All Theses & Dissertations   **Student Scholarship**

10-2014

Effects of 5-Aza-2' Deoxycitidine and Sulforaphane on Major Histocompatibility Complex (MHC) classes I and II surface expression in human acute T-cell leukemia cell Jurkat

Ali Salam Abdullah
University of Southern Maine

Follow this and additional works at: https://digitalcommons.usm.maine.edu/etd

Part of the Immune System Diseases Commons

Effects of 5-Aza-2' Deoxycitidine and Sulforaphane on Major Histocompatibility Complex (MHC) classes I and II surface expression in human acute T-cell leukemia cell Jurkat

By: Ali Salam Abdullah

M.B.Ch.B. University of Basra, College of Medicine/Iraq

A THESIS

Submitted in Partial Fulfillment of the Requirement for the Degree of

Master of Science

(In Applied Medical Sciences/ Immunology & Infectious Diseases)

October, 2014

University of Southern Maine

Advisory Committee:

Ah-Kau Ng, Ph.D. Professor, University of Southern Maine.

Stephen Pelsue, Ph.D. Associate Professor, University of Southern Maine.

Ronald Rubocki, Ph.D., D(ABHI), Director, Nordx Histocompatibilty Lab

Scarborough, Maine (an ASHI accredited lab).

THE UNIVERSITY OF SOUTHERN MAINE

/ 10 / 2014

We hereby recommend that the thesis of:

Ali Salam Abdullah

Entitled:

Effects of 5-Aza-2'Deoxycitidine and Sulforaphane on Major Histocompatibility Complex (MHC) classes I and II surface expression in human acute T-cell leukemia cell Jurkat

be accepted as partial fulfillment of the requirements for the Degree of Master of Science in Applied Medical Sciences.

Advisory Committee:

Ah-Kau Ng, Ph.D.

Chairperson

Stephen Pelsue, Ph.D.

Ronald Rubocki, Ph.D., D(ABHI)

ii

Dedication

To the Stars of my sky:

My father, my mother, and my brother

For all those times you stood by me

For all the truth that you made me see

For all the joy you brought to my life

For all the wrong that you made right

For every dream you made come true

For all the love I've found in you

I'm everything I am because you love me

By Dianne Warren (1995)

From the black sheep of our SAM family

Acknowledgments

Abstract

The immune system cells recognize and kill cancer cells by engaging the tumor antigens on the surfaces of cancer cells presented by the Major Histocompatibility Complex (MHC). Cancer cells can escape from immune system cells recognition and/or killing by down-regulating the MHC expression through genetic or epigenetic mechanisms. The epigenetic mechanisms affect the accessibility of DNA by histone deacetylation or DNA methylation. DNA methylation inhibitors have been studied and have been found to modulate epigenetic changes in cancer cells. There are three types of DNA methylation inhibitors: nucleoside analogues (e.g. 5- Aza-2'Deoxycitidine), antisense oligonucleotides, and non-nucleoside analogues inhibitors (e.g. Sulforaphane, an isothiocyanate). According to my knowledge there is no study about any isothiocyanate's effect on the MHC expression in cancer cells. Also, the only two studies I could find about the effect of 5-Aza-2'Deoxycitidine on the expression on MHC expression by human acute T-cell leukemia Jurkat cell line demonstrated its ability to up-regulate MHC class II expression at the mRNA level, with no information about its effect on MHC class II expression at the level of cell surface and no report about its effect on MHC class I expression by Jurkat cells. My study is the first about the effect of Sulforaphane on MHC expression and about the effect of 5-Aza-2'Deoxycitidine on MHC class I expression by Jurkat cells and on MHC class II expression at the level of cell surface of the same cells.

I treated Jurkat cells with 3 different concentrations of sulforaphane or 5-Aza-2'Deoxycitidine with 3 different durations of treatment in parallel with DMSO controls. Then I used indirect fluorescent staining of the cell samples with monoclonal antibodies to measure the degree of

surface expression of MHC classes I and II by flow cytometer. The results demonstrate that sulforaphane could down-regulate MHC class I cell surface expression by Jurkat cells while 5-Aza-2'Deoxycitidine could up-regulate MHC class I cell surface expression by Jurkat cells.

Both sulforaphane and 5-Aza-2'Deoxycitidine did not affect the MHC class II cell surface expression by Jurkat cells. I also studied the effect of both drugs on cells growth and viability and discovered that both could inhibit growth and reduced viability of Jurkat cells, in accordance with findings in previous studies by others..

Table of Contents

List of Tables

List of Figures

List of Abbreviations

- **MHC :** Major Histocompatibility Complex
- **HLA :** Human Leukocyte Antigen
- **CTL :** Cytotoxic T Lymphocyte
- **Kb :** Kilo base
- **TNF :** Tumor Necrosis Factor
- **INF :** Interferon
- **DNA :** Deoxyribonucleic acid
- **RNA :** Ribonucleic acid
- **miRNA :** Micro ribonucleic acid
- **CITA :** Class I transactivator
- **CIITA :** Class II transactivator
- **APC :** Antigen Presenting Cell
- **ER :** Endoplasmic Reticulum
- **RER :** Rough Endoplasmic Reticulum
- **Ii :** Invariant chain
- **ERAP :** Endoplasmic Reticulum Resident AminoPeptidase
- **HNSCC :** Head and Neck Squamous Cell Carcinoma
- **SCLC :** Small Cell Lung Carcinoma
- **NSCLC :** Non-Small Cell Lung Carcinoma
- **DNMT :** DNA MethylTransferase
- **HDAC :** Histone Deacetylase
- **MDS :** Myelodysplasia Syndrome
- **AML :** Acute Myeloid Leukemia
- **AZA :** 5-Aza-2'Deoxycitidine
- **SFN :** Sulforaphane
- **CLL :** Chronic Lymphocytic Leukemia
- **ALL :** Acute Lymphocytic Leukemia
- **ITC :** Isothiocyanate
- **AITC :** Allyl isothiocyanate
- **BITC :** Benzyl Isothiocyanate
- **PEITC :** Phenethyl isothiocyanate
- **Keap1 :** Kelch-like ECH-associated protein 1
- **Nrf2 :** Nuclear factor E2-factor related factor
- **NQO1 :** NAD(P)H:quinone reductase
- **ARE :** Antioxidant response element
- **HO1 :** Heme oxygenase 1
- **GCS :** Glutamylcysteine synthetase
- **GSH :** Glutathione

