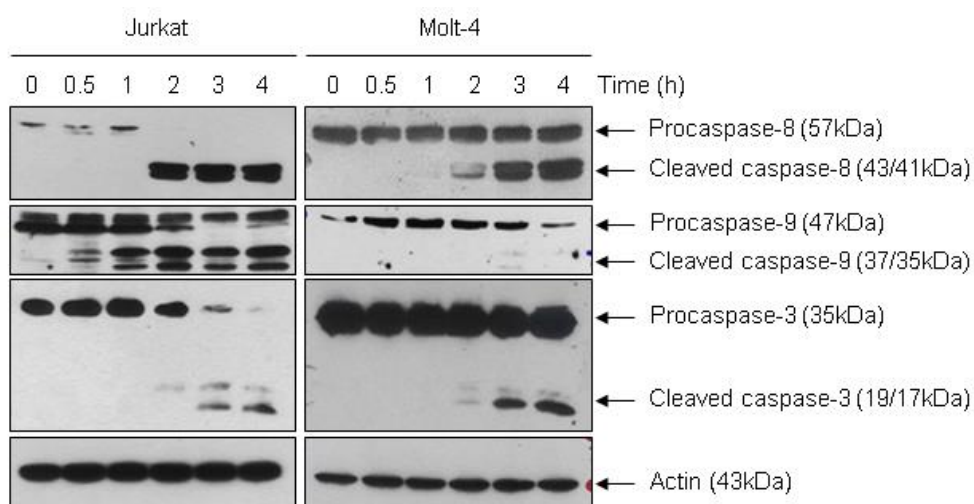
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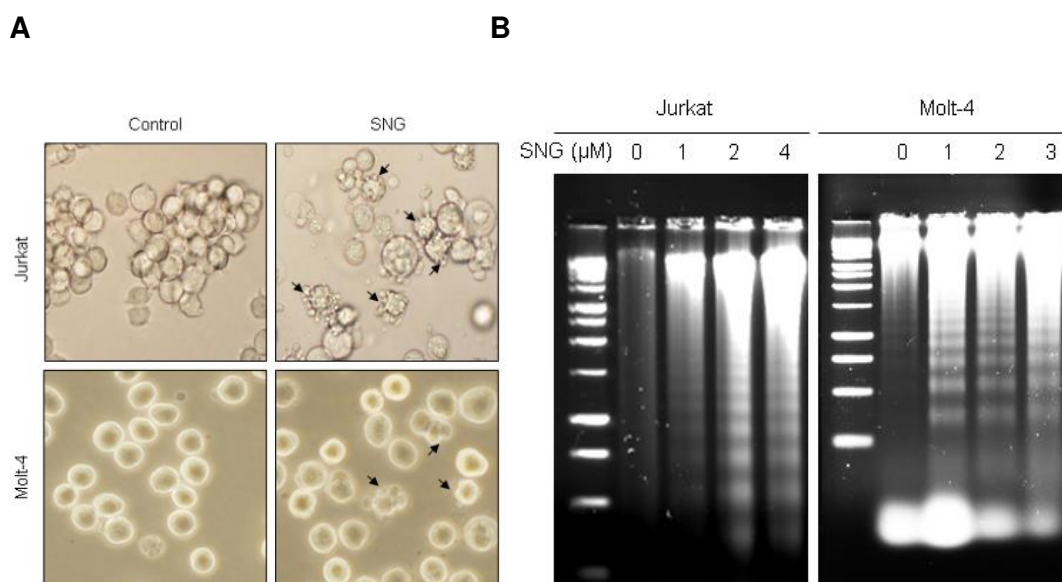
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Figure 4.2. Sanguinarine induces apoptosis in human leukemic cells. (A) Jurkat cells were treated with 3 µM and Molt-4 cells were treated with 1.5 µM SNG for 4 h. Images were taken under an inverted phase-contract microscope. Blebs are indicated with arrows. (B) Leukemic cells were treated with the indicated concentration of SNG for 4 h and DNA fragmentation assay was performed. (C) Cells treated with the indicated concentrations of SNG for 4 h, were stained with Annexin V-FITC/PI, and apoptosis was quantified using FACS analysis. Each quadrant represent cells in different stages; Q3 viable, Q4 early apoptosis, Q2 late apoptosis, and Q1 necrosis.

Both caspase-dependent and -independent mechanisms have been ascribed

to the membrane blebbing. For instance, membrane blebbing may be mediated by

DAPK via caspase-independent mechanism or by caspase-dependent cleavage of

PAK2, gelsolin, and ROCK I (Fischer et al., 2003; U. Ziegler & Groscurth, 2004).

Similarly, DNA fragmentation may be mediated by caspase-dependent cleavage of

ICAD or by EndoG and AIF via caspase-independent mechanism (Fischer et al.,

2003; Saraste & Pulkki, 2000). Cell surface exposure of phosphatidylserine can also

occur via caspase-dependent and –independent mechanisms (Fischer et al., 2003; Saraste & Pulkki, 2000). However, in most instances, apoptosis is initiated and executed by caspases. Next, we explored whether SNG-induced apoptosis was associated with caspase activation. Western blot analysis revealed that SNG treatment markedly increased the formation of active forms of cleaved caspase-8, -9, and -3 (Fig. 4.3.A). Consistently, the cleavage of caspase-3 substrate PARP was also increased in a dose- and time-dependent manner in SNG-treated cells (Fig. 4.3.B). Quantitative analysis of caspase-3 activation was also carried out as described in the materials and methods. Treatment with SNG prominently increases the activity of caspase-3 in both leukemic cells (Fig. 4.3.C).
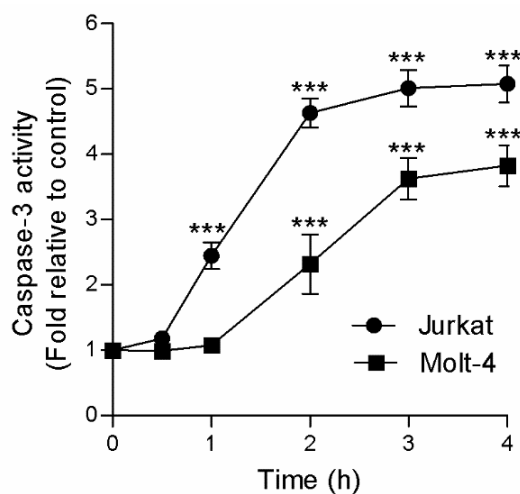
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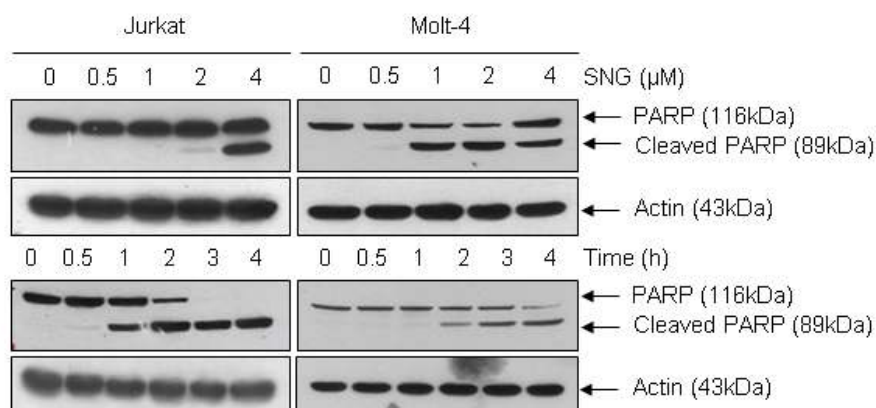
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Figure 4.3. Sanguinarine induces caspase-dependent apoptosis in human leukemic cells. (A) Jurkat and Molt-4 cells were treated with 3 µM and 1.5 µM SNG, respectively, for the indicated time period. After the treatment, Western blot analysis of caspase-8, -9, and -3 was performed. (B) Leukemic cells were treated with the indicated concentration of SNG for 4 h (upper panel), and Jurkat cells were treated with 3 µM and Molt-4 cells were treated with 1.5 µM SNG for the indicated time period (lower panel). PARP cleavage was analyzed by Western blot. Actin was used as a loading control (C) Jurkat and Molt-4 cells were treated with 3 µM and 1.5 µM SNG, respectively, for the indicated time period. Caspase-3 assay was performed. Data shown are means ± SD (n=3) (***p<0.001 vs control).

To confirm the role of caspases in SNG-induced apoptosis, cells were pre-treated with pan caspase inhibitor, Z-VAD-FMK and examined for apoptosis and cellular viability. Pre-treatment with Z-VAD-FMK significantly abolished SNG-induced caspase-3 activation (Fig. 4.4.A), apoptosis (Fig. 4.4.B and C), and loss of viability (Fig. 4.4.D), clearly demonstrating the role of caspase in SNG-induced apoptosis in human leukemic cells.

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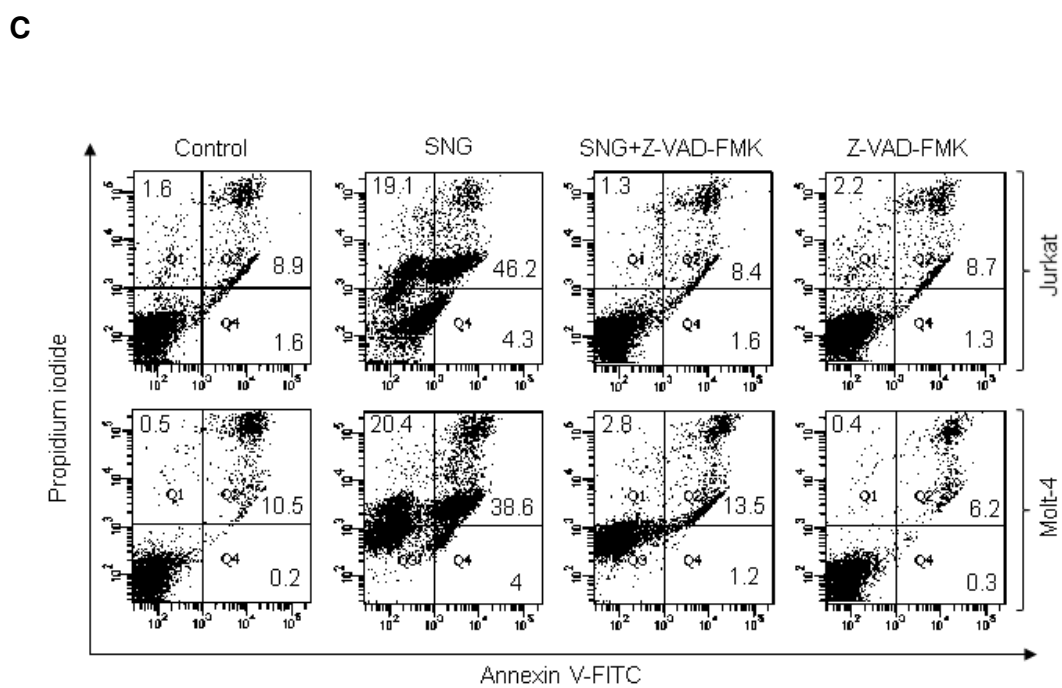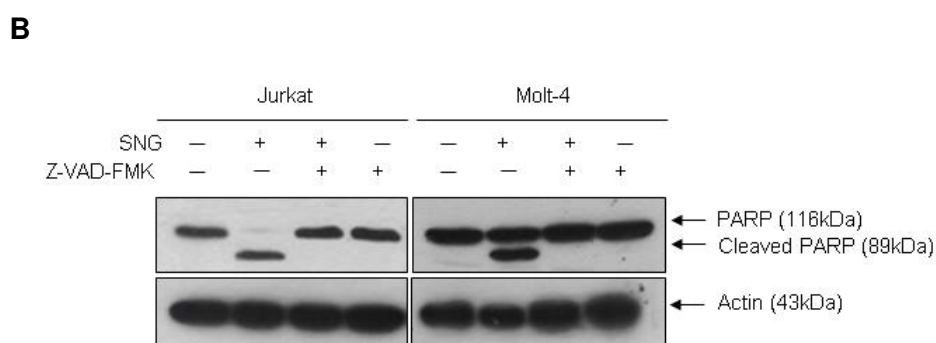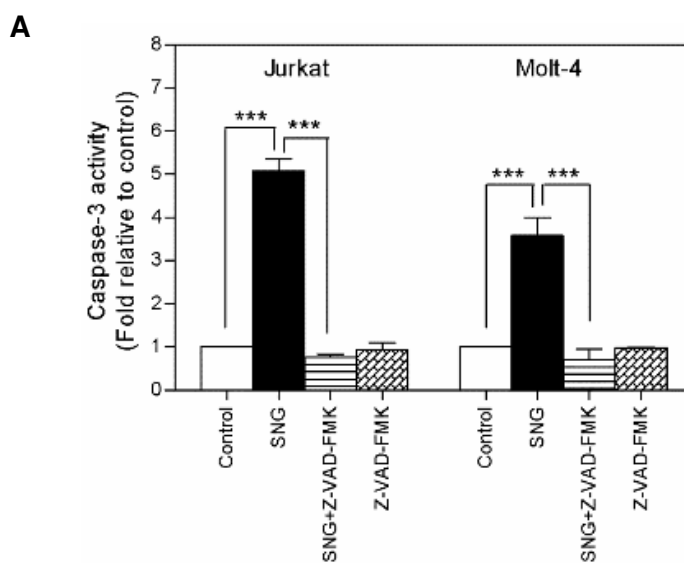
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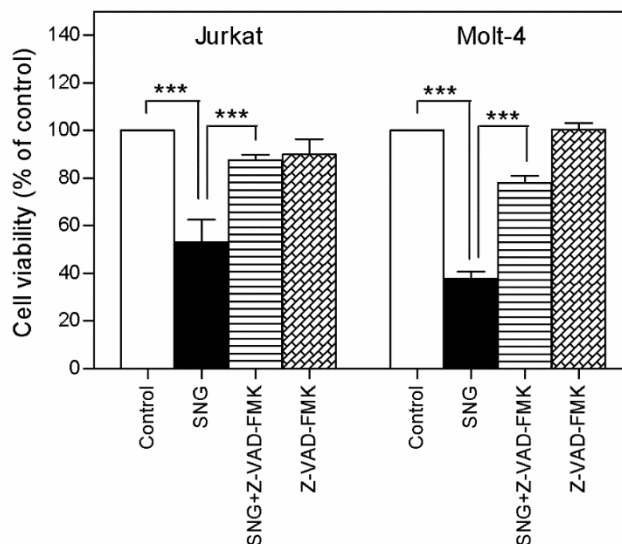
Figure 4.4. Caspase inhibitor attenuates SNG-induced apoptosis in human leukemic cells. Cells were pre-treated with 50 µM Z-VAD-FMK for 1 h followed by SNG (3 µM for Jurkat and 1.5 µM for Molt-4) treatment for 4 h. Following the treatment, (A) caspase-3 activity was measured. Data shown are means ± SD (n=3) (***p<0.001), (B) PARP cleavage was analyzed by Western blot. Actin was used as a loading control, (C) apoptosis was quantified using FACS analysis. Each quadrant represent cells in different stages; Q3 viable, Q4 early apoptosis, Q2 late apoptosis, and Q1 necrosis, and (D) cell viability was assessed by MTT assay. Data shown are means ± SD (n=3) (***p<0.001).

The process of apoptosis is negatively regulated by IAPs that inhibit apoptosis and promote resistance to chemotherapy via caspase-dependent and –independent mechanisms (Ceballos-Cancino et al., 2007; Dohi et al., 2007; Gyrd-Hansen & Meier, 2010). SNG was shown to inhibit the expression of IAPs namely cIAP-1, cIAP-2, XIAP, and survivin in human leukemic cells (Fig. 4.5). Expression of cIAP-2 in Molt-4 was not altered following SNG-treatment (Fig. 4.5). Thus, our results suggest that down-regulation of anti-apoptotic proteins is another mechanism by which SNG could induce apoptosis in human leukemic cells.

Figure 4.5. Sanguinarine induces inhibition of IAP proteins in human leukemic cells. Jurkat and Molt-4 cells were treated with 3 µM and 1.5 µM SNG, respectively, for the indicated time period. Western blot analysis of the indicated proteins were carried out. Actin was used as a loading control.
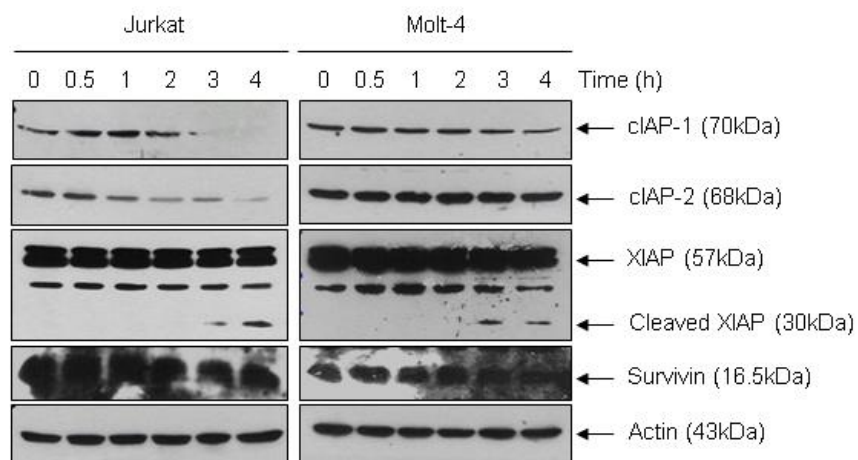
4.2 Sanguinarine-induced reactive oxygen species generation is critical for the induction of apoptosis

A large number of anticancer agents effectively kill cancer cells via increasing ROS generation or impeding the antioxidant defense mechanism (Miki et al., 2012; Thayyullathil, Chathoth, Hago, Patel, & Galadari, 2008; Thayyullathil, Rahman, Pallichankandy, Patel, & Galadari, 2014; Tsang, Chau, Kong, Fung, & Kwok, 2003; Woo et al., 2002). Previously, several reports have provided evidence for the role of ROS in the induction of apoptosis in response to SNG treatment (Dong et al., 2013; S. Kim et al., 2008; Matkar et al., 2008a, 2008b; Yin, Kim, Moon, & Lee, 2005). To the contrarily, some authors have observed that SNG-induced cytotoxicity is not associated with ROS generation (Debiton et al., 2003; Slunská, Gelnarová, Hammerová, Táborská, & Slaninová, 2010). Therefore, the second objective of our study was to investigate the role of ROS generation in SNG-induced apoptosis in human leukemic cells. To examine this, ROS generation was analyzed in SNG-treated cells by performing H_2DCFDA staining followed by fluorescent microscopy. Treatment of leukemic cells with SNG resulted in the generation of H_2DCFDA-derived fluorescence, as observed under a fluorescent microscope (Fig. 4.6.A), suggesting that SNG induces ROS generation. Simultaneously, fluorescent intensity was quantitatively measured spectrophotometrically. A dose-dependent increase in H_2DCFDA-derived fluorescence intensity was observed in both leukemic cell lines following SNG treatment (Fig. 4.6.B).