- **PARP :** Poly (ADP-ribose) polymerase
- **MAPK :** Mitogen-activated protein kinases
- **ERK :** Extracellular signal-regulated kinases
- **ROS :** Reactive Oxygen Species
- **IAP :** Inhibitor of apoptosis
- **JNK :** c-Jun NH_2-termial kinases
- **NF-κB :** Nuclear Factor Kappa B
- **VEGF :** Vascular Endothelial Growth Factor
- **RPMI :** *Roswell Park Memorial Institute*
- **DMSO :** Dimethyl sulfoxide
- **FBS :** Fetal Bovine Serum
- **ATCC :** American Type Cell Culture
- **µM :** Micro molar
- **mM:** Mill molar
- **ml :** Milliliter
- **µl :** Microliter
- **DPBS :** Dulbecco's Phosphate Buffer Saline
- **Ig :** Immunoglobine
- **FITC :** Florescent Isothiocyanate
- **nm :** Nanometer
- **Ab :** Antibody
- **mAb :** Monoclonal Antibody
- **FSC :** Forward Scatter
- **SSC :** Side Scatter

1. Introduction and background:

1.1. The Major Histocompatibility Complex (MHC) :

1.1.1. MHC definition and discovery :

The MHCs are transmembranous protein molecules expressed on cell surfaces. They are the key molecules associated with organ rejections in transplantation. Human MHC was discovered by Jean Dausset and his colleagues in 1965 and the name Hu-1 was given first which later has become Human Leukocyte Antigen (HLA). The discovery happened while Dausset and his colleague discovered high rates of kidney rejection among patients who previously received blood transfusion or were multiparous women, and that these patients' blood contained antibodies that recognized specific proteins on cell surfaces of the donated organs. These proteins were the MHC gene products, and were found to be present on all mammalian cell surfaces [1, 2].

In 1970, Baruj Benacerraf and his colleagues discovered what they called immune response (Ir) genes in mice which control immune responses and were mapped to the murine MHC gene loci [2]. Later, in 1974, Rolf Zinkernagel and Peter Doherty concluded the phenomenon of MHC restriction which means that cytotoxic T lymphocytes (CTLs) only recognize and kill virally infected cells expressing MHC class I shared by these T lymphocytes.

1.1.2. The MHC genes [1] :

The MHC gene complex is composed of two polymorphic gene systems for classes I and II, and non-polymorphic gene cluster for the antigen presentation machinery components. The class I molecules are expressed in all nucleated cells and are associated with CD8$^+$ CTLs' function. The class II are expressed on dendritic cells, B lymphocytes, macrophages, and few other cells, and are associated with differentiation and functions of CD4$^+$T helper cells.

The inheritance of MHC alleles is an autosomal dominant Mendelian inheritance which means that half of the linked alleles, i.e. haplotypes, are from each of the parents, and both haplotypes are expressed. The MHC genes are the most polymorphic in the genome and about 3,500 HLA alleles have been estimated serologically.

The molecular studies have shown that each serologically distinct allele may consist of multiple variants that differ from each other slightly. In humans, the MHC gene is located on the short arm of chromosome 6, and is about 4,000 kilobases in length.

In general, the MHC gene can be divided into 3 loci (see Figure I in Appendix I):

- Class II locus: the first 1000 kilobases (kb) encode for the following genes (with locations indicated by the positions of bases on the MHC gene complex): Tapasin (50-100 kb), DP A1 and B1 (around the 350 kb location), DM A&B (around the 500 kb), genes from location 475 to 600 kb in order (Proteasome genes, TAP1, Proteasome genes, TAP2, and DOB), DQ A2&B2 around 650 kb, DQ A1&B1 around 750 kb, DRB1 at about 825 kb , DRB3-5 at 900 kb, and DRA at 1000 kb.

- Class III locus: the second 1000 kb encodes C4B somewhere between 1400 and 1450 kb, C4A around 1450 kb, factor B and C2 are around 1500 kb, around 1850 kb in order are LT-beta, TNF-alpha, and LT-alpha, and MIC-B between 1900 and 1950 kb.

- Class I locus: the last 2000 kb encodes MIC-A between 2000 and 2050 kb, HLA-B between 2050 and 2100 kb, HLA-C between 2150 and 2200 kb, HLA-E between 2850 and 2900 kb, HLA-A between 2500 and 2550 kb, HLA-G around 2750 kb, and HLA-F around 2850 kb.

1.1.3. The MHC transcription factors and transactivators [3-5]:

Bernard Mach at the University of Geneva discovered the first transactivator of MHC genes called MHC class II transactivator or CIITA. It has no DNA binding domain; instead, it interacts with transcription factors at the promoter of MHC class II gene to generate an active protein-DNA complex called "MHC enhanceosome". The latter activates the MHC class II genes. CIITA is required for the expression of MHC class II (HLADP, -DQ and -DR) genes and for the regulatory molecules involved in the MHC class II antigen pathway including the invariant chain, HLA-DO and -DM for both constitutive and inducible expression of MHC class II. CIITA can also activate the promoters of MHC class I genes but it is not the only transactivator for MHC class I. Seventeen years after the discovery of CIITA, the MHC class I transactivator (CITA or NLRC5) was identified. CITA can also induce class I related genes required in MHC class I antigen presentation, such as $\beta 2$-microglobulin, TAP1 and LMP2. Both CIITA and CITA are members of NLR (nucleotide binding domain-leucine rich repeats containing) family of proteins.

3

By detailed phylogenetic analysis of the nucleotide binding domain, CITA is the most closely related to CIITA among all NLR proteins.

MHC class I genes share similar cis-regulatory elements in their proximal promoters, termed W/S, X1, X2 and Y-box motifs (see Figure II in Appendix I). CITA can associate with the RFX transcription factor complex at the X1 box and can cooperate with ATF1/CREB family transcription factor at the X2 box of MHC class I promoter. NFY transcription factor binds to Y box. MHC class II gene has conserved upstream sequences with cis-regulatory elements: S (binds to RFX), X (binds to RFX, AP1, X2BP, and CREB), and Y (binds to NFY). All form MHC enhanceosome that requires CIITA to be active.

In innate and adaptive immune responses, cytokines (mostly interferons IFN α, β, and γ for class I, and IFN-γ for class II) and signals like Toll-like receptor TLR signals activate transcription factors to increase the level of expression of MHC molecules.

1.1.4. MHC class I structure [1] (see Figure III in Appendix I):

MHC class I molecule is composed of two non-covalently linked subunits: an alpha chain (MHC I-encoded 44-47 kDa polypeptide) and a beta2-microglobulin (non-MHC-encoded, 12 kDa polypeptide) chain. Three quarters of the alpha chain extends extracellularly. The other quarter composes of a short hydrophobic segment (25 amino acids) inside the cell membrane and ends with the intracellular carboxyl-terminal residues (30 amino acids).

The N-terminal end of MHC class I molecule composes of two segments, alpha-1 and -2, each is about 90 residues long and they interact with each other to form a platform of

eight-stranded antiparallel beta pleated sheet that supports two parallel strands of alpha helix. This forms the peptide binding cleft of a size ~25 x 10 x 11 A° that binds to 8-11 amino acids peptides in a flexible extended conformation. Each MHC molecule has only one peptide binding cleft which can bind to many different peptides but it binds to one peptide at a time in a non-covalently binding. The alpha-1 and -2 represent the polymorphic portion of the MHC class I molecule and responsible for the variation among MHC class I alleles in peptides binding and T cells recognition. Next to the alpha-2 domain, proximal to cell surface, is the alpha-3 segment which folds into an Ig domain which represents the binding site for CD8 on T cells. The amino acids sequence of the alpha-3 segment is conserved among all MHC class I alleles. Next to the alpha-3 domain is the hydrophobic transmembrane segment. The last part of a complete MHC class I molecule is the beta-2 microglobulin, which is encoded by a gene distinct from the MHC, and, like alpha-3 domain, is conserved in all MHC class I molecules. Beta-2 microglobulin interacts with alpha-3 domain non-covalently.

1.1.5 MHC class II structure [1](see Figure III in Appendix I):

MHC class II is composed of non-covalently associated 32-34 kDa alpha and 29-32 kDa beta polypeptide chains. Both are encoded in polymorphic genes in the MHC II locus. Each chain is divided into amino (polymorphic α1 and β1) and carboxyl (non-polymorphic α2 and β2) terminal segments. α1 and β1 interact to form the peptide binding cleft (four strands of the floor and a side of the wall are formed by each of α1 and β1). The polymorphic residues are located in and around the cleft and mostly in the beta chain. The ends of the cleft are open so that peptides of 30 amino acids or larger

5

can fit in. The extracellular parts of α2 and β2 are folded to form Ig domains that of beta 2 contain the binding site for CD4 on T cell. The transmembranous hydrophobic segments are of 25 amino acids length and have intracellular short hydrophilic carboxyl ends.

1.1.6. MHC class I and antigen processing for presentation [1], (see Figure IV in Appendix I):

In an antigen presenting cell (APC), TAP, a transporter protein, mediates the transportation of degraded proteins by proteasomes from cytosol to endoplasmic reticulum (ER) lumen, in an ATP-dependent manner. In the ER, class I alpha chains and beta-2-microglobulines are synthesized and folded by the aid of chaperon proteins like calnexin and the calreticulin to form an empty class I dimer.

In the ER lumen, a protein named Tapasin brings TAP into a complex with a newly synthesized empty MHC class I molecule. Also, oxireductase, Erp57, and the chaperons mentioned above, all interact with the formed complex. Then, an ER-resident aminopeptidase (ERAP) enzyme trims the peptides brought by TAP and other peptides formed in the ER to produce smaller peptides in a size of 8 to 16 amino acids suitable for MHC-clefts binding. Once binding occurs, the affinity of binding to Tapasin is lost and the MHC-peptide complex is released from the ER and is transported to the cell surface through the Golgi complex and by exocytic vesicles. The MHC-peptide complex will be presented by APC to a CD8$^+$ T cell expressing with a proper T cell receptor (TCR). If no

peptide binds to the MHC dimer, the latter will be transported to the cytosol to be degraded by proteasomes.