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Figure 4.6. Sanguinarine induces intracellular reactive oxygen species generation in human leukemic cells. (A) Jurkat and Molt-4 cells were treated with 3 µM and 1.5 µM SNG, respectively, for 30 min. ROS-mediated H_2DCFDA-derived fluorescence was observed under fluorescent microscopy. H_2O_2 (300 µM) was used as a positive control. (B) H_2DCFDA-derived fluorescence was quantified in leukemic cells treated with indicated concentration of SNG. Data shown are means ± SD (n=3) (*p<0.05 and ***p<0.001 vs control).

In order to establish the role of ROS in SNG-induced apoptosis, both leukemic cells were pre-treated with thiol-based ROS scavengers, namely GSH and NAC, prior to treatment with SNG. GSH is the major non-protein thiol antioxidant, which directly removes ROS or may act as a substrate for GPx and Prx antioxidant system. The rate-limiting step in the GSH synthesis is the availability of cysteine. NAC provides an alternate means of boosting intracellular GSH via elevating the intracellular cysteine pools (Lushchak, 2012). Pre-treatment with both ROS scavengers significantly attenuated SNG-induced ROS generation (Fig. 4.7.A), apoptosis as analyzed by Annexin V-FITC/PI staining and Western blot analysis of PARP (Fig. 4.7.B and C), and loss of viability as measured by MTT assay (Fig. 4.7.D). Moreover, other well-established thiol- and non-thiol-ROS scavengers such as α-lipoic acid and L-ascorbic acid also significantly reduced SNG-induced loss of viability (Fig. 4.7.E).

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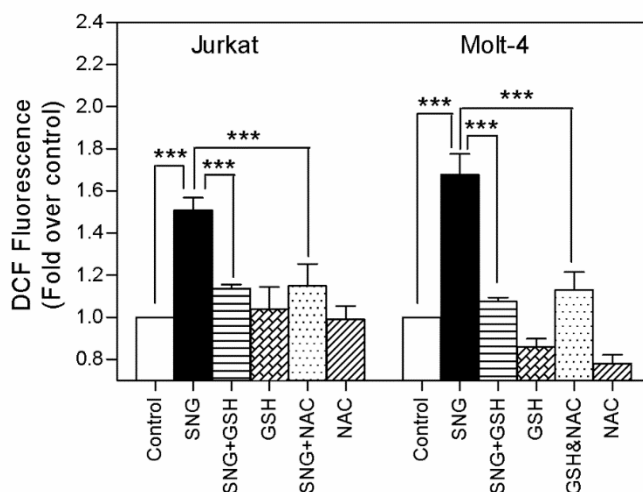
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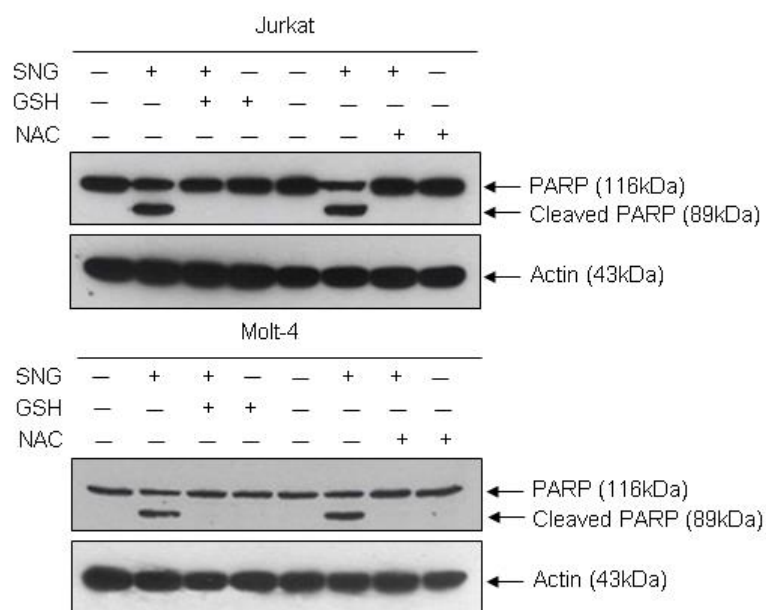
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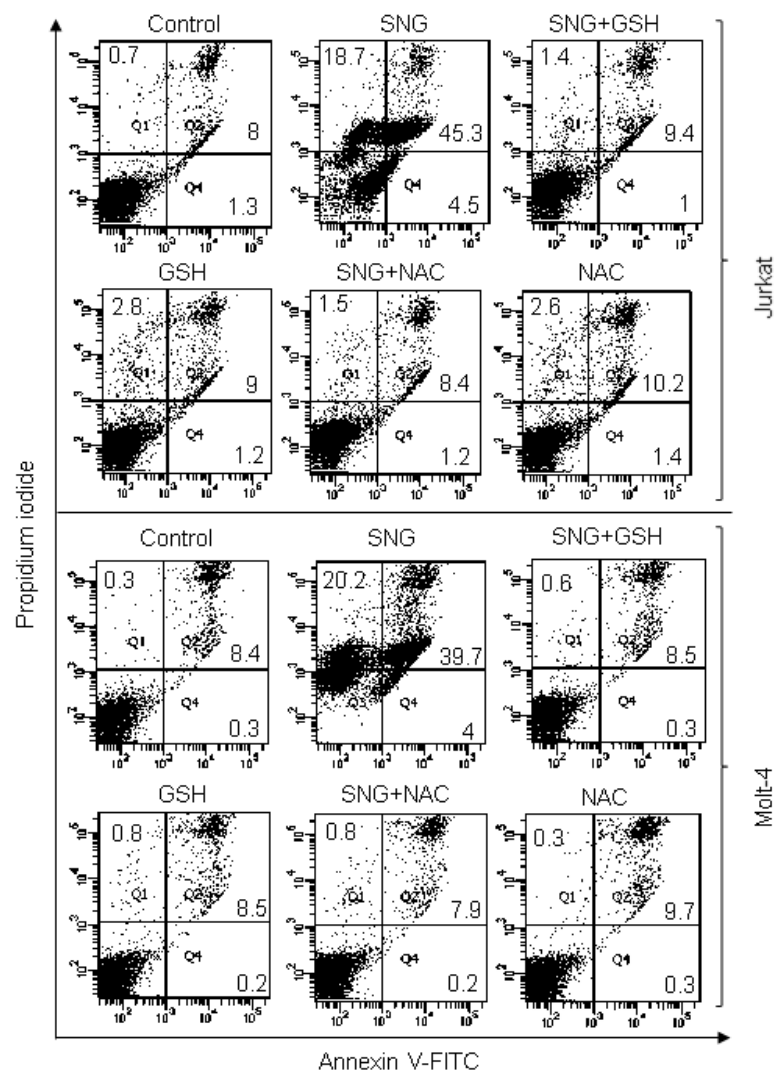
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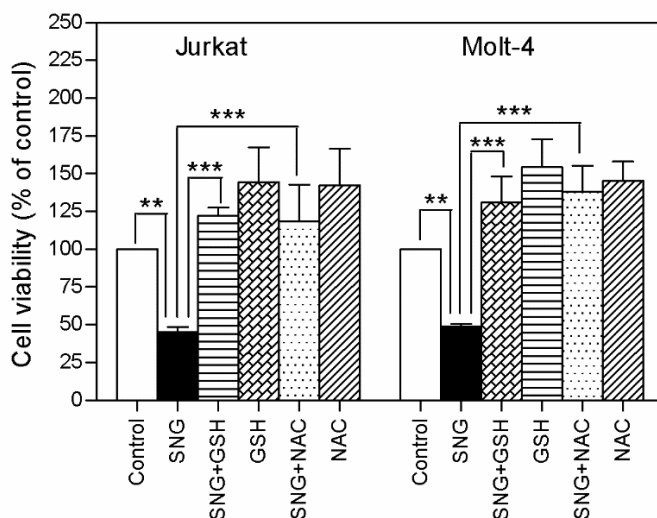
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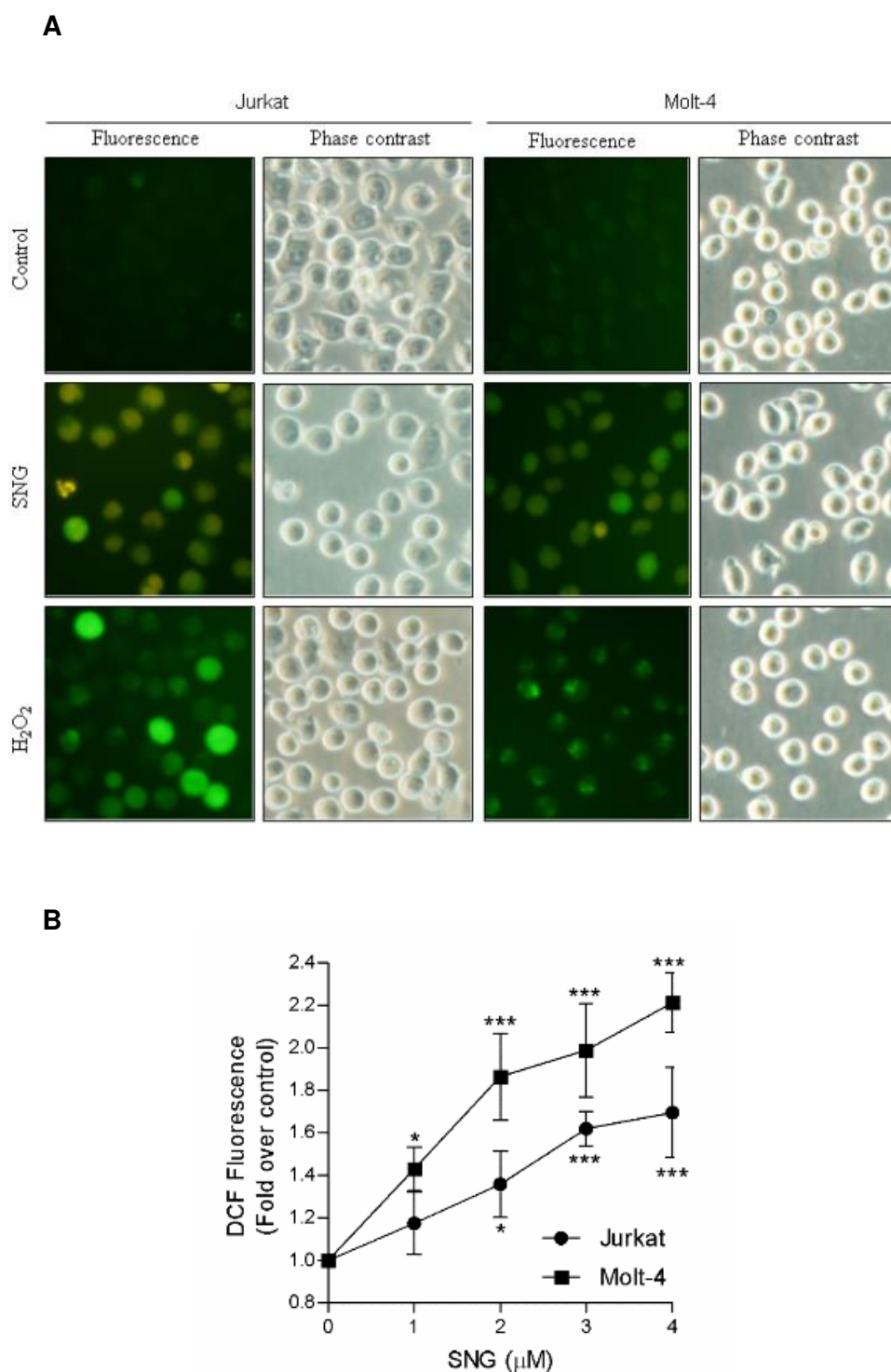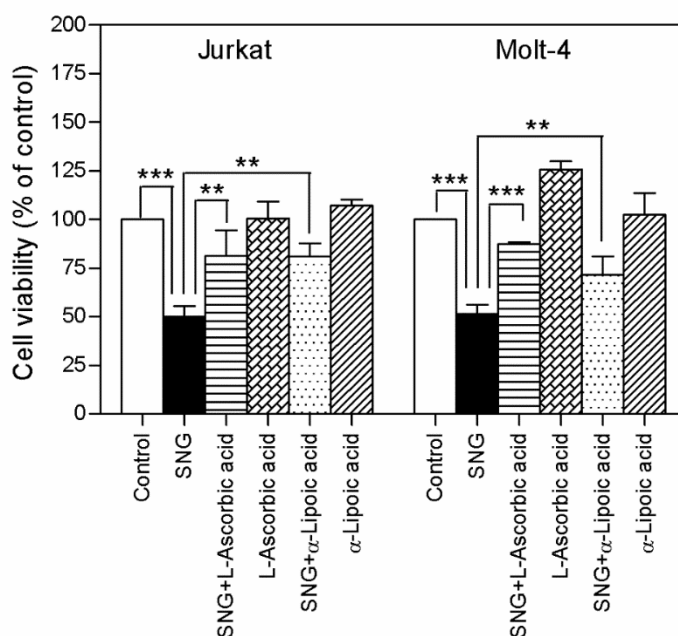

Figure 4.7. Sanguinarine induces reactive oxygen species-dependent apoptosis in human leukemic cells. Cells were pre-treated with GSH (10 mM) and NAC (10 mM) for 1 h followed by SNG (3 µM for Jurkat and 1.5 µM for Molt-4) treatment. Following the treatment (A) H$_2$DCFDA-derived florescence was analyzed by fluorometry. Data shown are means ± SD (n=3) (***p<0.001), (B and C) apoptosis was evaluated by Western blot analysis of PARP and Annexin V-FITC/PI staining, and (D) cell viability was analyzed by using MTT assay. Data shown are means ± SD (n=3) (**p<0.01 and ***p<0.001). (E) Cells were pre-treated with L-ascorbic acid (50 µM) and α-lipoic acid (50 µM) for 1 h followed by SNG (3 µM for Jurkat and 1.5 µM for Molt-4) treatment for 4 h. Following the treatment, cell viability was measured by using MTT assay. Data shown are means ± SD (n=3) (**p<0.01 and ***p<0.001)

H$_2$DCFDA-based fluorescence generation is non-specific for a particular kind of ROS. In order to distinguish the type of ROS generated by SNG, the effect of SNG in the presence of Sod Pyr and catalase (H$_2$O$_2$ scavengers), and SOD (O$_2$•−scavenger) was tested. As shown in Fig. 4.8.A and B, pre-treatment with H$_2$O$_2$ scavengers significantly inhibited the enhancement of H$_2$DCFDA-derived fluorescence and loss of viability caused by SNG, while O$_2$•− scavenger failed to do so. To confirm H$_2$O$_2$ generation further, Amplex red, a H$_2$O$_2$-specific fluorogenic probe was used. As shown in Fig. 4.8.C, SNG treatment of Jurkat cells resulted in a significant increase of Amplex red-derived fluorescence. Specific H$_2$O$_2$ scavengers such as catalase and Sod Pyr significantly abolished SNG-induced fluorescence generation (Fig. 4.8.C). Together, these data suggest that ROS plays a critical role in SNG-induced apoptosis in human leukemic cells. Since, Sod Pyr and catalase are specific scavengers for H$_2$O$_2$, these observations provide a strong basis for the involvement of H$_2$O$_2$ in SNG-induced apoptosis.