1.1.7. MHC class II and antigen processing for presentation [1] (see Figure IV in Appendix I):

In the ER of an APC, alpha and beta chains of MHC class II are synthesized coordinately and associate to each other to form dimers. Dimers then undergo folding and assembly promoted by the Invariant chain (Ii) protein and by the aid of ER-resident chaperons like calnexin to form stable structures. Ii also directs the newly formed MHC class II molecules to the late endosomes after leaving ER by exocytic vesicles and it blocks the peptide binding clefts to prevent it accepting peptides before reaching endosomes.

The vesicles fuse with endosomes which contain peptides from various sources (intra and extracellular), and by endosomal proteases enzymes, Ii is degraded and a 24 amino acids peptide is left called CLIP (class II associated invariant chain peptide).

Degraded peptides inside endosomes are associated with a non-polymorphic MHC molecule called HLA-DM. The latter exchanges their peptides with CLIP and accelerates peptides binding to MHC class II. After binding to MHC class II, the large peptides are trimmed by proteolytic enzymes to result in 10-30 amino acids long peptides.

Peptide-loaded MHC class II are then delivered to cell membrane by fusion of endosomes with the membrane. The peptide-MHC II complex will be presented by the APC to a CD4$^+$ T cell expressing a proper TCR.

1.2.   MHC and immune response against cancer

1.2.1. Immune response to cancer:

The immune response against transformed tumor cells is achieved by both innate and adaptive immune systems. The initial step is to recognize the cancer cells by the cancer antigens on their cell surfaces presented by the Major Histocompatibility Complexes (MHCs), and this is called the immune surveillance [6]. MHC class I presents tumor antigens to be recognized by the CD8$^+$ Cytotoxic T Lymphocytes (CTLs), and CTLs will kill the cancer cells. While MHC class II presented tumor antigens will be recognized by CD4$^+$ T helper cells which will facilitate the activation and proliferation of the CTLs by the cytokines they secrete.

Cancer cells have different mechanisms by which they can escape immune system recognition and/or killing [1]. One of the important mechanisms by which the cancer cells can escape the immune cells is the down-regulation of expression or the absence of necessary molecules for immune cells activities, and these molecules include MHC classes I and II [6].

MHC class I expression has been found down-regulated in different human cancers like bladder cancer [7], lung cancer [8], prostate cancer [9], cervical cancer [10], leukemia [11], lymphoma [12,13], melanoma [14,15], gastric cancer [16], endometrial cancer [17], ovarian cancer [18], breast cancer [19], and colon cancer [20].

The association of MHC class I expression down-regulation in cancers with clinical prognosis was found conflicting in different studies [21].Poor prognosis was found in

8

HNSCC, breast carcinoma, SCLC, prostate carcinoma, bladder carcinoma, cervical squamous cell carcinoma, and cutaneous melanoma. Good prognosis was found in uveal melanoma and colon carcinoma, and there was no association with prognosis in NSCLC, pulmonary adenocarcinoma, cervical adenocarcinoma, cutaneous squamous cell carcinoma, and large cell lymphoma.

MHC class II down-regulation was found in different types of cancers [22] like carcinomas of the head and neck, esophagus, lung, breast, colon, liver, kidney, bladder, ovary, cervix, cutaneous and uveal melanoma, and leukemia, as well as lymphoma cells [23-25]. The clinical prognosis association with MHC class II studies have also shown conflicting results and more researches are required in this field [22].

1.2.2. Epigenetic mechanisms in MHC expression down-regulation in cancers[22,26] :

The mechanisms by which cancer cells down-regulate the expression of MHC molecules can be classified into genetic and epigenetics. For mammalian cells, 3 categories of factors affect the transcription initiation: promoters, promoter-specific regulatory factors, and the accessibility to DNA (regulated by epigenetic mechanisms). The latter operates through changing the chromatin structure by posttranslational modifications of histones and postreplicational modification of DNA. Epigenetic mechanisms are involved in genomic imprinting, gene silencing, X chromosome inactivation, reprogramming in transferred nuclei, and some elements of carcinogenesis.

Because of the fact that epigenetic mechanisms are reversible, they have been extensively studied to develop immune therapies, and different compounds have been

9

found to be able to reverse the epigenetic changes in cancer cells and support the immune system in its battles against cancer cells. The epigenetic changes regarding the expression of MHC molecules either silence the MHC genes themselves or the transcription regulatory factors or both.

1.2.3. DNA methylation [26]:

DNA methylation, an epigenetic mechanism, is mediated by DNA methyl transferases (DNMTs). In mammalian cells, it occurs at the C5 of cytosine (5mC) usually within CpG dinucleotide. The mechanism of DNA methylation starts with DNMT binding to the DNA and eversion of target nucleotide to project out of the DNA helix (base flipping), then the DNMT attacks cytosine C6 and results in the transferring of the methyl group from S-adenosyl-l -methionine (AdoMet) to the activated cytosine C5 (see figure V in Appendix I). DNA methylation is also associated with phenomena such as DNA repair, initiation of sexual dimorphism, progression through cell division checkpoints, and suppression of the huge number of transposable and retroviral elements in the mammalian genome.