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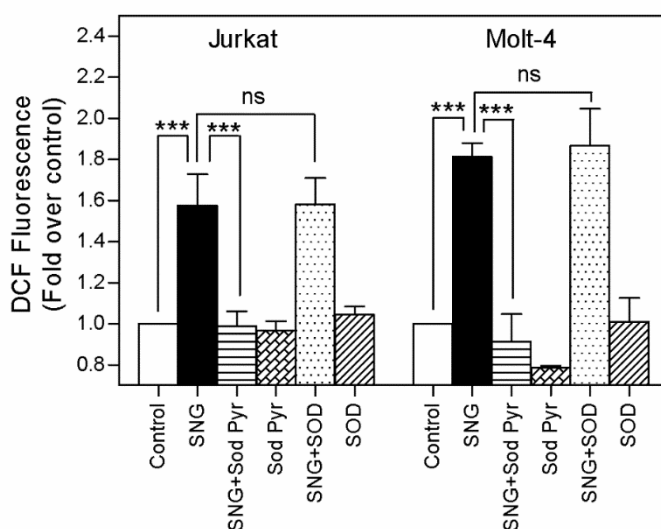
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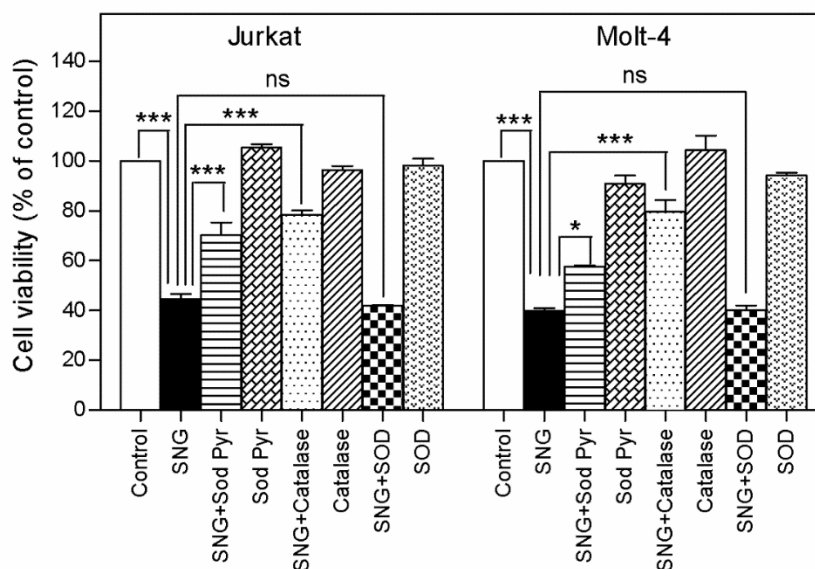
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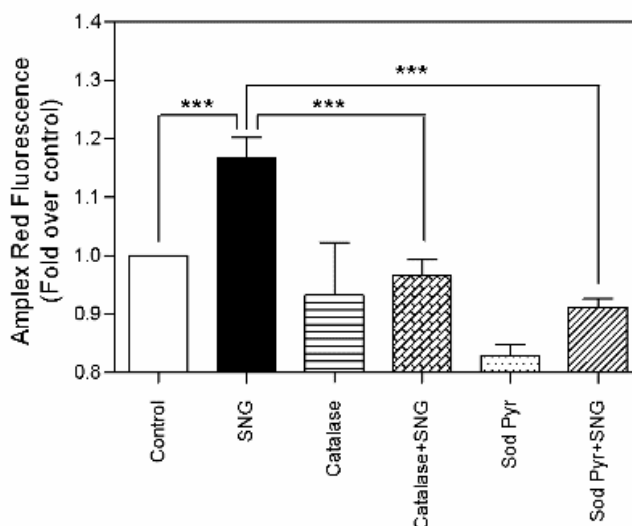
Figure 4.8. Sanguinarine induces hydrogen peroxide-dependent cell death in human leukemic cells. (A) Cells were pre-treated with Sod Pyr (500 µM) and SOD (1000 U/mL) for 1 h followed by SNG (3 µM for Jurkat and 1.5 µM for Molt-4) treatment for 4 h. H_2DCFDA-derived florescence was analyzed by fluorometry. Data shown are means ± SD (n=3) (***p<0.001 and ns: not significant). (B) Cells were pre-treated with Sod Pyr (500 µM), catalase (catalase (5000 U/mL), and SOD (1000 U/mL) for 1 h followed by SNG (3 µM for Jurkat and 1.5 µM for Molt-4) treatment for 4 h. Cell viability was analyzed by MTT assay. Data shown are means ± SD (n=3) (*p<0.05, ***p<0.001, and ns: not significant). (C) Jurkat cells pre-treated with Sod Pyr (500 µM) and catalase (5000 U/mL) were exposed to 3 µM SNG and 50 µM Amplex red. Amplex red-derived florescence was analyzed by fluorometry. Data shown are means ± SD (n=3) (***p<0.001).

The mitochondrial respiratory chain is a major source of ROS production within most mammalian cells (Sena & Chandel, 2012). MitoPY1 is a mitochondria-specific fluorescent probe that selectively responds to H_2O_2 by fluorescence enhancement. To determine whether SNG induces mitochondrial-derived H_2O_2, MitoPY1 was used. When leukemic cells were induced to undergo apoptosis by SNG, elevated levels of MitoPY1-derived fluorescence was observed, and this increase was significantly reduced by Sod Pyr and catalase (Fig. 4.9). Similar results were observed in H_2O_2-treated cells which was used as a positive control (Fig. 4.9).
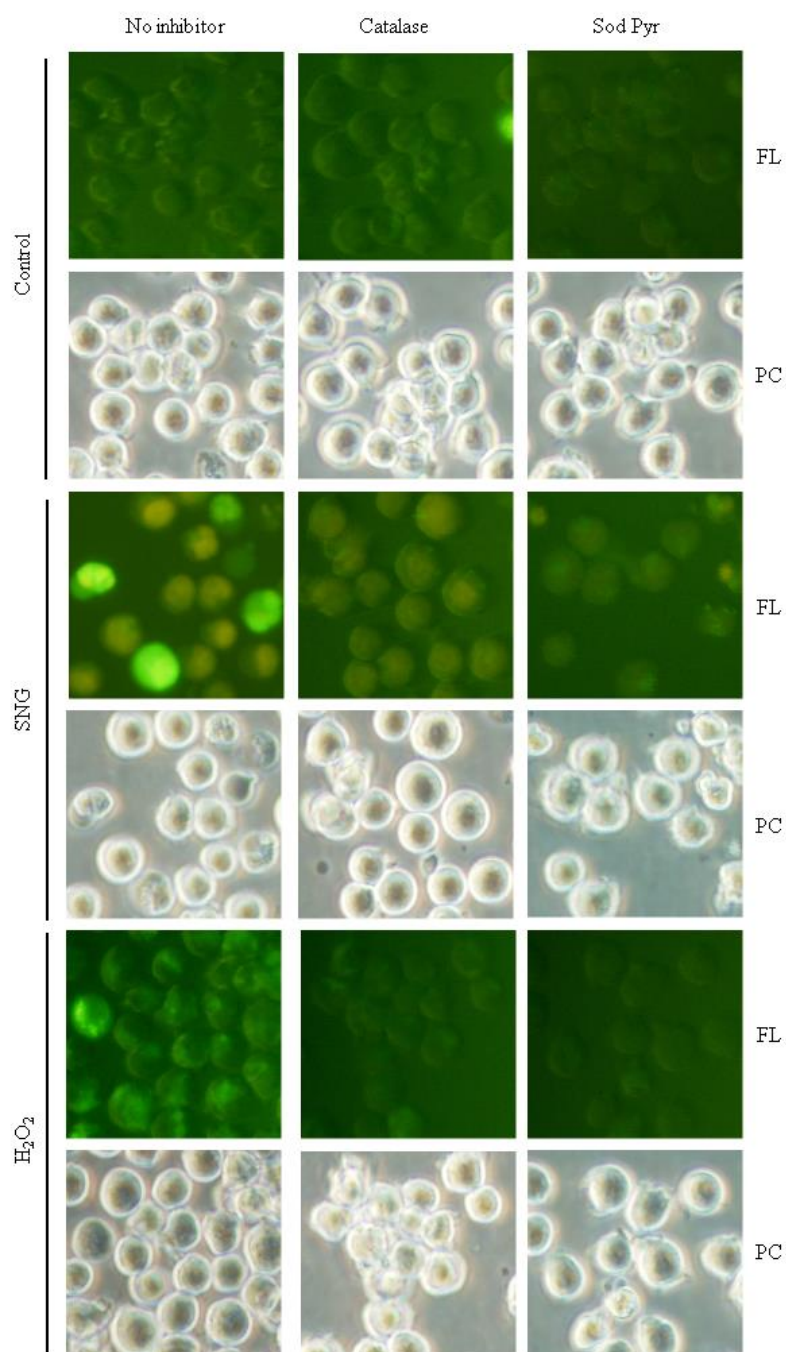
Figure 4.9. Sanguinarine induces mitochondrial-hydrogen peroxide generation in human leukemic cells. Fluorescent microscopy images were taken of Jurkat cells pre-treated with 10 µM MitoPY1 followed by SNG (3 µM) treatment in the presence and absence of Sod Pyr (500 µM) and catalase (5000 U/mL). H_2O_2 (300 µM) was used as a positive control. FL, fluorescence; PC, phase contrast.

Catalase is an important antioxidant enzyme that catalyzes the dissociation of H_2O_2 directly into H_2O and O_2 (Scibior & Czeczot, 2006). Accumulation of H_2O_2 due to catalase suppression has been implicated in the apoptosis of a variety of cancers, including cervix, ovarian, and lung cancer (Iwai et al., 2003; Yang et al., 2011). Simultaneously, catalase has been shown to protect tumor cells from ROS-mediated apoptosis (Bechtel & Bauer, 2009). In this context, enzymatic activity of catalase was measured following SNG treatment. As shown in Fig. 4.10, catalase enzymatic activity was significantly reduced upon SNG treatment in Jurkat cells by four hours post treatment.
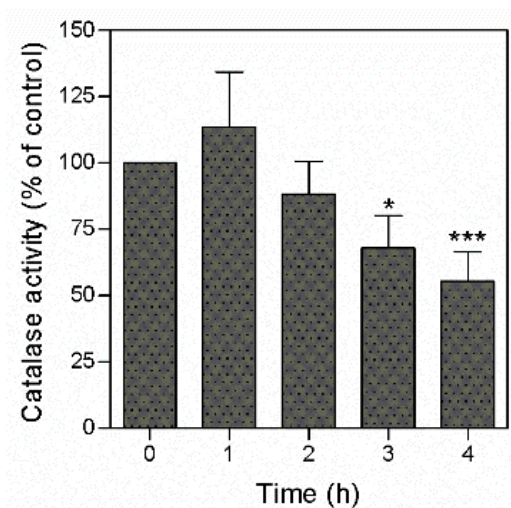
Figure 4.10. Sanguinarine decreases catalase enzymatic activity. Jurkat cells were treated with 3 µM SNG for the indicated time period and catalase activity was measured. Data shown are means ± SD (n=3) (*p<0.05 and ***p<0.001 vs control).

Depletion of intracellular GSH is considered as an early hallmark of H_2O_2-mediated oxidative stress, and a prime signaling event associated with the induction of apoptotic cell death (Franco & Cidlowski, 2009). Treatment of leukemic cells with different doses of SNG resulted in a dose-dependent decrease in cellular GSH content (Fig. 4.11.A). In order to examine if H_2O_2 generation is responsible for the observed GSH depletion, cells were pre-treated with catalase followed by SNG

treatment and cellular GSH level was quantified. As shown in Fig. 4.11.B, scavenging of H_2O_2 using catalase significantly inhibited the GSH depletion, suggesting that SNG-induced H_2O_2 generation leads to the intracellular GSH depletion.
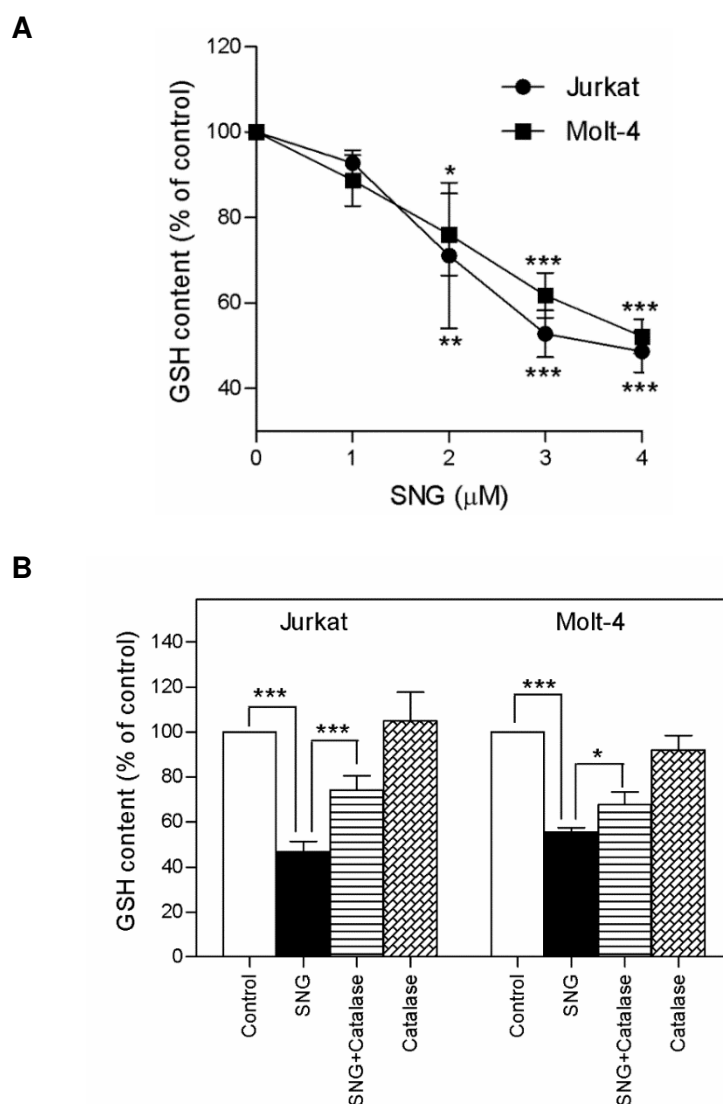
Figure 4.11. Sanguinarine induces hydrogen peroxide-dependent glutathione depletion. (A) GSH content were measured in leukemic cells treated with indicated concentration of SNG for 4 h. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, and ***p<0.001 vs control) (B) Cells pre-treated with catalase (5000 U/mL) were treated with SNG (3 µM for Jurkat and 1.5 µM for Molt-4), and examined for total GSH content. Data shown are means ± SD (n=3) (*p<0.05 and ***p<0.001).

4.3 Sanguinarine induces reactive oxygen species-dependent ceramide generation in human leukemic cells

Previously, we and others have established that ceramide is an important pro-apoptotic lipid activated in response to various anticancer agents (Galadari et al., 2015; Pettus, Chalfant, & Hannun, 2002; Thayyullathil, Chathoth, Kizhakkayil, et al., 2011; Yabu et al., 2015). In addition, a growing body of evidence suggests that ROS enhances ceramide generation through modulating ceramide biosynthetic and degradative enzymes (Galadari et al., 2013). Therefore, the third objective of our study was to elucidate the role of ceramide in SNG-induced cell death. Initially, we investigated whether ceramide is generated upon SNG treatment in leukemic cells. As shown in Fig. 4.12.A and B, SNG treatment resulted in a significant dose- and time-dependent induction of ceramide in both leukemic cell lines.
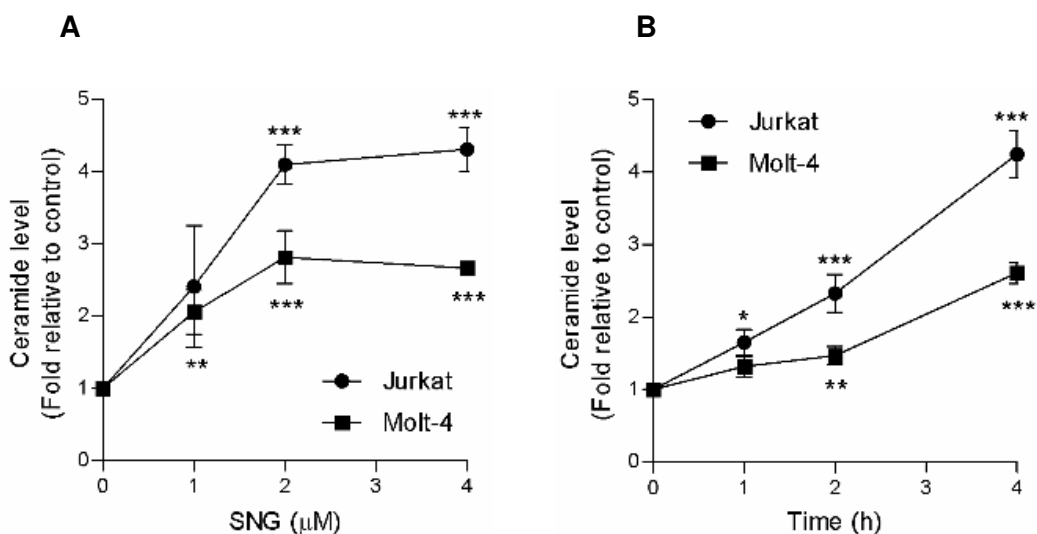
Figure 4.12. Sanguinarine induces ceramide generation in human leukemic cells. (A) Cells were treated with indicated concentration of SNG for 4 h, (B) Jurkat and Molt-4 cells were treated with 3 μM and 1.5 μM SNG, respectively, for the indicated time period. Ceramide levels were measured as described in the Materials and methods. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, and ***p<0.001 vs control).

As mentioned earlier, a plethora of studies have demonstrated that ROS induces ceramide generation, and once generated, both of them contribute to

apoptosis induction (Dumitru, Zhang, Li, & Gulbins, 2007; Galadari et al., 2013; Thayyullathil et al., 2014). Since, SNG induces both ROS generation and ceramide accumulation, the possibility of ROS-induced ceramide generation was examined. Pre-treatment with GSH and NAC completely abrogated ceramide accumulation in Jurkat and Molt-4 cells, as depicted in Fig. 4.13.A and 4.13.B, respectively. This data clearly demonstrate that SNG induces ROS-dependent ceramide generation in both Jurkat and Molt-4 human leukemic cells.
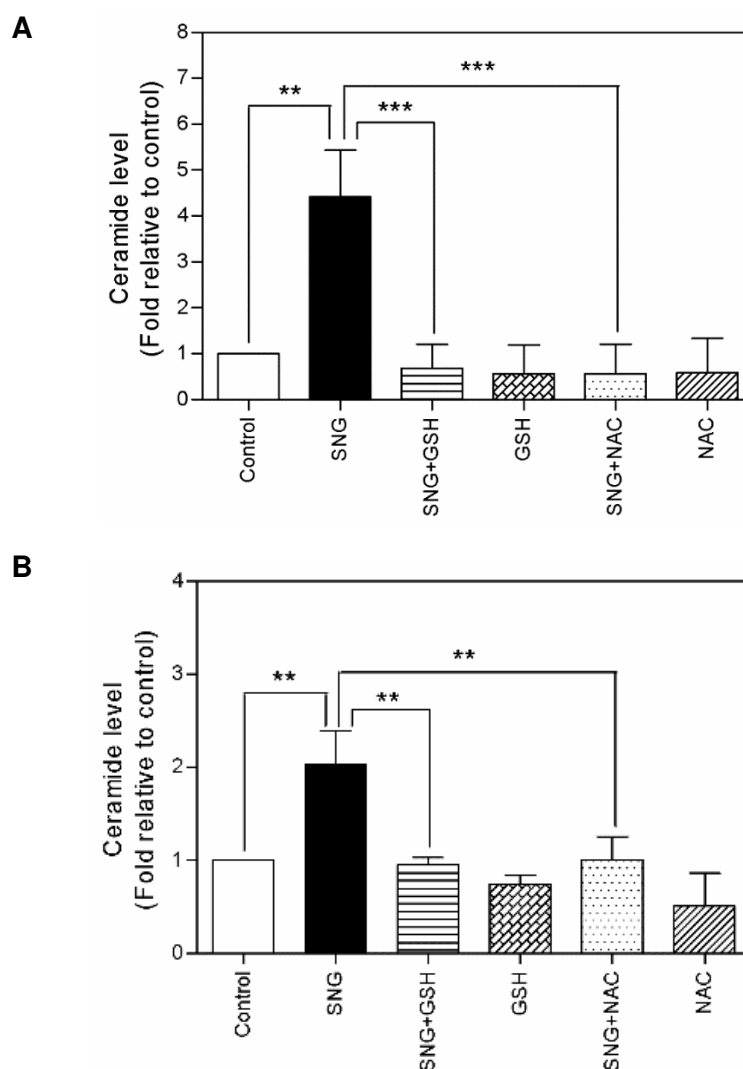
Figure 4.13. Sanguinarine induces ROS-dependent ceramide generation in human leukemic cells. (A) Jurkat and (B) Molt-4 cells were pre-treated with GSH (10 mM) and NAC (10 mM) for 1 h, followed by the SNG treatment (3 µM for Jurkat and 1.5 µM for Molt-4) for 4 h. Ceramide levels were measured as described in the Materials and methods. Data shown are means ± SD (n=3) (**p<0.01 and ***p<0.001)

Extracellular addition of H_2O_2 may be used to mimic the effects of intracellularly produced ROS (Y. Chen, Azad, & Gibson, 2009). In order to further confirm the involvement of ROS in SNG-induced ceramide generation and apoptosis, Jurkat cells were treated with 400 µM H_2O_2 for 12 h. As expected, H_2O_2 treatment resulted in an increase in ceramide generation (Fig. 4.14.A), apoptosis (Fig. 4.14.B), and loss of cell viability (Fig. 4.14.C).

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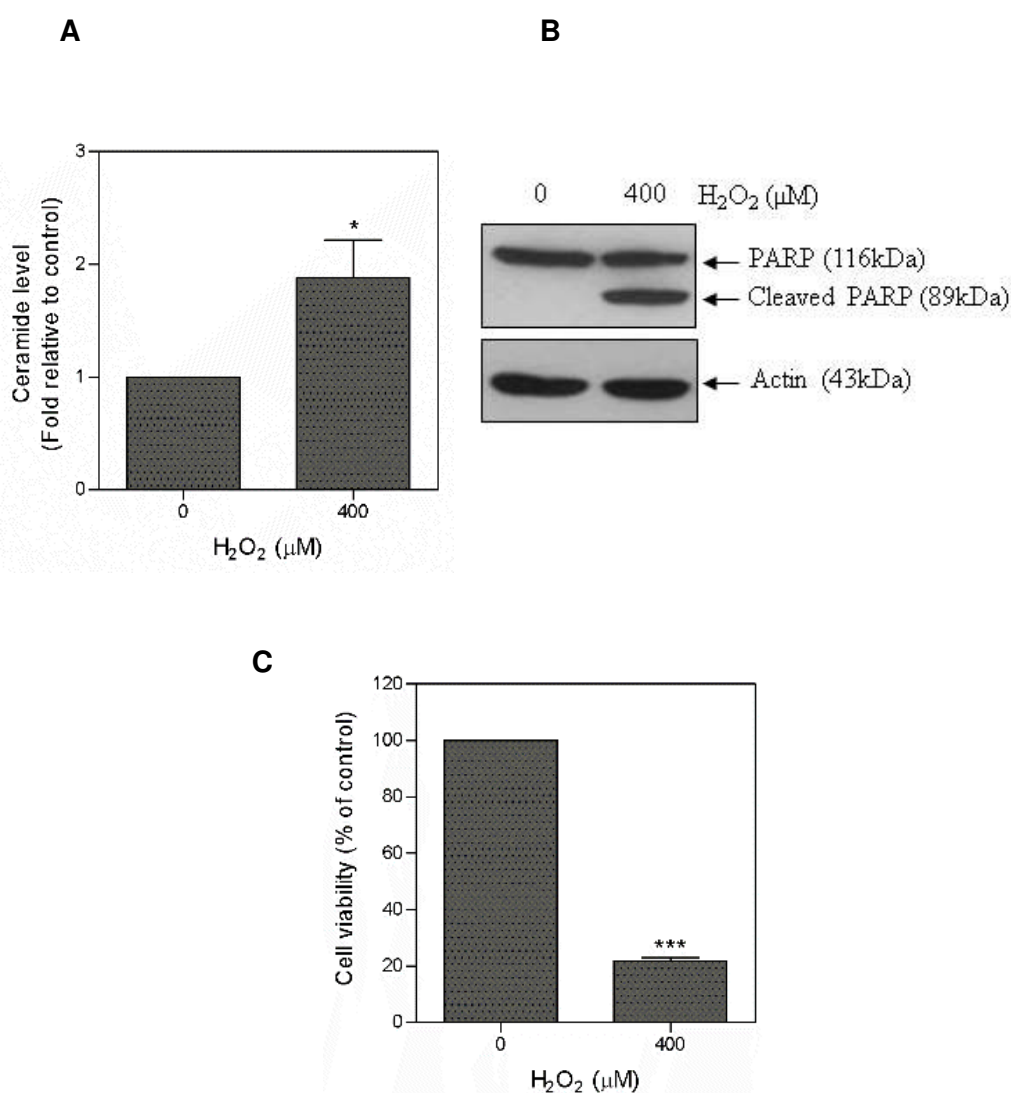
Figure 4.14. Effect of hydrogen peroxide on ceramide generation and apoptosis. Jurkat cells were treated with 400 µM H_2O_2 for 6 h. Following treatment, (A) ceramide levels were measured by HPLC, (B) PARP cleavage was analyzed by Western blot, and (C) cell viability was measured by using MTT assay. Data shown are means ± SD (n=3) (*p<0.05 and ***p<0.001 vs control).

Ceramide can be generated in the cells either via *de novo* synthesis, or via sphingomyelin hydrolysis (Galadari et al., 2013; Galadari et al., 2015). In order to distinguish which of these pathways is involved in SNG-induced ceramide generation, and to decipher whether the ceramide that is generated has any role in SNG-induced loss of viability, leukemic cells were pre-treated with inhibitors targeting these pathways prior to SNG treatment, and the cells were analyzed for ceramide generation and viability. The *de novo* ceramide synthesis inhibitors (Myr and FB1) (Fig. 4.15.A and B), and the nSMase inhibitor (GW4869) (Fig. 4.15.C and D) had no effect on SNG-induced ceramide generation and loss of viability. Interestingly, aSMase inhibitor (Desip), partly, but significantly, blocked the SNG-induced ceramide generation and loss of viability in Jurkat cells, but not in Molt-4 cells (Fig. 4.15.C and D). The reason for this disparity may well be due to the fact that Molt-4 cells have very little aSMase activity (B. Liu & Hannun, 1997). Moreover, Desip significantly protected SNG-induced apoptosis in Jurkat cells (Fig. 4.15.E), when none of the other inhibitors, namely Myr, FB1, and GW4869 produced any protection (Fig. 4.15.F, G, and H). Increase in the activity of aSMase was further confirmed by measuring its enzymatic activation following SNG treatment in Jurkat cells, while no change in the aSMase enzymatic activity was observed in Molt-4 cells (Fig. 4.15.I). SNG had no effect on nSMase activity in both Jurkat and Molt-4 cells (Fig. 4.15.J). Altogether, these data clearly demonstrate that SNG induces aSMase activation, leading to ceramide generation and subsequent induction of apoptosis, at least in Jurkat cells. In Molt-4 cells, SNG-induced ceramide generation was not mediated via aSMase activation. Moreover, the results also demonstrate that SNG has no effect on the activation of *de novo* ceramide biosynthetic enzymes (SPT and ceramide synthase) and nSMase enzyme in both Jurkat and Molt-4 cells.

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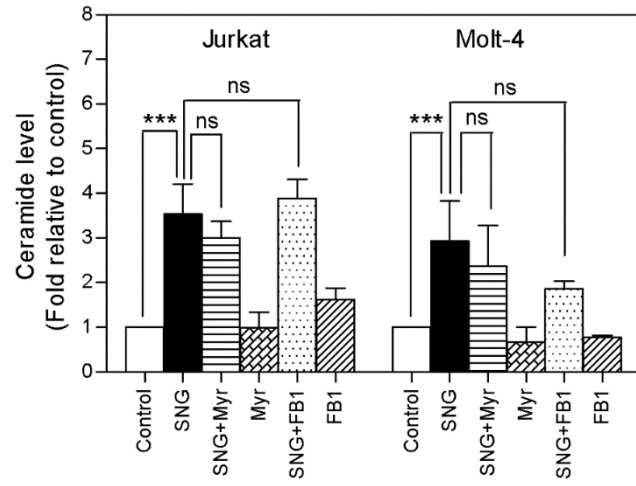
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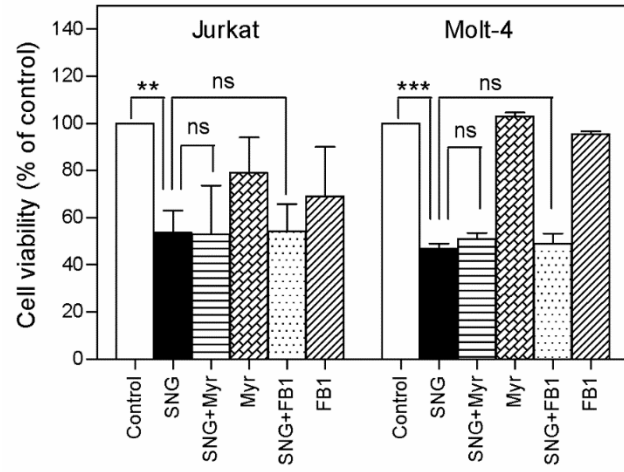
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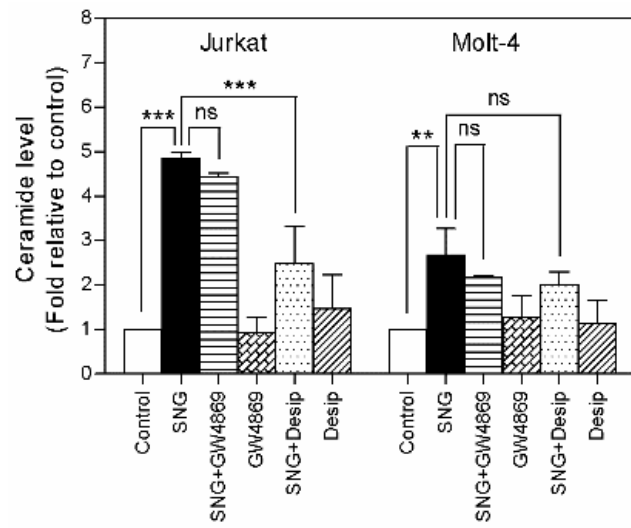
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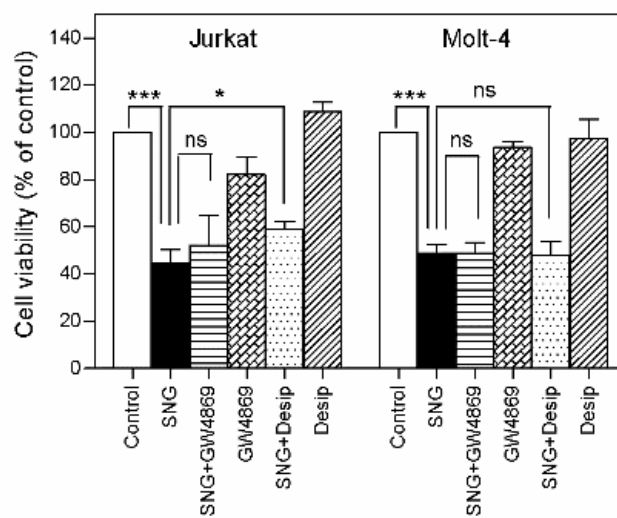
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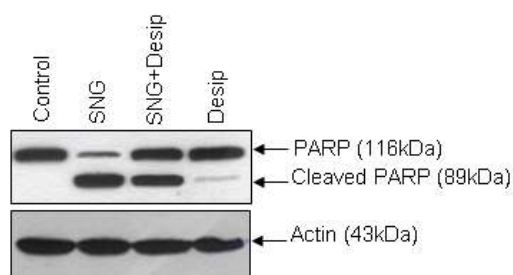
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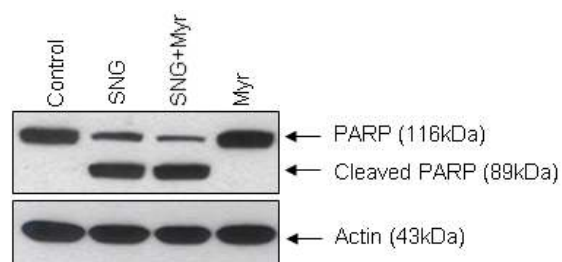
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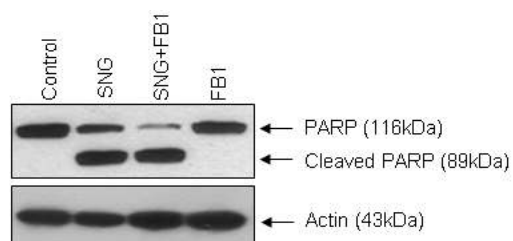
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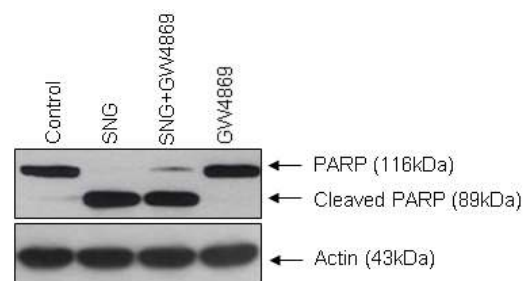
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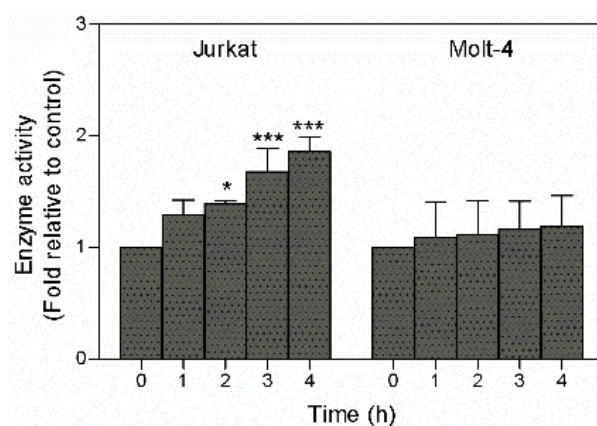
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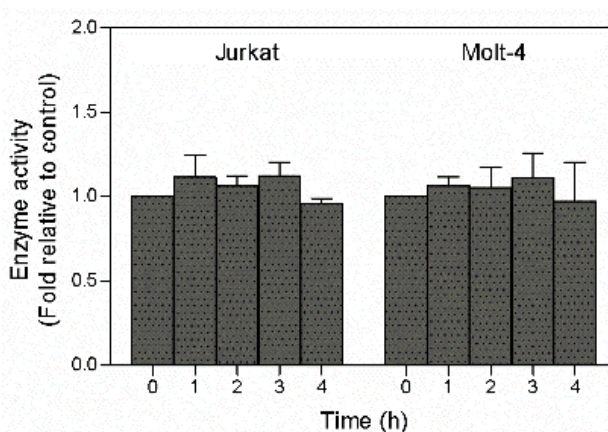
Figure 4.15. Sanguinarine modulates the activity of ceramide biosynthetic enzymes. Cells were pre-treated with Myr (5 µM) and FB1 (50 µM) for 1 h, followed by incubation with SNG (3 µM for Jurkat and 1.5 µM for Molt-4) for 4 h. (A) Ceramide levels were measured as described in the Materials and methods and (B) cell viability was measured by using MTT assay. Data shown are means ± SD (n=3) (**p<0.01, ***p<0.001, and ns: not significant). Cells were pre-treated with GW4869 (25 µM) and Desip (10 µM) for 1 h, followed by incubation with SNG (3 µM for Jurkat and 1.5 µM for Molt-4) for 4 h. (C) Ceramide levels were measured as described in the Materials and methods and (D) cell viability was measured by using MTT assay. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, ***p<0.001, and ns: not significant). Jurkat cells were pre-treated with (E) Desip (10 µM), (F) Myr (5 µM), (G) FB1 (50 µM), and (H) GW4869 (25 µM) for 1 h, followed by incubation with 3 µM SNG for 4 h. Western blot analysis of PARP was carried out. Actin was used as loading control. Cells were treated with SNG (3 µM for Jurkat and 1.5 µM for Molt-4) for indicated time period. The activity of (I) aSMase and (J) nSMase were performed as described in the Materials and methods. Data shown are means ± SD (n=3) (*p<0.05 and ***p<0.001 vs respective control).

Next, to determine whether SNG modulate activity of ceramide degradative enzymes, we studied activities of ceramide degradative enzymes such as aCDase, nCDase, and GCS following SNG treatment. As shown in Fig. 4.16.A and B, activities of both aCDase and GCS were decreased in time-dependent manner in both Jurkat (Fig. 4.16.A) and Molt-4 (Fig. 4.16.B) cells, while nCDase activity was not altered in either cell lines. It is noteworthy that the GCS inhibition was highly significant and happened within 1 h of SNG treatment in both leukemic cells. These finding imply that inactivation of GCS and aCDase might play an important role in SNG-mediated apoptosis, and thus, we hypothesized that inhibition of these enzymes might enhance the cytotoxic activity of SNG. To test this hypothesis, Jurkat cells pre-treated with GCS inhibitor (PDMP) or aCDase inhibitor (NOE) and were subjected to SNG treatment. Following the treatment, cells were examined for ceramide generation, apoptosis and cell viability. Pre-treatment with both PDMP and NOE, followed by treatment with modestly toxic concentration of SNG resulted in a pronounced increase in ceramide generation (Fig. 4.16.C), PARP cleavage (Fig. 4.16.D), and loss of viability (Fig. 4.16.E).