The DNMTs in mammals are DNMT3 (a and b, both are responsible for initial de novo CpG methylation which requires a regulatory factor, DNMT3L) and DNMT1 which is responsible for maintaining methylation pattern during chromosome replication and repair and for de novo methylation at non-CpG islands (and possibly even in CpG islands to some extent).

1.2.4. DNA methylation inhibitors and their effects on cancer:

When cancer develops, abnormal hypermethylation of CpG islands in the promoters of tumor suppressor genes usually occurs and leads to silencing of these genes. Thus, reactivation of tumor suppressor genes by DNA methylation inhibitors has been widely studied [27].

There are three types of DNA methylation inhibitors [28]: (I)nucleoside analogues (like azacytidine or 5-azacytidine, decitabine or 5-aza-2`-Deoxycitidine, fazarabine or 1-β-D-arabinofuranosyl-5 azacytocine, and DHAC or dihydro-5-azacytidine) which structurally resemble cytidine and require to be incorporated into DNA to function, (II) the antisense oligonucleotides which inhibits DNMTs (1,3a, and 3b), and (III) non-nucleoside analogues (like hydralazine, procainamide, and procaine) which inhibit the expression of DNMT.

Nucleoside analogues were found to induce cell death by different mechanisms [29] such as by covalently linking with DNMT which causes obstruction of DNA synthesis, induction of DNA damage through structural instability at the site of incorporation, significant demethylation after repeated replication. Their incorporation into RNA causes ribosomal disassembly, defective tRNA function and inhibition of protein production.

Decitabine or 5-aza-2`-Deoxycitidine (see figure VI in Appendix), used in this research, is currently used to treat myelodysplasia syndrome (MDS). It is under investigation for

treating acute myeloid leukemia (AML) and other malignancies [30]. Thus far, it is the most studied DNA methylation inhibitor.

5-AZA-CdR has been found able to inhibit cell growth, induce apoptosis, and up regulate MHC class I expression: in melanoma cells (tested by flow cytometry analysis) [31, 32], in ovarian cancer cells (tested by flow cytometry) [33, 34], and in human esophageal squamous cell carcinoma cells (tested by mRNA RT-PCR) [35-37]. It has been also found able to inhibit cell growth, induce apoptosis, and up-regulate MHC class II in lymphoma cells (tested by flow cytometry) [38, 39].

In leukemia cells, 5-Aza-CdR induced expression of both MHC classes I and II in chronic lymphocytic leukemia (CLL) cells (tested by flow cytometry)[40], up-regulated MHC class I in k562 leukemia cells (tested by flow cytometry) [41], and induced MHC class-II expression in acute lymphocytic leukemia (ALL) Jurkat cells (tested by mRNA RT-PCR) [42]. It also caused cell growth inhibition and apoptosis in HL-60, Raji, Jurkat, and ML-1 leukemic cell lines [43].

1.2.5. Sulforaphane , a non-nucleoside analogue DNA methylation inhibitor:

Isothiocyanates (ITC) are dietary compounds present in cruciferous vegetables like broccoli, cabbage and cauliflower. They are characterized by a sulfur containing functional group (N=C=S). The widely studied and commonly known ITC are allylisothiocyanate (AITC), benzyl isothiocyanate (BITC), phenethylisothiocyanate (PEITC), and Sulforaphane (SFN) [44].

Consumption of cruciferous vegetables was found conversely related to cancer incidence in different epidemiological studies for different cancer types like prostate, colorectal, breast, and lung cancers [45, 46].

ITC have been found to have different anticancer functions (cell cycle arrest or apoptosis) in different cancer types (leukemia, prostate, breast, lung, cervical cancer, and colorectal cancers)[47] through different mechanisms such as modulation of biotransformation enzymes, inhibition of NF-κB pathways, modulation of micro RNA (miRNA) regulation, stimulation of cancer cells cell cycle arrest and apoptosis, modulation of hormone receptors expression, anti-angiogenic and anti-metastatic effects, and epigenetic regulation by inhibiting DNMTs or HDACs [48].

ITC down-regulate DNMTs expression, thus they can be considered as non-nucleoside analogues DNA methylation inhibitors [48].

Among the isothiocyanates, sulforaphane (SNF) (see figure VII in Appendix I) was widely studied, it has been found able to inhibit cell growth, induce apoptosis, inhibit DNA methylation and histone deacetylation in cancer cells [44, 49]. Its growth inhibition and apoptosis induction were found in prostate cancer, breast cancer, colon cancer, leukemia, medulloblastoma [49], ovarian cancer, salivary gland cancer, and liver cancer [50].

Sulforaphane is also an epigenetic modulator; it inhibited histone deacetylase in prostate, colon, and breast cancers [48, 49]. It also inhibited DNA methylation in breast

and colon cancers (decreased DNMT1 and 3a and caused site specific CpG islands demethylathion) [49], and in prostate cancer [51].

In a review [52], anti-cancer mechanisms of SFN were discussed and can be classified into the followings:

1. SFN inhibits phase I enzymes which are usually involved in oxidation, reduction, or hydrolysis and are involved in converting procarcinogens to carcinogens.

   This inhibition is thought to be through direct interactions with cytochrome P450 enzymes (CYP).

2. SFN induce phase II enzymes: SFN is able to react with Keap1 (Kelch-like ECH-associated protein 1) and promotes Nrf2 (nuclear factor E2-factor related factor) dissociation from Keap1.

   Nrf2 binds to ARE (antioxidant response element) sites and activates ARE-driven gene expression which includes NAD(P)H:Quinone reductase (NQO1), hemeoxygenase 1 (HO1), and gamma-glutamylcysteinesynthetase (γ-GCS), a rate-limiting enzyme in glutathione (GSH) synthesis. This will induce the production of detoxifying phase II enzymes like glutathione peroxidase, glutathione reductase, ferritin, and haptaglobin.