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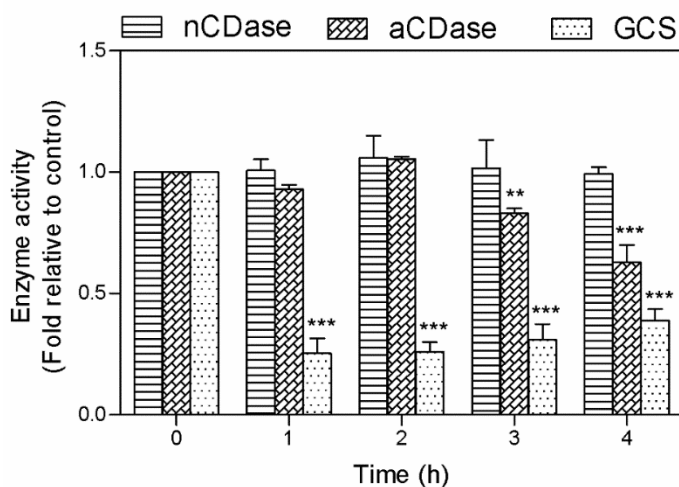
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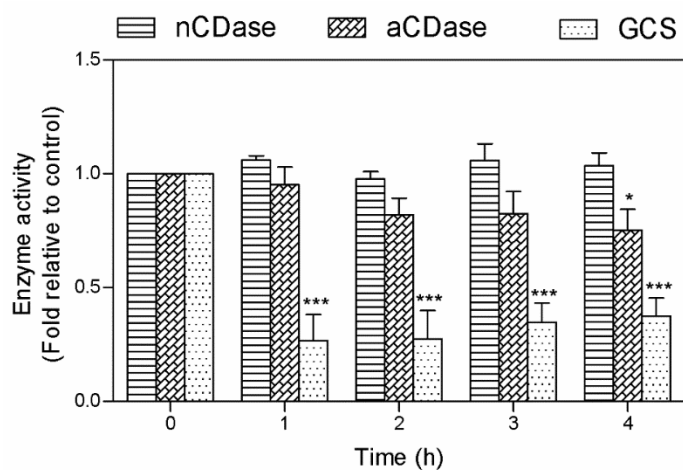
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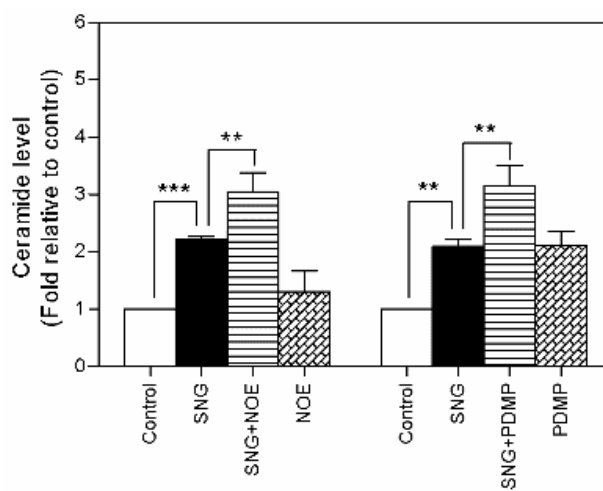
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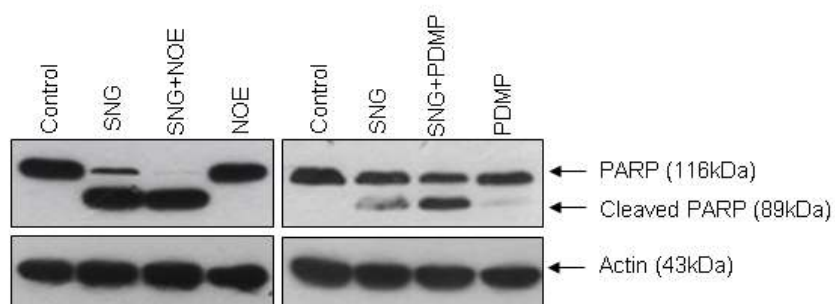
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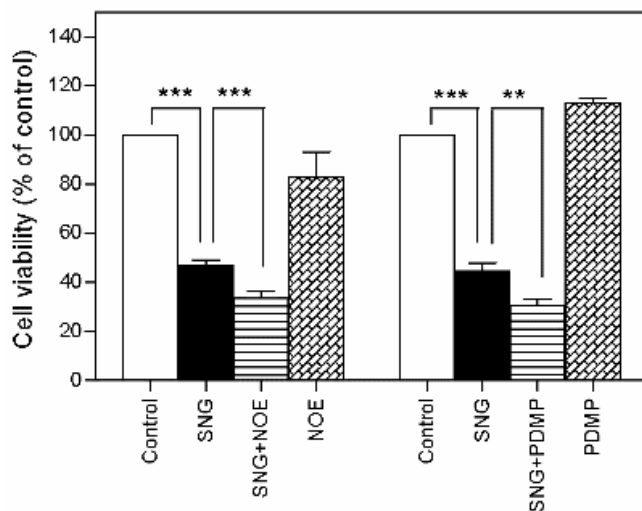
Figure 4.16. Sanguinarine modulates the activity of ceramide degradative enzymes. (A) Jurkat cells were treated with 3 µM SNG and (B) Molt-4 cells were treated with 1.5 µM SNG for the indicated time period. Activity of nCDase, aCDase, and GCS were performed as described in the Materials and methods. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, and ***p<0.001 vs respective control). Jurkat cells were pre-treated with PDMP (30 µM) and NOE (50 µM) for 1 h, followed by incubation with 2 µM SNG. After the treatment, (C) ceramide levels were measured. Data shown are means ± SD (n=3) (**p<0.01, and ***p<0.001), (D) Western blot analysis of PARP was carried out. Actin was used as loading control, and (E) cell viability was measured. Data shown are means ± SD (n=3) (**p<0.01, and ***p<0.001)

From the previous results, it is clear that SNG induces aSMase activity in Jurkat cells, while it inhibits aCDase and GCS activity in both Jurkat and Molt-4 cells. To examine if these modulations are ROS-dependent, the activities of these enzymes were measured following NAC pre-treatment. Blockade of SNG-induced ROS generation by NAC essentially abrogated the activation of aSMase in Jurkat cells (Fig. 4.17.A) and inactivation of aCDase and GCS in both leukemic cells (Fig. 4.17.A and B). Altogether, these results highlight the pivotal role of ROS-dependent ceramide generation in regulating the apoptotic action of SNG in human leukemic cells.

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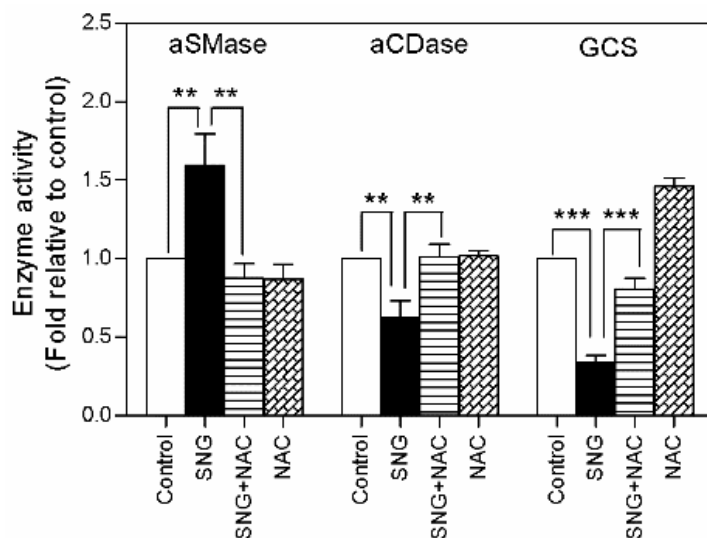
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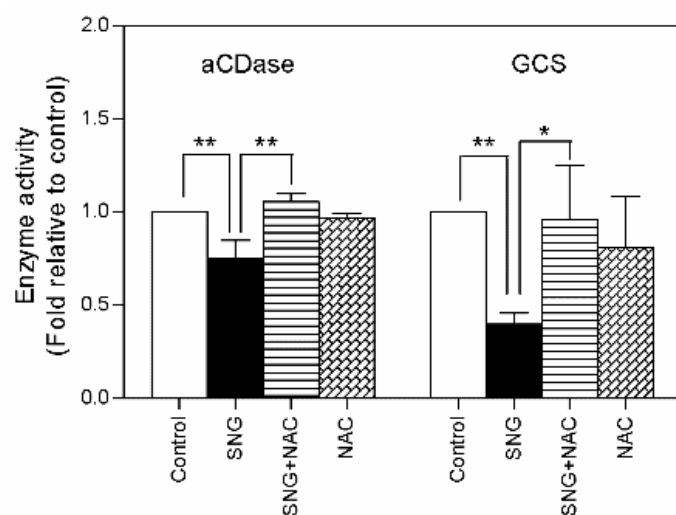
Figure 4.17. Sanguinarine induces reactive oxygen species-dependent modulation of ceramide biosynthetic and degradative enzyme activities. (A) Jurkat and (B) Molt-4 cells were pre-treated with NAC (10 mM) for 1 h, followed by SNG (3 µM for Jurkat and 1.5 µM for Molt-4) treatment for 4 h. Following the treatment, activity of indicated enzymes (aSMase, aCDase, and GCS) were performed as described in the Materials and methods. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, and ***p<0.001)

4.4 Sanguinarine inhibits Akt signaling pathway downstream to the reactive oxygen species-dependent ceramide generation

Akt is one of the most important and versatile protein kinase involved in the regulation of multiple tumorigenic cellular processes such as proliferation, cell cycle progression, inhibition of apoptosis, induction of cell motility, angiogenesis and metastasis (Brazil et al., 2002; Qiao et al., 2008). Akt inhibits apoptosis through direct phosphorylation and inactivation of pro-apoptotic proteins, down-regulation of pro-apoptotic proteins, and increased expression of anti-apoptotic proteins. Because Akt is deregulated in a wide range of malignancies, it is considered a major potential target for novel anticancer therapies (Brazil et al., 2002). Simultaneously, many compounds such as perifosine, MK2206, GDC-0068, and GSK2110183 that target Akt signaling pathway are in clinical trial or in the development pipeline (Dienstmann et al., 2014). Considering the fact that mutation and aberrant activation of PI3K/Akt signaling pathway are frequent in acute leukemia (Fransecky, Mochmann, & Baldus, 2015), the fourth objective of our study was to examine the role of Akt in SNG-induced apoptosis in human leukemic cells. Thus, we investigated whether SNG has any effect on phosphorylation (activation)-dephosphorylation (inactivation) status of Akt. As shown in Fig. 4.18.A and B, Akt is constitutively phosphorylated at Ser473 in the untreated leukemic cells. However, SNG treatment resulted in both a dose- and a time-dependent dephosphorylation of Akt at Ser473 (Fig. 4.18.A and B).

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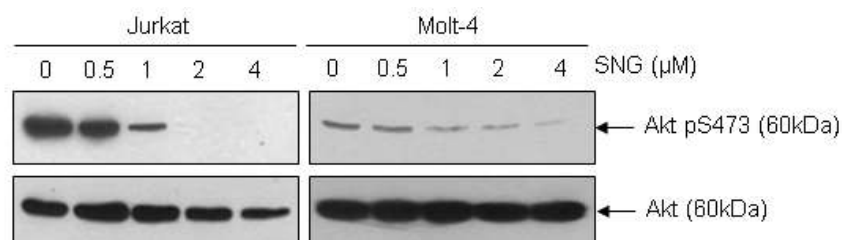
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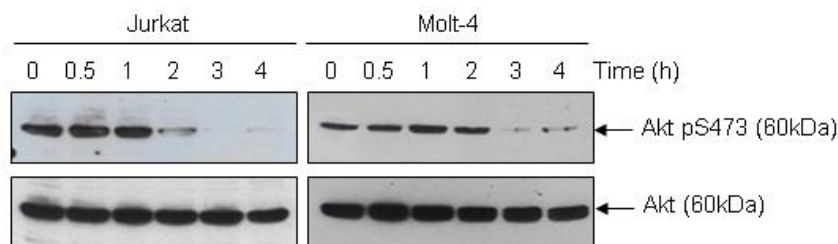
Figure 4.18. Sanguinarine induces Akt dephosphorylation at Ser473. (A) Leukemic cells were treated with indicated concentration of SNG for 4 h, (B) Jurkat and Molt-4 cells were treated with 3 µM and 2 µM SNG, respectively, for the indicated time period. Western blot analysis for phospho-Akt Ser473, and total Akt was carried out.

Sanguinarine-induced Akt dephosphorylation is not associated with changes in its upstream kinases, as evidenced by no changes in the expression of PI3K (both the regulatory subunit p85 and the catalytic subunit p110β), and phospho PDK1 Ser241 in both leukemic cells following SNG treatment (Fig. 4.19).
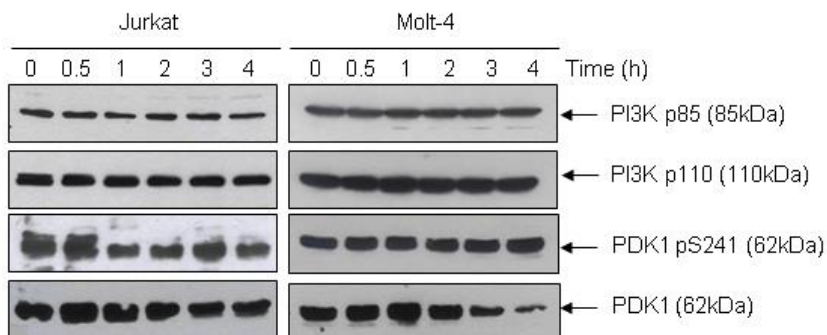
Figure 4.19. Sanguinarine-induced Akt dephosphorylation is not associated with changes in its upstream kinase. Jurkat and Molt-4 cells were treated with 3 µM and 2 µM SNG, respectively, for the indicated time period. Western blot analysis for PI3K p85, PI3K p110, phospho-PDK1 Ser241, and PDK1 was carried out.

Meanwhile, Akt downstream kinases, such as mTOR and GSK3β, were dephosphorylated following exposure of leukemic cells to SNG (Fig. 4.20). It is well established that mTOR itself is an important cell growth and pro-survival protein that is activated by Akt-mediated phosphorylation at Ser2448, whereas, GSK3β mainly

acts as a pro-apoptotic protein that is inactivated by Akt-mediated Ser9 phosphorylation (Ciuffreda, Di Sanza, Incani, & Milella, 2010; Jacobs et al., 2012; Manning & Cantley, 2007; Pópulo, Lopes, & Soares, 2012). Moreover, mTOR downstream target 4EBP1, was also dephosphorylated at Thr37/46 following SNG treatment of leukemic cells (Fig. 4.20). These results clearly demonstrate that SNG inhibits Akt signaling pathway, which is reported to be highly activated during the development and progression of leukemia (Fransecky et al., 2015; Okumura et al., 2012).
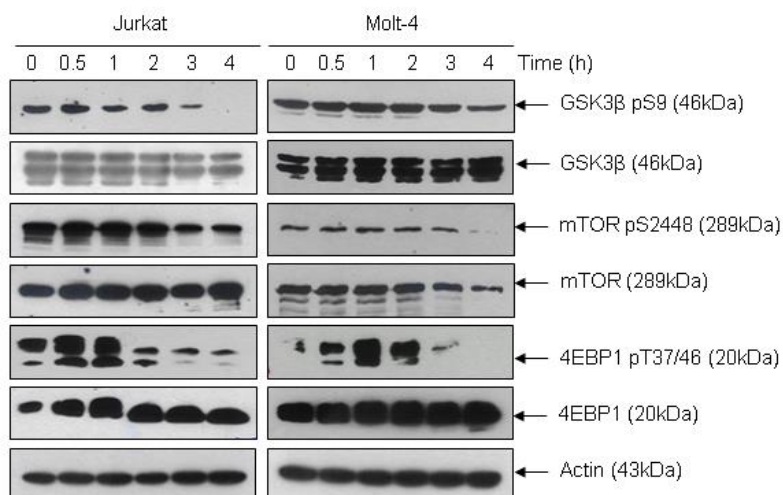
Figure 4.20. Sanguinarine induces dephosphorylation of kinases downstream of Akt. Jurkat and Molt-4 cells were treated with 3 µM and 2 µM SNG, respectively, for the indicated time period. Western blot analysis for indicated proteins were carried out. Actin was used as loading control.

In order to determine whether Akt dephosphorylation is ROS-dependent, leukemic cells pre-treated with GSH and NAC were treated with SNG, and monitored for Akt phosphorylation status. As shown in Fig. 4.21, both ROS scavengers significantly abrogated SNG-induced Akt dephosphorylation in both Jurkat and Molt-4 cells, demonstrating that Akt dephosphorylation is a ROS-dependent process.
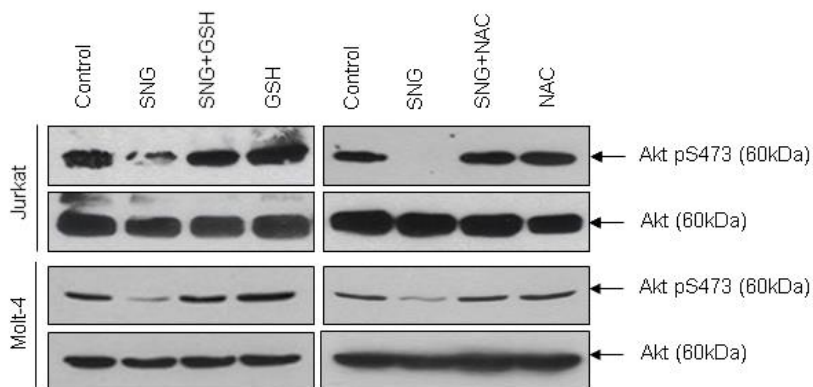
Figure 4.21. Sanguinarine induces reactive oxygen species-dependent Akt dephosphorylation in human leukemic cells. Cells were pre-treated with GSH (10 mM) and NAC (10 mM) for 1 h, followed by SNG treatment (3 µM for Jurkat and 2 µM for Molt-4) for 4 h. Western blot analysis for phospho-Akt Ser473, and total Akt was carried out.