3. SFN caused cell cycle arrests in prostate and colon cancers:

   - G2/M cell cycle arrest (in prostate and colon cancers) with decreasing levels of cyclin B, Cdc25 (cell division cycle 25 which activates Cdk), and Cdk (cyclin-dependent kinase), and inducing Chk (which inactivates Cdc25 by

14

phosphorylation). Active Cdk/Cycline complex are required for cell cycle progression in G2 phase to M phase.

- G_1 cell cycle arrest occurred concomitantly with an increase in p21 (cell cycle inhibitor protein), and a decrease in cyclin and c-myc. P21 induction by SFN is independent of p53, the latter could also be induces by SFN.

4. SFN induced apoptosis in colon and prostate cancer cells through the death receptor and mitochondrial pathways by increased PARP [poly (ADP-ribose) polymerase] cleavage, increased release of histone associated DNA fragments, activation of caspase 3, 7, 8, and 9, change in Bax and Bak to Bcl-X_L protein ratios in favor of the pro-apoptotic factors Bax and Bak, and the release of cytochrome C from mitochondria.

5. SFN is a HDAC inhibitor: SFN increases global histone acetylation, acetylated histones interactions with the P21 and Bax promoter, induction of p21 and Bax mRNA and protein levels.

6. SFN shows effect on Mitogen-activated protein kinases (MAPKs) [extracellular signal-regulated kinases (ERK), c-Jun NH_2-termial kinases (JNK), and p38]:

In HT-29 colon cancer cells activation of AP-1 luciferase activity occurred at low concentrations of SFN treatment while it was inhibited at high concentration.

AP-1 activity and cyclin D1 protein levels increased at lower concentrations (≤ 35 µM) and decreased at higher concentrations (≥ 50 µM), but cell viability decreased in a direct dose dependent manner. Activation of p54-JNK isoform may be responsible for activation of AP-1.

SFN was also able to decrease cell viability and activate the MAPK pathways ERK, JNK, and p38; and activated JNK was able to decrease cyclin D1 levels at high concentrations of SFN.

SFN treatment in human colon adenocarcinoma cells Caco-2 induced ERK activation but not JNK or p38.

SFN can also inhibit p38 activity which phosphorylates Nrf2 and promotes its association with Keap1 thereby suppressing Nrf2 translocation to the nucleus, thus inhibiting the activation of phase II enzymes.

7. SFN can affect proliferation signals and apoptotic signals via modulation of NF-κB (nuclear factor kappa-B) activity: constitutive activation of NF-κB is common in various human malignancies and leads to up-regulation of the inhibitor of apoptosis (IAP) family genes encoding adhesion molecules, inflammatory cytokines, growth factors, and anti-apoptotic genes. Down-regulation of NF-κB activity by SFN coincided with expression of downstream targets, VEGF, cyclin D1, and Bcl-X$_L$, and this was attributed to the inhibition of IKK phosphorylation, thereby attenuating IκBα phosphorylation and degradation.

8. The production of reactive oxygen species (ROS) is thought to be a key mechanism by which SFN induces apoptosis and it is accompanied by disruption of mitochondrial membrane potential and cytosolic release of cytochrome C. Conjugation of SFN with GSH which is a necessary step in SFN metabolism depletes the intracellular concentration of GSH and potentially lowers the oxidative stress threshold of the cell.

1.2.6. Predicting the effects of SFN and 5-Aza-2' Deoxycitidine on MHC expression in cancer cells:

According to my knowledge, there is no reported study on the effects of isothiocyanates, including SFN, on MHC expression in cancer cells. However, from the already known effects of SFN on factors that are known to play roles on MHC expression in cancer cells we can try to envision what might be the mechanisms and the kind of effects of SFN on MHC in these cells.

In treated cancer cells showing down-regulation of MHC expression due to DNA methylation or histone deacetylation, SFN could act by up-regulating MHC expression in these cells through its ability to inhibit epigenetic changes that affects MHC gene expression. In addition, SFN might affect MHC expression via its effects on four other important factors with effect on MHC expression. The factors are:

1- Reactive oxygen species (ROS): as we mentioned above, SFN induce ROS production, and ROS has been found to up-regulate MHC class I in Osteocarcinoma cell line [53] and MHC class-II in dendritic cells (DC) [54-57]. Thus, it is possible for SFN to up-regulate both classes of MHC via this mechanism.

2- Nuclear Factor kappa B (NF-kB): SFN inhibits NF-kB which is an enhancer for MHC class I expression as shown in studies on neuroblastoma and pluripotent stem cells [58, 59]. In this way, SFN is expected to down-regulate MHC class I.

3- Mitogen-activated protein kinases (MAPKs): SFN activates MAPKs and the inhibition of MAPKs in esophageal and gastric cancers was found to up-regulate MHC class I expression [60]. In this way, SFN is expected to down-regulate MHC class I expression.

For the 5-Aza-2' Deoxycitidine effects on MHC expression in cancer cells, besides its DNA methylation inhibition, it could also affect MHC classes I and II in cancer cells via mechanisms similar to SFN effects such as ROS production in leukemia cells [61,62], as well as through an opposite mechanism of up-regulating NF-kB activity in leukemia cells [63].