Next, an attempt was made to determine whether Akt dephosphorylation is downstream to the ROS-induced ceramide generation. Pre-treatment with Desip, which had previously shown to inhibit SNG-induced ceramide generation and apoptosis (Fig. 4.15.C, D, and E), also blocked SNG-induced Akt dephosphorylation (Fig. 4.22.A). Furthermore, *de novo* ceramide biosynthetic enzymes inhibitors (Myr and FB1) and the nSMase inhibitor (GW4869), did not inhibit SNG-induced Akt dephosphorylation (Fig. 4.22.B). These inhibitors had previously shown to have no effect on SNG-induced ceramide generation, loss of viability and apoptosis (Fig. 4.15.A, B, F, G and H). Moreover, pre-treatment with PDMP or NOE, which had previously shown to sensitize SNG-induced ceramide generation and apoptosis (Fig. 4.16.C, D, and E), also sensitized SNG-induced Akt dephosphorylation (Fig. 4.22.C).
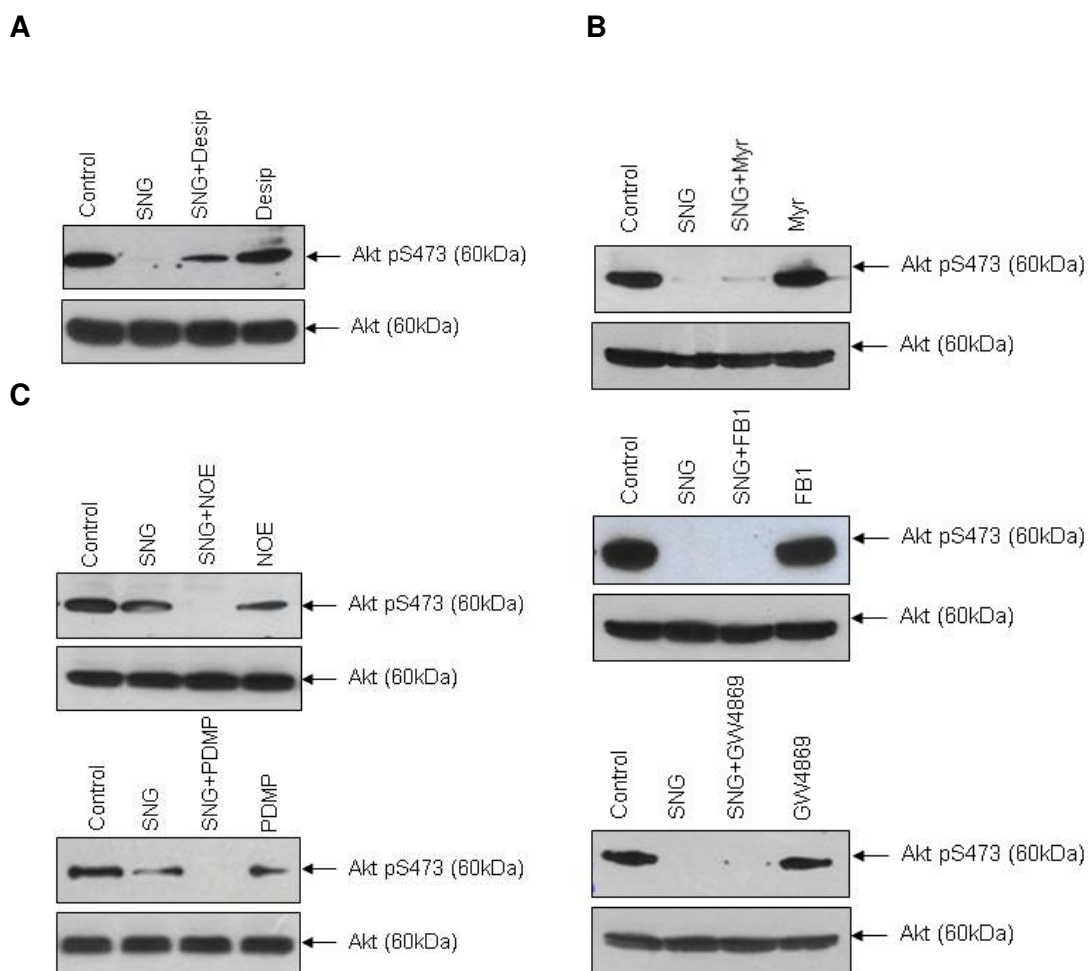
Figure 4.22. Sanguinarine induced Akt dephosphorylation is downstream to the reactive oxygen species-dependent ceramide generation. Jurkat cells were pre-treated with (A) Desip (10 µM), and (B) Myr (5 µM), FB1 (50 µM), and GW4869 (25 µM) for 1 h, followed by 3 µM SNG treatment for 4 h. Following the treatment, Western blot analysis for phospho-Akt Ser473, and total Akt was carried out. (C) Jurkat cells were pre-treated with PDMP (30 µM) and NOE (50 µM) for 1 h, followed by the 2 µM SNG treatment for 4 h. Following the treatment, Western blot analysis for phospho-Akt Ser473, and total Akt was carried out.

These data clearly indicate that SNG induces Akt-dephosphorylation downstream to the ROS-induced ceramide generation. In order to confirm this further, Jurkat cells were treated with C_6-ceramide and examined for Akt dephosphorylation, PARP cleavage, and loss of viability. As shown in Fig. 4.23.A and B, ceramide treatment resulted in dose-dependent Akt dephosphorylation,

PARP cleavage, and loss of viability. Altogether, these results suggest that SNG mediates ROS-dependent ceramide generation, leading to Akt dephosphorylation and apoptosis in human leukemic cells.

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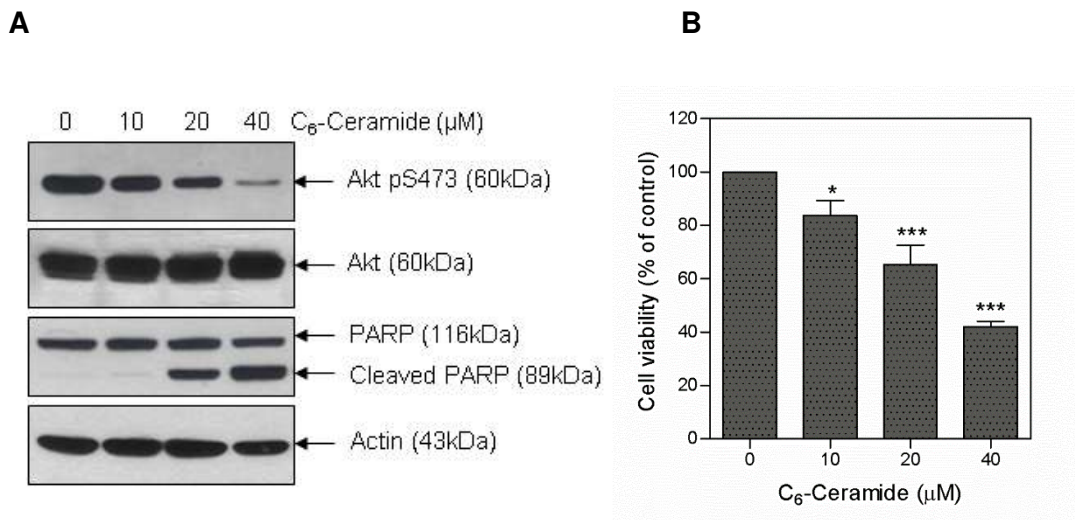
Figure 4.23. Effect of ceramide on Akt phosphorylation status and apoptosis. Jurkat cells were treated with indicated concentration of C_6-ceramide and (A) Western blot analysis for phospho-Akt Ser473, total Akt, and PARP was carried out. Actin was used as loading control and (B) cell viability was measured by using MTT assay. Data shown are means ± SD (n=3) (*p<0.05 and ***p<0.001 vs control).

Two important serine/threonine phosphatases (PP1 and PP2A) allostericaly activated by ceramide, collectively termed as CAPP are shown to dephosphorylate Akt (Salinas et al., 2000; Schubert et al., 2000; Wolff, Dobrowsky, Bielawska, Obeid, & Hannun, 1994). To examine if any of these CAPPs are involved in SNG-induced Akt dephosphorylation, Jurkat cells were pre-treated with an increasing concentration of CalA (a well-established CAPP inhibitor) for 1 h followed by SNG treatment for 4 h. As shown in Fig. 4.24.A, CalA dose-dependently inhibited SNG-induced Akt dephosphorylation. More importantly, CalA pre-treatment partially, but significantly, protected Jurkat cells from SNG-induced apoptosis and loss of viability (Fig. 4.24.A and B). These findings clearly indicate the involvement of CAPP in SNG-induced Akt dephosphorylation and apoptosis.

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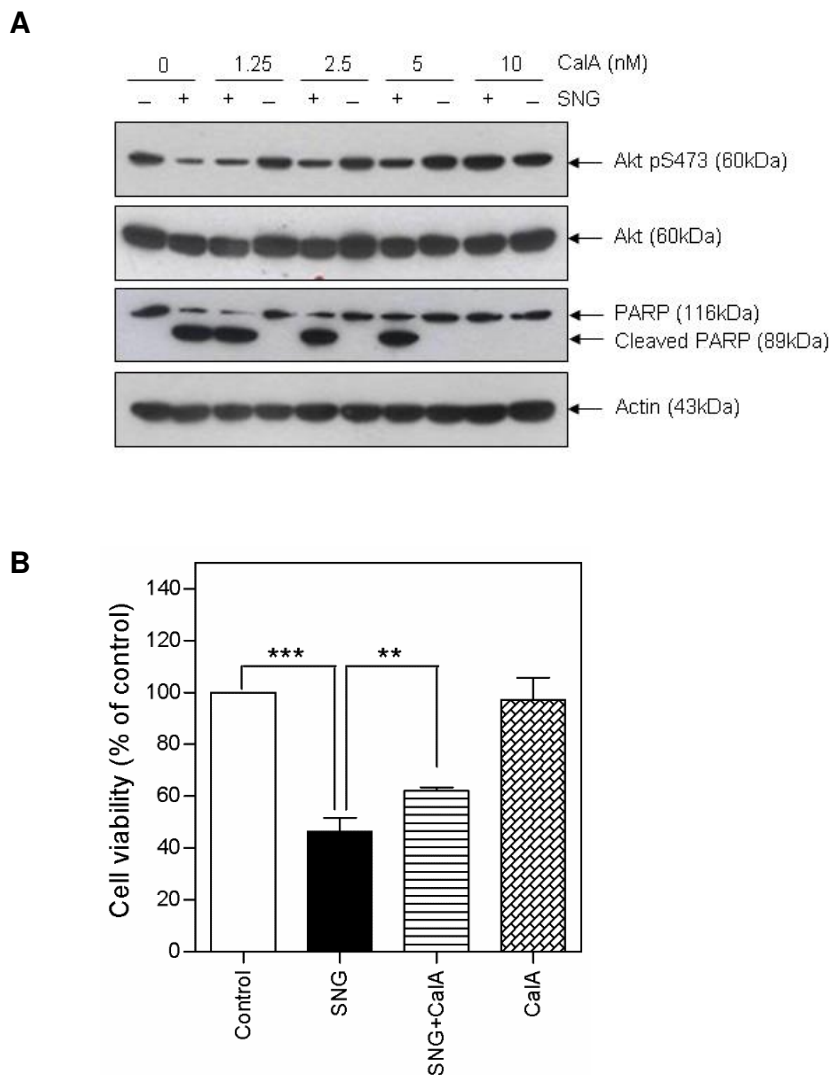
Figure 4.24. Sanguinarine induces Akt dephosphorylation via ceramide activated protein phosphatase. Jurkat cells were pre-treated with indicated concentrations of CalA for 1 h, followed by SNG treatment for 4 h. After the treatment, (A) Western blot analysis of phospho-Akt Ser473, total Akt, and PARP was performed. Actin was used as a loading control and (B) cell viability was measured by using MTT assay. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, and ***p<0.001).

In order to distinguish between PP1 and PP2A involvement in SNG-induced apoptosis, another CAPP inhibitor with unique activity profile against CAPP was used. OA inhibits PP2A at low concentration (IC_{50}< 0.1 nM), but it inhibits both PP2A and PP1 at higher concentration (IC_{50}> 150 nM) (Thayyullathil, Chathoth, Shahin, et al., 2011). Jurkat cells were pre-treated with 1 nM and 300 nM OA, followed by treatment with SNG for 4 h. As shown in Fig. 4.25.A and B, OA at low concentration

(1 nM) did not affect SNG-induced Akt dephosphorylation, PARP cleavage, and loss of viability. However, at the higher concentration (300 nM), OA significantly blocked the SNG effect (Fig. 4.25.A and B). This activity profile strongly supports a role for PP1, but not PP2A in mediating SNG-induced Akt dephosphorylation. Indeed, this is further confirmed by using PA, a potent and selective inhibitor of PP1 (Kishikawa, Chalfant, Perry, Bielawska, & Hannun, 1999). Pre-treatment with PA significantly abolished SNG-induced Akt dephosphorylation, PARP cleavage and loss of viability (Fig. 4.25.C and D). Taken together, these results clearly indicate that SNG promotes Akt dephosphorylation and apoptosis via activating the PP1 form of CAPP.

A

B

C   **D**
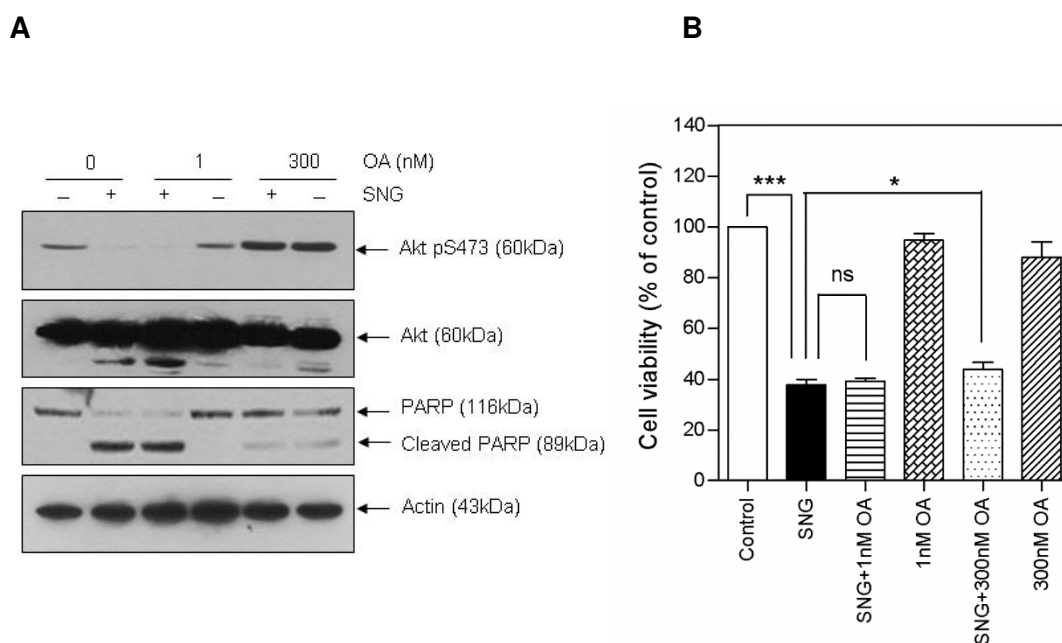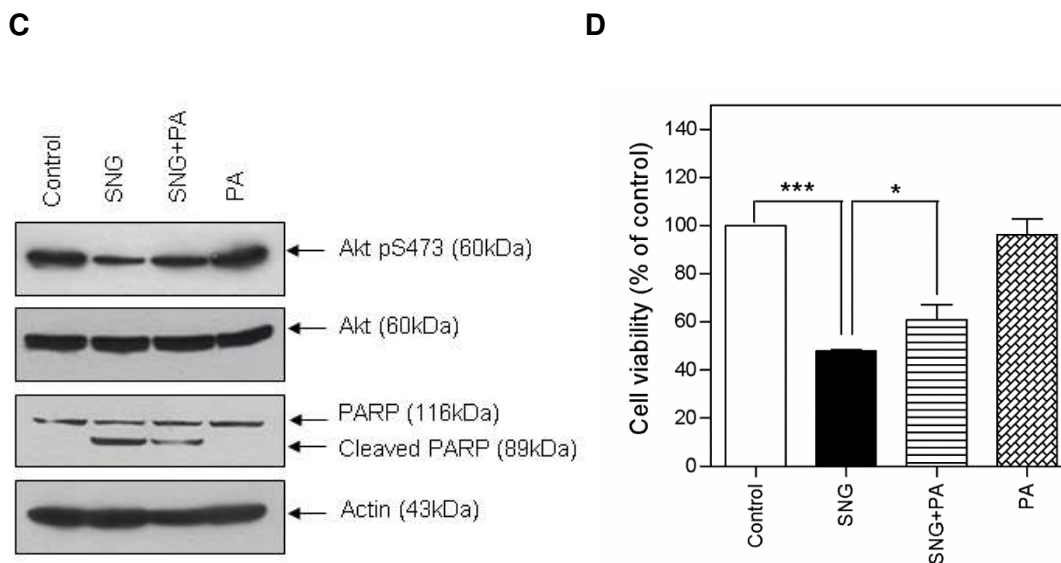
Figure 4.25. Sanguinarine induces Akt dephosphorylation via protein phosphatase 1 type of ceramide activated protein phosphatase. Jurkat cells were pre-treated with (A and B) indicated concentrations of OA, and (C and D) 30 µM PA for 1 h, followed by SNG treatment for 4 h. After the treatment, Western blot analysis for phospho-Akt Ser473, total Akt, and PARP was performed. Actin was used as a loading control. Cell viability was measured by using MTT assay. Data shown are means ± SD (n=3) (*p<0.05 and ***p<0.001).

This was further validated by exposing Jurkat cells to CAPP inhibitors followed by treatment with C_6-ceramide. As shown in Fig. 4.26.A, B and C, both CalA and PA protected the C_6-ceramide-induced Akt dephosphorylation, while OA failed to do so.

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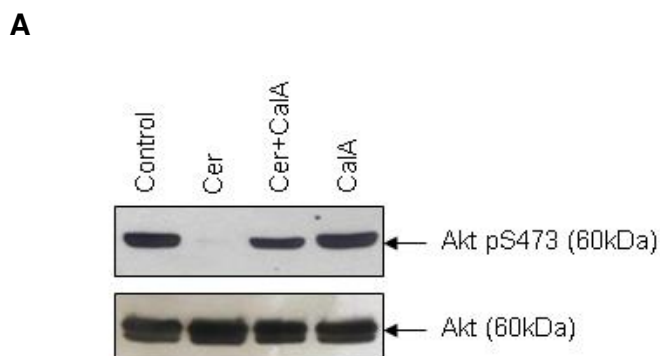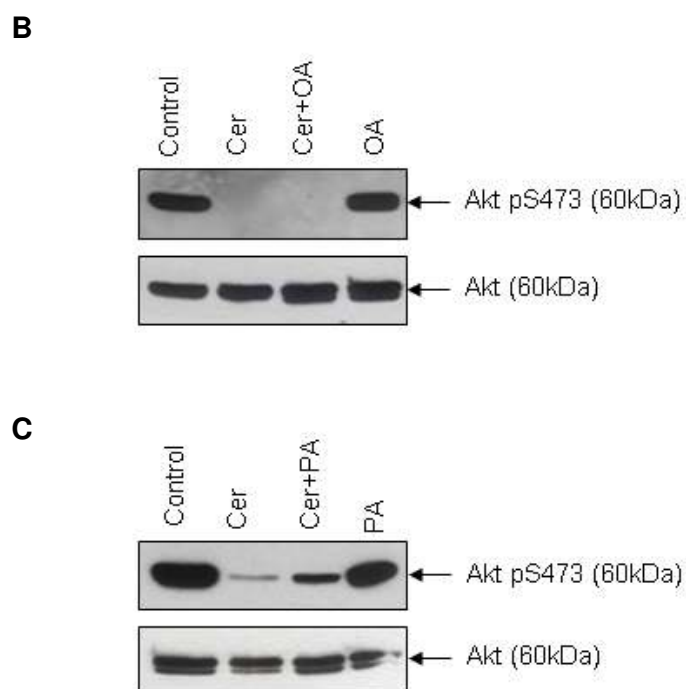
Figure 4.26. Ceramide induces ceramide activated protein phosphatase-dependent Akt dephosphorylation. Jurkat cells were pre-treated with (A) CalA, (B) OA, and (C) PA, followed by C_6-ceramide treatment. Western blot analysis for phospho-Akt Ser473 and total Akt was carried out.

4.5 Sanguinarine-induced apoptotic cell death is associated with reactive oxygen species-dependent activation of extracellular signal-regulated kinase 1/2 in human leukemic cells.

Mitogen activated protein kinases, including ERK1/2, JNK, and p38, are critical kinases that regulate a variety of biological process, such as cell survival, proliferation, differentiation, and apoptosis (Lu & Xu, 2006). Since these kinases are involved in the regulation of variety of proliferative and tumor suppressive signals, our fifth objective is to delineate the involvement of MAPKs in SNG-induced apoptosis. To determine the role of these kinases in SNG-induced loss of viability, leukemic cells were pre-treated with inhibitors of these kinases, and their influence on cell viability was evaluated following SNG treatment. As shown in Fig 4.27.A and B, pre-treatment with ERK1/2 inhibitor (U0126), significantly abrogated SNG-induced loss of viability in both Jurkat (Fig 4.27.A) and Molt-4 (Fig 4.27.B) cells, while JNK inhibitor (SP600125) and p38 inhibitor (SB202190) had no effect on SNG-induced loss of viability in both cells (Fig. 4.27.A and B).

A
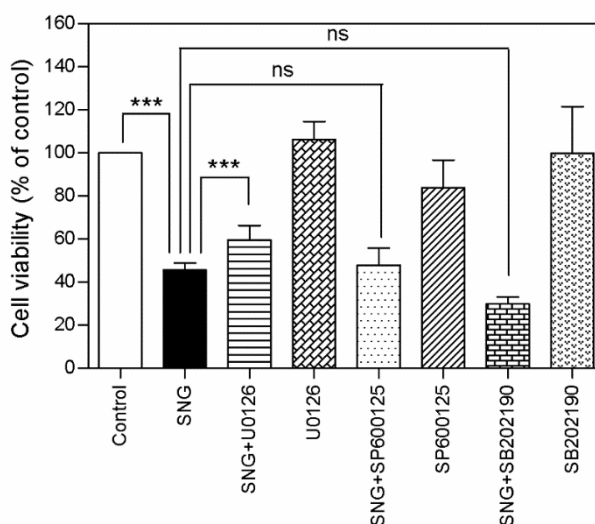
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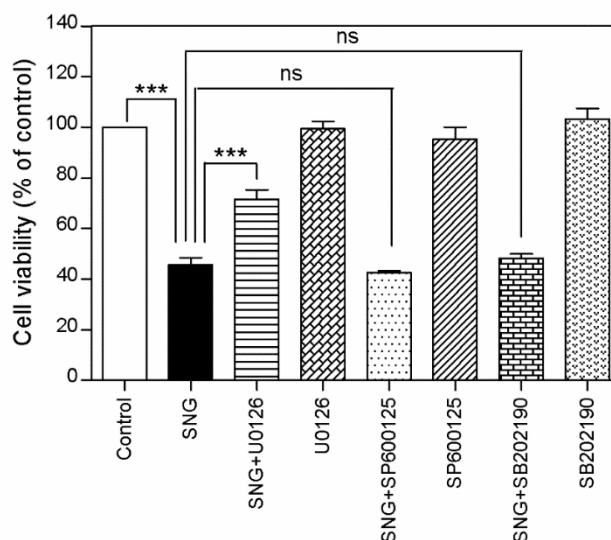
Figure 4.27. Effect of mitogen activated protein kinase inhibitors on sanguinarine-induced loss of viability. (A) Jurkat and (B) Molt-4 cells were pre-treated with U0126 (30 μM), SP600125 (20 μM), and SB202190 (20 μM) for 1 h, followed by SNG treatment (3 μM for Jurkat and 1.5 μM for Molt-4) for 4 h. Cell viability was measured by using MTT assay. Data shown are means ± SD (n=3) (***p<0.001 and ns: not significant).

The biological activity of ERK1/2 can be regulated via its phosphorylation (activation) and dephosphorylation (inactivation) status. To determine, if ERK1/2 is activated in response to SNG treatment, phosphorylation status of ERK1/2 was evaluated following SNG treatment. As shown in Fig. 4.28, treatment with SNG demonstrated an increase in ERK1/2 phosphorylation in both Jurkat and Molt-4 cells. In Jurkat cells, maximum phosphorylation was seen at 30 min, while in Molt-4 cells, phosphorylation gradually increased until 3 h (Fig. 4.28).
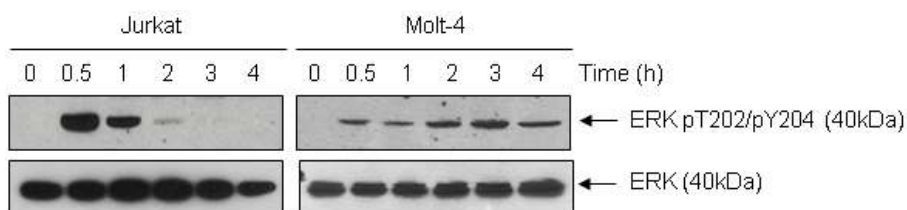
Figure 4.28. Sanguinarine induces extracellular signal-regulated kinase 1/2 phosphorylation in human leukemic cells. Jurkat cells were treated 3 μM and Molt-4 cells were treated with 1.5 μM SNG for the indicated time period. Western blot analysis for phospho-ERK1/2, and total ERK1/2 was carried out.

In order to understand the biological significance of ERK1/2 phosphorylation in SNG-induced apoptosis, leukemic cells were pre-treated with ERK1/2 inhibitor U0126, prior to exposing the cells to SNG. Pre-treatment with U0126 not only inhibited SNG-induced ERK1/2 phosphorylation, but also decreased SNG-induced PARP cleavage in Jurkat (Fig. 4.29.A) and Molt-4 (Fig. 4.29.B) cells, demonstrating that ERK1/2 phosphorylation is involved in SNG-induced apoptosis.

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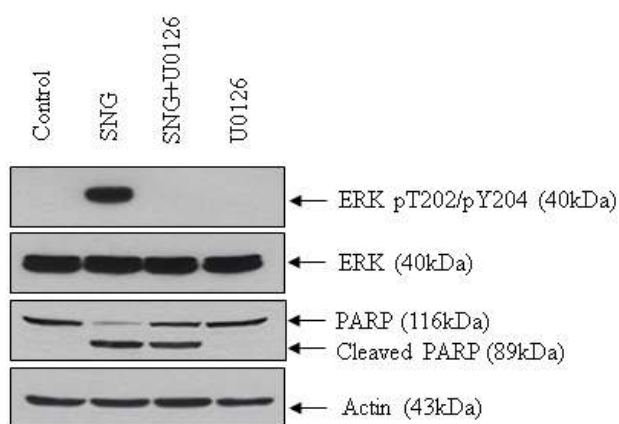
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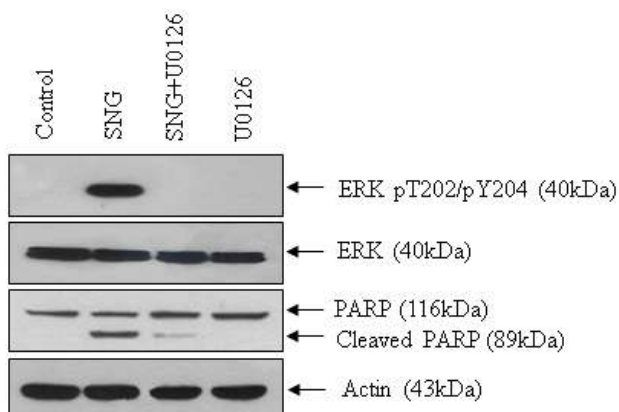
Figure 4.29. Effect of extracellular signal-regulated kinase 1/2 inhibitor on sanguinarine-induced apoptosis. (A) Jurkat and (B) Molt-4 cells were pre-treated with U0126 (30 µM), followed by the SNG (3 µM for Jurkat and 1.5 µM for Molt-4 cells) treatment. Western blot analysis for phospho-ERK1/2, total ERK1/2, and PARP was carried out. Actin was used as a loading control.

The intracellular generation of ROS has been shown to phosphorylate ERK1/2, and leads to the induction of apoptosis (X. Zhang et al., 2003). In addition, we have recently shown that SNG induces ROS-dependent ERK1/2 phosphorylation and subsequent induction of autophagic cell death in human malignant glioma cells (Pallichankandy et al., 2015) In order to examine if SNG-induced ERK1/2 phosphorylation is downstream to ROS generation, cells pre-treated with ROS scavengers were treated with SNG, and examined for ERK1/2 phosphorylation status. Pre-treatment with GSH and NAC completely abrogated SNG-induced ERK1/2 phosphorylation (Fig. 4.30), demonstrating that ERK1/2 phosphorylation is a ROS-dependent process.
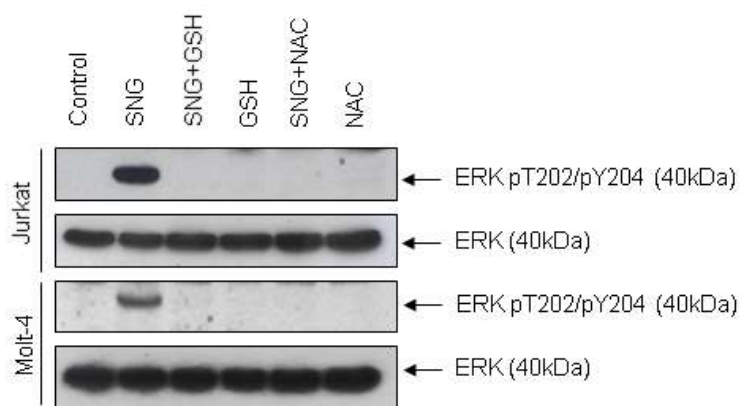
Figure 4.30. Effect of reactive oxygen species scavengers on sanguinarine-induced extracellular signal-regulated kinase 1/2 phosphorylation. Cells were pre-treated with GSH (10 mM) and NAC (10 mM) for 1 h, followed by SNG (3 µM for Jurkat and 1.5 µM for Molt-4 cells) treatment. Western blot analysis for phospho-ERK1/2, and total ERK1/2 was carried out.

In order to examine if ERK1/2 activation is responsible for ceramide generation, intracellular ceramide generation was measured in leukemic cells pre-treated with U0126, followed by SNG treatment. As show in Fig. 4.31, pre-treatment with U0126, had no effect on SNG-induced ceramide generation, demonstrating that ceramide generation is not regulated by ERK1/2.
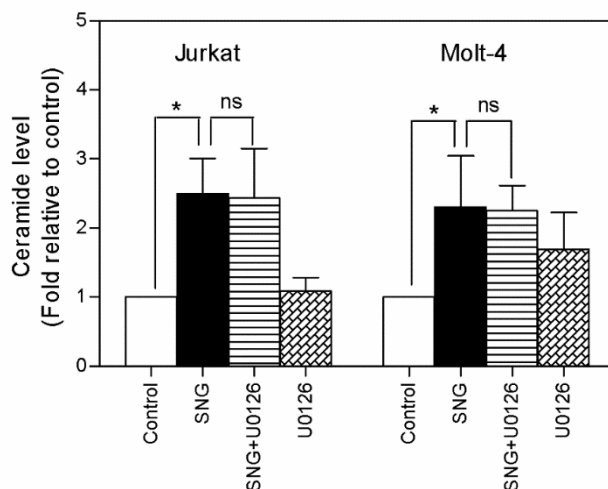
Figure 4.31. Sanguinarine-induced ceramide generation is not regulated by extracellular signal-regulated kinase 1/2 activation. Cells were pre-treated with U0126 (30 µM), followed by the SNG (3 µM for Jurkat and 1.5 µM for Molt-4) treatment. Ceramide levels were measured as described in the Materials and methods. Data shown are means ± SD (n=3) (*p<0.05 and ns: not significant).

Moreover, the possibility of ERK1/2 activation downstream to ceramide generation was also evaluated. Pre-treatment with aSMase inhibitor (Desip), did not inhibit SNG-induced ERK1/2 phosphorylation (Fig. 4.32), indicating that ERK1/2 activation is not regulated by ceramide generation.
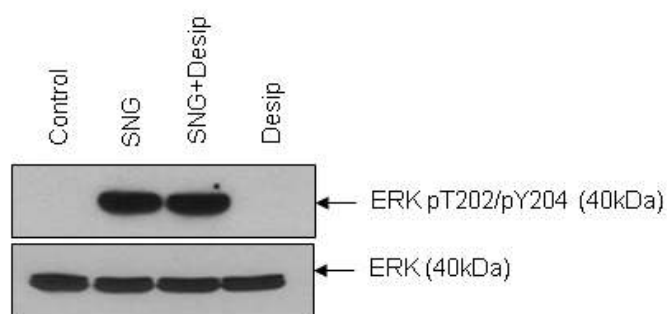
Figure 4.32. Sanguinarine induced extracellular signal-regulated kinase 1/2 activation is not regulated by ceramide generation. Jurkat cells were pre-treated with Desip (10 µM) for 1 h, followed by incubation with 3 µM SNG. Western blot analysis for phospho-ERK1/2 and total ERK1/2 was carried out.

In addition, pre-treatment with U0126, did not inhibit SNG-induced Akt dephosphorylation (Fig. 4.33), signifying that Akt dephosphorylation is not regulated by ERK1/2.
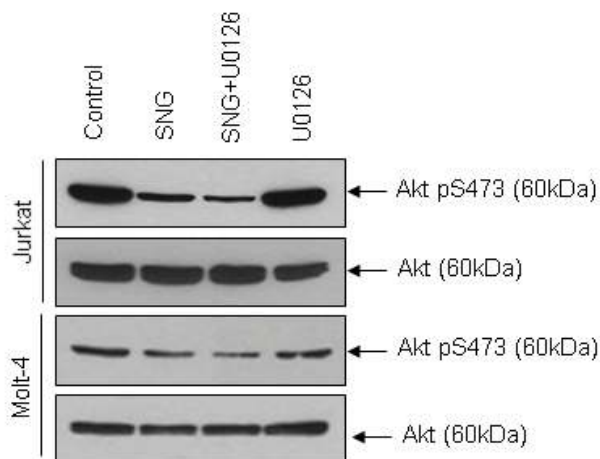
Figure 4.33. Sanguinarine induces reactive oxygen species-dependent extracellular signal-regulated kinase 1/2 activation independent of the ceramide and Akt pathway. Cells were pre-treated with U0126 (30 µM), followed by the SNG (3 µM for Jurkat and 2 µM for Molt-4) treatment. Western blot analysis for phospho-Akt Ser473 and total Akt was carried out.

Taken together, these findings clearly indicate that ERK1/2 activation is not downstream to ceramide generation and both ceramide generation and Akt dephosphorylation is not regulated by ERK1/2.

Chapter 5: Discussion and conclusion

The present study investigated the antileukemic potential of SNG *in vitro* and further examined the molecular signaling mechanisms involved in this process. The results from this study demonstrate that SNG exhibits a potent pro-apoptotic effect on leukemic cells via two different mechanisms. In the first mechanism, SNG induces excessive ROS generation, leading to ceramide accumulation, which further mediates the inhibition of Akt signaling pathway, and activation of the caspase cascade. In the second mechanism, SNG mediates ROS-dependent ERK1/2 activation, leading to the induction of apoptosis. In recent studies, SNG has been shown to induce apoptosis in many cancer cells such as skin (Hammerová et al., 2011; Rosen et al., 2015), breast (Dong et al., 2013; S. Kim et al., 2008), lung (S. Gu et al., 2015; Jang et al., 2009), bladder (Han, Park, et al., 2013), prostate (Adhami et al., 2004), cervix (Xu et al., 2012), as well as, primary effusion lymphoma (Hussain et al., 2007) through the activation of diverse signaling mechanisms, including ROS generation and ER stress, as well as inhibition of NFκB (Chaturvedi et al., 1997; Duvoix et al., 2004; S. Gu et al., 2015; Hussain et al., 2007; S. Kim et al., 2008; Matkar et al., 2008a; S. Y. Park et al., 2014; Xu et al., 2012; Yin et al., 2005). However, none of these studies explored the functional role of ROS-ceramide-Akt signaling axis in SNG-induced tumor suppression. This is the first study demonstrating that SNG mediates caspase activation and apoptosis in human leukemic cells via ROS-dependent ceramide accumulation and inactivation of Akt signaling pathway (Fig. 5.1). Furthermore, our study also demonstrates involvement of ROS-dependent ERK1/2 phosphorylation in SNG-induced apoptosis (Fig. 5.1). In particular, the demonstration that SNG induces ceramide accumulation is extremely important, since ceramide plays a critical role in the activation and execution of

various signal transduction pathways, leading to tumor suppression (Galadari et al., 2015).