1.2.7. Effects of SFN and 5-Aza-2' Deoxycitidine on Jurkat cells:

Sulforaphane could cause cell growth inhibition, cell-cycle arrest and apoptosis in leukemia cells in time and dose dependent manner. Maximum effects could be observed after 48h of 30 µM treatment, with cell number reducing from ~6 to ~1 million and up to 38% apoptotic cells [64]. Treatment with 0.1µM 5-Aza-2' Deoxycitidine for 72h was found able to inhibit cell proliferation and growth by 20%, as well as to induce apoptosis in a dose dependent manner in Jurkat cells [65].

Regarding the effects on MHC expression, my literature search has failed to find any study on SFN effect on MHC expression in any kind of cells. However, for Jurkat cells, there are two studies on the effects of 5-Aza-2' Deoxycitidine on MHC class II expression. One study showed that treatment with 1 µM 5-Aza-2' Deoxycitidine for 72h could induce the expression of CIITA-PIII and HLA-DRA mRNAs [42] in Jurkat cells. The

other study reported that combined treatment with 100U of IFN-γ and 1μM 5-Aza-2' Deoxycitidine for 72h could induce CIITA and HLA-DR mRNAs expression, whereas 5-Aza-2' Deoxycitidine or IFN-γ alone did not yield the same effect [66].

1.3.   Aim of my study:

This study investigates the effects of Sulforaphane (SFN) and 5-Aza-2' Deoxycitidine (Aza) on both MHC classes I and II surface expression in human T leukemia Jurkat cells by flow cytometry analysis. The effects of SFN and Aza on cell growth and viability are also studied microscopically by trypan blue exclusion assay.

To my knowledge this will be the first study of SFN effect on MHC expression. It is also the first study on the effects of 5-Aza-2' Deoxycitidine on MHC class I expression in Jurkat cells as well as on cell surface expression of the MHC class II molecules in these cells.

2. Materials and Methods:

2.1. Materials:

A) Cell Culture [67] :

1- Jurkat cell line: Clone E6-1 (ATCC® TIB-152™), purchased from American Type Cell Culture (ATCC), is a human acute T cell leukemia cell line. The cell line was established from the peripheral blood of a 14 year old boy by Schneider et al., and was originally designated JM. I obtained the cell stock from USM liquid nitrogen tank.

The sample was dated 10/03/2006.

2- Cell Culture Medium: RPMI (Roswell Park Memorial Institute) medium 1640, (Mediatech, Inc. Manassas VA 20109) lot#15040293. I enriched it with 10% Fetal Bovine Serum (FBS), 2mM L-glutamine (Mediatech), and 1%pencilline/streptomycin (100X, Mediatech).

The medium is used as culture medium and as a washing buffer for cell preparation for assays. The media used for culturing were made and handled under sterile condition (bottles were never opened outside the protective cabinet), but media used for washing were unsterile and only L-glutamate were added (1%). It was kept in 4°C refrigerator.

B) Chemicals:

1- D, L-Sulforaphane [68]: Sigma Aldrich product# S4441. 5 mg as yellow liquid, and stored at -20°C. Molecular Formula: $C_6H_{11}NOS_2$, Molecular Weight: 177.29. Synonym: 1-isothiocyanato-4-(methylsulfinyl)-butane. It is not soluble in water but dissolved in DMSO (40 mg/mL).

Aliquots of SFN in DMSO (Sigma Aldrich product#15438) were prepared as stocks for storage at -20°C. Based on its molecular weight and the total 5 mg amount weight of the purchased SFN, the final molar quantity was estimated to be ~0.043 mill moles. This small liquid sample was diluted in 4.3 ml DMSO to have a sample of 0.01M or 10mM. When 10µl of such a solution is added to 10 ml of medium, the final concentration will be 10 µM, 20 µl for 20 µM, and 30 µl for 30 µM. The 4.3 ml sample was divided to 10 aliquots each contain ~ 430 µl and kept in -20°C refrigerator. No aliquot was refrozen after use.

2- **5-Aza-2'Deoxycytidine [68]:** Sigma Aldrich product#A3656, 5 mg.

Synonym: 2'Deoxy-5-azacytidine, 4-Amino-1-(2-deoxy-β-D-ribofuranosyl)-1, 3, 5-triazin-2(1H)-one, and Decitabine. Molecular Formula $C_8H_{12}N_4O_4$. Molecular Weight = 228.21. Soluble in 50 mg/ml in 50% acetic acid, 50 mg/ml in DMSO, or 0.25 mg/ml in water.

It is toxic, mutagenic, and carcinogenic and should always be handled with no skin exposed, with facial masks, and with eyes protection.

The sample purchased was 5 mg, and according to the molecular weight (228.21) and the final concentrations desired (1, 2, and 3 µM), the sample was dissolved in 2.2 ml of DMSO to prepare 10 mM stock aliquots (each 200 µl).

The preparation was sterilized before use by passing through Drummond self-locking 0.8 µM double layer filters. A 1:10 dilution of each aliquot was made in RPMI culture medium for injection through the filters before use in cell treatment experiment. The diluted aliquots are 1 mM in concentration.

From an aliquot, 10 µl added to 10 ml of medium would result in 1 µM, 20 µl for 2 µM, and 30 µl for 3 µM final concentrations. The aliquots were kept at -20°C and each aliquot would only be used once after thawing.

3- **Dimethyl sulfoxide 99.9% ACS spectrophotometric grade (DMSO) [68]:** Sigma Aldrich product#D2560, 500 ml. Molecular Weight 78.13 and Molecular formula: C_2H_6SO. Kept at room temperature. It was used for preparing aliquots of both SFN and 5-aza-2'Deoxycytidine and was added with similar volumes as controls to the parallel treated cells with SFN and 5-aza-2'Deoxycytidine (10, 20, or 30 µl parallel to SFN and 1, 2, or 3 in parallel to AZA) .