Figure 5.1. Proposed mechanism of sanguinarine-induced apoptosis in human leukemic cells. SNG induces ROS-dependent ceramide up-regulation, which in turn activates CAPP (PP1), leading to Akt dephosphorylation and subsequent induction of caspase-dependent apoptosis in human leukemic cells. Alternatively, SNG induces ROS-dependent ERK1/2 activation, leading to the induction of apoptosis.

Micromolar concentration of SNG has been shown to impart apoptosis in A431 human squamous carcinoma cells, but not in normal human epidermal keratinocytes at similar concentrations (Ahmad et al., 2000). Differential toxicity of SNG was also observed by Kaminskyy et al, where DNA damaging effects of SNG was more selective towards mouse leukemic cells compared to primary mouse spleen cells (Kaminskyy, Lin, et al., 2008). Sun et al. also observed profound cytotoxicity of SNG in DU145 and C4-2 prostate cancer cells compared to PZ-HPV7 normal prostate epithelial cells (Sun et al., 2010). On the other hand, others have shown that SNG does not exhibit more sensitivity against cancer cells versus normal cells (Debiton et al., 2003; Malíková et al., 2006; Slunská et al., 2010). Knowing that ROS generation is one of its prime signaling mechanisms, it is possible that SNG is more selective towards tumor cells as opposed to normal cells. Cancer cells have elevated basic ROS level compared to normal cells. It can be therapeutically utilized by further increasing ROS generation to such a toxic level which may specifically induce death in cancer cells (Fig. 5.2).
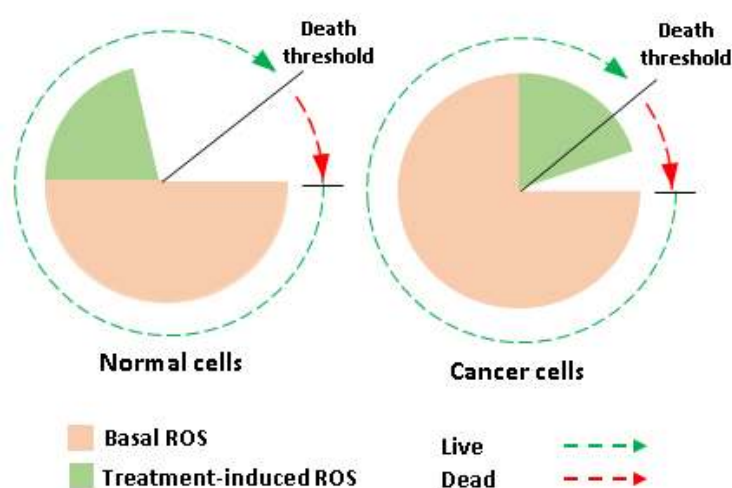
Figure 5.2. ROS levels in normal versus cancer cells. Cancer cells have elevated basal ROS levels as opposed to normal cells. Treatment with anticancer agents may increase ROS generation in cancer cells above the death threshold and induce death specifically in cancer cells, but not in normal cells.

In line with this, Leung et al. demonstrated that tyrosine kinase inhibitor-resistant non-small cell lung cancer (with T790M mutation in EGFR) have increased NOX2 activity and substantially higher basal ROS levels compared to normal lung tissues (Leung et al., 2015). SNG was shown to induce NOX3 activity in EGFR mutant cells, leading to EGFR over-oxidation, degradation and apoptosis (Leung et al., 2015). Such response were not observed in EGFR wild-type cells, demonstrating that ROS generation may be one of the key factors that determine the tumor selectivity of SNG; however, this needs to be further investigated.

ROS-mediated cytotoxicity may be achieved by using agents that either increase ROS generation, or inhibit antioxidant defense, or a combination of both. Thus, use of a compound that causes further increases in intracellular ROS, in conjunction with inhibitors of antioxidant system, might be a promising approach towards developing more successful anticancer therapy. For instance, a combination of arsenic trioxide (a ROS-generating agent) and 2-methoxyestradiol (a SOD inhibitor) were demonstrated to have synergistic activity against human leukemia (Y. Zhou, Hileman, Plunkett, Keating, & Huang, 2003). In this context, SNG is a 'multifaceted ligand', where it induces both ROS generation and diminution of the cellular antioxidant system (GSH and catalase). We show several lines of evidence that demonstrate the vital involvement of ROS in SNG-induced apoptotic cell death. First, SNG induces a dose-dependent ROS production (Fig. 4.6.A and B). Second, pre-treatment of cells with ROS scavengers (GSH and NAC) almost completely abolished SNG-induced ROS production and apoptosis (Fig. 4.7.A, B, C and D). Third, the cytotoxic effect of SNG was significantly abolished in the presence of a variety of antioxidants (Fig. 4.7.E). In particular, our results demonstrate that SNG-induced apoptosis was mediated, at least in part, by an increase in the cellular levels of H_2O_2, as evidenced by the attenuation of ROS-

derived fluorescence and loss of viability following pre-treatment with H_2O_2-specific scavengers (Fig. 4.8.A, B, and C and Fig. 4.9).

The present study also underscores involvement of the tumor suppressor lipid ceramide downstream to ROS in SNG-induced apoptosis. It is now well-established that ceramide modulates diverse intracellular signaling pathways that increase the sensitivity of human tumor cells to various anticancer agents (Galadari et al., 2015). Furthermore, defects in ceramide generation and metabolism could potentially contribute to the tumor cell survival and resistance to chemo and radiotherapy (Galadari et al., 2015). In the present study, we validate that SNG induces ROS-dependent ceramide generation (Fig. 4.13.A and B), and also demonstrate that increased ceramide generation plays a functional role in SNG-induced apoptosis (Fig. 4.15.E and Fig. 4.16.D). It is noteworthy that ROS production is required for the accumulation of ceramide in response to SNG-treatment. A direct link between ROS and ceramide production has been established in several cancer cell lines including leukemia (Gouazé et al., 2001; Mansat-de Mas et al., 1999). We also demonstrate that extracellular supplementation of antioxidants to human leukemic cells inhibited SNG-induced ceramide production (Fig. 4.13.A and B).

Most of the anticancer agents which induce endogenous ceramide generation act by interfering with the enzymes of ceramide biosynthetic or degradative pathway. Ceramide accumulation mainly occurs *via* (1) *de novo* pathway, (2) SMase-mediated hydrolysis of sphingomyelin, and (3) inhibition of CDase- or GCS-mediated conversion of ceramide into sphingosine and glucosylceramide, respectively. In the present study, SNG was shown to induce the activity of aSMase in Jurkat cells (Fig. 4.15.I), while inhibiting the activity of both aCDase and GCS in both Jurkat and Molt-4 cells (Fig. 4.16.A and B). No significant increase in aSMase activity was observed in Molt-4 cells (Fig. 4.15.I), which are

inherently known for having very little aSMase activity (B. Liu & Hannun, 1997). It is noteworthy that GCS inhibition by SNG was significant and occurred within 1 h of SNG treatment in both cell lines (Fig. 4.16.A and B). Mechanism of SNG-induced GCS inhibition needs further investigation. Such studies are underway in our laboratory. The involvement of ceramide biosynthetic and degradative enzymes in SNG-induced apoptosis was further confirmed by using various inhibitors that manipulate the activities of these enzymes. Pre-treatment with aSMase inhibitor (Desip) resulted in the attenuation of both ceramide generation and apoptosis (Fig. 4.15.C, D and E), while, the aCDase inhibitor (NOE) and GCS inhibitor (PDMP) promoted it (Fig. 4.16.C, D and E). The manipulation of ceramide biosynthetic enzymes by anticancer agents have also been reported previously. Several pro-apoptotic stimuli, such as FAS, TNF-α, and UV irradiation activate aSMase-mediated ceramide generation in a variety of cancer cell types to induce apoptosis (Gulbins & Grassmé, 2002; Y. Zhang et al., 2001). Similarly, tamoxifen inhibits ceramide glucosylation (mediated by GCS), leading to the accumulation of intracellular ceramide and induction of apoptosis in adriamycin-resistant MCF-7 human breast cancer cells (Lavie et al., 1997). Moreover, inhibition of aCDase was found to be essential for the anticancer activity of Carmofur, a clinically used antineoplastic drug for the treatment of colorectal carcinoma (Realini et al., 2013). In this context, our results show that SNG induces ROS-dependent intracellular accumulation of ceramide via activation of aSMase (in Jurkat) and inhibition of GCS and aCDase (in Jurkat and Molt-4), and induces apoptosis, supporting a significant role for ceramide in triggering apoptosis.

Akt is crucial to many aspects of cell growth, survival and apoptosis, and is frequently activated in many cancers including leukemia (Chang et al., 2003; Fransecky et al., 2015; Manning & Cantley, 2007). Akt activation in turn signals a variety of key downstream targets, including GSK3β and mTOR, which

consequently inhibit apoptosis and promote cell survival (Manning & Cantley, 2007). Previously, others have shown that inhibition of Akt signaling pathway is consistently associated with the induction of apoptosis in cancer cells (K. F. Chen et al., 2008; Hennessy, Smith, Ram, Lu, & Mills, 2005; Manning & Cantley, 2007). Our results show that SNG inhibits Akt phosphorylation at Ser473 (Fig. 4.18.A and B). The inhibitory effect of SNG on Akt phosphorylation is also correlated with the dephosphorylation of its downstream targets, GSK3β and mTOR (Fig. 4.20). It is well established that mTOR itself is an important cell growth and pro-survival protein that is activated by Akt-mediated phosphorylation at Ser2448, whereas, GSK3β mainly acts as a pro-apoptotic protein that is inactivated by Akt-mediated Ser9 phosphorylation (Ciuffreda et al., 2010; Jacobs et al., 2012; Manning & Cantley, 2007; Pópulo et al., 2012). SNG-mediated dephosphorylation of Akt may reactivate the tumor suppressive functions of GSK3β, while inactivating the pro-tumorigenic functions of mTOR. In addition, SNG treatment has also been shown to reduce the phosphorylation status of the mTOR downstream target, 4EBP1 (Fig. 4.20). A plethora of studies have demonstrated the link between ceramide and Akt in the regulation of apoptosis (Salinas et al., 2000; Schubert et al., 2000). Several studies have demonstrated that CAPPs (PP1 and PP2A) dephosphorylate Akt at Ser473 and induce apoptosis (Salinas et al., 2000; Schubert et al., 2000). This effect was reversed by pre-treatment with CAPP inhibitors such as CalA and OA (Salinas et al., 2000; Schubert et al., 2000). In our study, Akt dephosphorylation did not initiate until there was significant ceramide generation, suggesting the possible involvement of CAPPs in SNG-induced Akt dephosphorylation. Moreover, in this study, several lines of evidence demonstrate the vital involvement of ceramide in SNG-induced Akt dephosphorylation. First, aSMase inhibitor (Desip) significantly blocked SNG-induced Akt dephosphorylation and apoptosis (Fig. 4.15.E and 4.22.A). Second, enhancement of ceramide generation using aCDase inhibitor (NOE) and GCS

inhibitor (PDMP) sensitized SNG-induced Akt dephosphorylation and apoptosis (Fig. 4.16.D and 4.22.C). Third, direct treatment of leukemic cells with C_6-ceramide resulted in Akt dephosphorylation (Fig. 4.23.A). Forth, using various CAPPs inhibitors (CalA, OA, and PA), it was clearly demonstrated that activation of the PP1 form of CAPP was responsible for SNG-induced Akt dephosphorylation (Fig. 4.24.A and B and Fig. 4.25.A, B, C and D). In addition, ROS scavengers such as GSH and NAC also significantly abolished SNG-induced Akt dephosphorylation, demonstrating the role of ROS in Akt dephosphorylation (Fig. 4.21). Thus, these results clearly demonstrate that SNG induces ROS-dependent ceramide generation, which in turn activates PP1, leading to Akt dephosphorylation and apoptosis in human leukemic cells (Fig. 5.1).

Importantly, our study has also identified a crucial role of ERK1/2 in the facilitation of SNG-induced apoptotic cell death. Antioxidants, such as GSH and NAC, significantly abrogated ROS production and ERK1/2 activation, demonstrating the key role of ROS in ERK1/2 activation. Using ERK1/2 inhibitor (U0126), we identified that neither ceramide generation nor Akt dephosphorylation are regulated by ERK1/2. Moreover, ceramide inhibitor (Desip) did not attenuate ERK1/2 activation, demonstrating that ERK1/2 follows an alternative pathway of cell death, independent of ceramide-Akt axis. The mechanism involved in the ERK1/2-mediated apoptosis by SNG needs further investigation.

In summary, our study demonstrates that a hierarchy of signaling events are activated by SNG in order to induce apoptosis in human leukemic cells. Briefly, SNG induces caspase-dependent apoptosis in human leukemic cells, which is regulated by ROS-dependent ceramide up-regulation, which in turn activates PP1, leading to Akt dephosphorylation (Fig. 5.1). In an alternative pathway, SNG induces ROS-dependent ERK1/2 activation, leading to the induction of apoptosis in human leukemic cells (Fig. 5.1). Further investigations to understand the mechanisms by

which SNG induces apoptosis in human leukemic cells, tumor xenografts, and leukemic mice models should provide a rationale for the application of SNG-based pro-apoptotic drug in the treatment of leukemia. Accordingly, such studies are currently underway in our laboratory.

In conclusion, our data demonstrate that SNG induces:

- Caspase-dependent apoptosis in human leukemic cells.

- Mitochondrial H_2O_2 generation and subsequent induction of apoptosis in human leukemic cells.

- ROS-dependent ceramide generation via aSMase activation (in Jurkat cells) and aCDase and GCS inhibition (in Jurkat and Molt-4 cells).

- Ceramide-dependent PP1 activation and subsequent induction of Akt dephosphorylation at Ser473 in human leukemic cells.

- ROS-dependent ERK1/2 activation, leading to the induction of apoptosis independent of the ceramide activation and Akt dephosphorylation.

This conclusion is illustrated pictorially in Fig. 5.1.

Chapter 6: Future directions

Three independent findings by Ahmad et al., Kaminskyy et al., and Sun et at., demonstrate that cytotoxicity of SNG is more selective against cancer cells compared to normal cells (Ahmad et al., 2000; Kaminskyy, Lin, et al., 2008; Sun et al., 2010). In contrast, others have shown that SNG does not exhibit preferential sensitivity against cancer cells versus normal cells (Debiton et al., 2003; Malíková et al., 2006; Slunská et al., 2010). In future experiments, by using variety of normal and cancer cells, we will evaluate whether SNG exhibits any kind of selectivity against cancer cells. Two possible explanation can be drawn for preferential sensitivity of SNG against cancer cells:

(1) In our study, we have shown that SNG induces ROS generation which is critical for the induction of apoptosis. Furthermore, several previous reports have provided strong evidence for the involvement of ROS in the facilitation of SNG-induced apoptosis (Dong et al., 2013; S. Kim et al., 2008; Matkar et al., 2008a, 2008b; Yin, Kim, Moon, & Lee, 2005). Knowing that cancer cells already have increased basal ROS level, SNG-induced ROS generation may further intensify intracellular ROS levels in cancer cells to such a toxic level, which may specifically induce death in cancer cells (Fig. 5.2).

(2) We have identified that SNG induces expression and activation of tumor suppressor protein, prostate apoptosis response-4 (Par-4) in Jurkat cells (data not shown). Par-4 is known to selectively activate and induce apoptosis in cancer cells, but not in normal cells (Thayyullathil et al., 2014). In future, we will evaluate whether ROS generation or Par-4 activation is involved in the SNG-induced tumor selectivity.

In the present study, we identified that SNG induces rapid and significant inhibition of GCS enzyme in both Jurkat and Molt-4 cells. Similar results were also

observed in PC-3 and DU145 prostate cancer cells (data not shown). However, it has not been studied as to how GCS enzyme activity is inhibited by SNG. The mechanism(s) involved in this process will be evaluated in future experiments by employing suitable *in vivo* and *in vitro* models. Additionally, a plethora of studies demonstrate that, as opposed to conventional chemotherapeutic agents, SNG is effective against various multidrug resistant cancer cells overexpressing P-gp protein (Ding et al., 2002; Eid et al., 2013; Gatti et al., 2014; Weerasinghe et al., 2006). It is noteworthy that GCS activity is very well associated with P-gp expression and resistance to chemotherapy (Gouazé et al., 2005; Gouazé et al., 2004; Gupta & Liu, 2013; Lavie, Cao, Bursten, Giuliano, & Cabot, 1996; Y. Y. Liu et al., 2010; Y. Y. Liu, Han, Giuliano, & Cabot, 1999; Y. Y. Liu, Han, Giuliano, Hansen, & Cabot, 2000; Y. Y. Liu, Hill, & Li, 2013; Lucci et al., 1998). In future, we would like to examine whether increased sensitivity of SNG against P-gp overexpressing multidrug resistant cancer cells is mediated via its GCS inhibitory activity.

In this study, we have identified that SNG-induces ROS-dependent ERK1/2 phosphorylation leading to the induction of apoptosis in human leukemic cells. Previously, we have also identified that SNG-induces ROS-dependent ERK1/2 phosphorylation, leading to the induction of autophagic cell death in human malignant glioma cells (Pallichankandy et al., 2015). The mechanism by which SNG regulates ERK1/2 activity requires further investigation. The upstream and downstream regulators in this process will be studied in future.

Further investigation to understand the mechanisms by which SNG induces apoptosis in human leukemia cells using tumor xenografts will provide a rationale for the application of SNG-based pro-apoptotic drug in the treatment of leukemia. Accordingly, such studies are currently underway in our laboratory.

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