4- **Dulbecco's Phosphate Buffer Saline (DPBS), 1X:** (Mediatech, Inc. Manassas VA 20109) lot#21031448. Cold PBS was used for washing cells in assays and to prepare the final antibody-stained cells suspension for flow cytometry analysis. It is kept at 4°C in refrigerator.

C) **Antibodies :**

   a) Mouse monoclonal antibody against human MHC class I: W6/32. It is an IgG2a that reacts with monomorphic determinants on HLA A, B, C molecules. It was produced by fusing P3X63Ag8 myeloma cells with spleen cells from a BALB/c mouse immunized with cells from human tonsils. It was obtained as HB95 from ATCC [69].

   b) Mouse monoclonal antibody against human MHC class II: L243. It is an IgG2a that reacts with a non-polymorphic antigen of MHC class II on human cells. It was produced by fusing NS1 myeloma cells with spleen cells from a BALB/c

22

mouse immunized with human RPMI 8866 cells. It was obtained as HB55 from ATCC [70].

c) Mouse monoclonal antibody IE3 used as an isotype-matched control antibody. Monoclonal antibody IE3 is an IgG2a that reacts with human CD62-P, a platelet activation antigen. It was produced by fusing NS1-Ag4/1 myeloma cells with spleen cells from a BALB/c mouse immunized with human platelets. It was produced in Dr. Ah-kau Ng's lab [71].

Hybridoma clones HB95 (W6/32), and IE3 were cultured individually in DMEM medium (supplemented with 10%FBS /gentamycin) in 37°C incubator with 5% CO_2. Spent culture media were collected and tested for antibody activity. Active supernatants were kept frozen at -20°C before use without dilution. HB55 (L243) clone was cultured as above and injected into mineral oil (Pristane)-primed Balb/c mice to produce ascetic tumors. Ascetic fluids were collected, cleared and kept frozen at -20°C before use at a 1:50 dilution.

d) Secondary antibody: Fluorescein-5-Isothiocyanate (FITC-isomer1)-conjugated AffiniPureF (ab') 2 Frag Goat Anti-Mouse IgG, Fcγ Frag Specific [72]: It has minimal cross reaction to human, bovine, and horse serum proteins. It was purchased from Jackson ImmunoResearch Laboratories, Inc. Code# 115-096-071 . Its A_{max} = 292 nm and E_{max}= 520 nm. The optimal amount for the immune fluorescence assay was determined by Jennifer Walker in Dr. Stephen Pelsue's lab to be 1 to 1.5µg for each $1x10^6$ of target cells to be stained. The purchased product, a powder preparation of 1 mg,

was dissolved in 750 µl of dH2O (Milli-Q) in a dark room, and aliquots of 35 µl/vial were made and kept in -20°C refrigerator with a light protection cover (each had a concentration of 1.3 µg/µL of Ab). As a working solution, a 1:100 dilution of the FITC-2° Ab stock was made in complete RPMI medium, and 100 µl was added to every assay sample, resulting in 1.3 µgFITC-2° antibodies per $1x10^6$ cells.

2.2. Methods:

2.2.1. Overview of Experimental design :

Jurkat cells were subcultured on a 72 hour interval period to maintain high concentration of viable cells. Freshly harvested Jurkat cells were treated cells with SFN or 5-Aza-2' Deoxycitidine at 3 different concentrations for 3 different durations (24h, 48h, and 72h), in parallel with controls with the same concentrations of DMSO as in the SFN or 5-Aza-2' Deoxycitidine solutions used.

Then, the treated Jurkat cells were reacted with primary antibody to MHC class I or II, followed by a secondary Ab conjugated with FITC, to measure the level of surface expression of MHC classes I and II by flow cytometry. For comparison, Jurkat cells were also treated with two negative controls, one was only culture medium with no primary Ab, and the other was an irrelevant isotype-matched primary antibody.

All assays were carried out in standard flow cytometry test tubes in duplicate.

Every Jurkat cell treatment experiment was repeated three times to demonstrate test reproducibility and to exclude faulty results.

2.2.2. Preparation of Jurkat cell culture :

Frozen Jurkat stock culture in liquid nitrogen tank was thawed and grown in RPMI medium (with 10%, FBS, 2 mM L-glutamine, and 1 % penicillin/strep antibiotics) at 37°C in a 5% CO_2 incubator. As recommended by the American Type Cell Culture (ATCC), the Jurkat cell culture was kept between 1×10^5 and 3×10^6 viable cells/mL [69].

Because ~200×10^6 Jurkat cells were needed for every treatment experiment (explained later), two T-75 flasks of cultures at log phase of growth were set up to be ready for each experiment. At the same time, additional Jurkat cultures were seeded in new T-75 flasks, with ~50×10^6 cells in each flask, in order to repeat the experiment 48-72 h later. In this arrangement, use of Jurkat cultures of long and distant passages could be avoided.

2.2.3. Cell counting and viability determination :

Cell count was performed using the haemacytometer chamber. Cell viability was determined by trypan blue exclusion test. 20 µl of cell suspension after being mixed by pipetting were added to equal volume of 0.4% trypan blue in PBS. The mixture was mixed well by pipetting and 20 µl was taken and loaded into a haemacytometer chamber. Live (unstained) and dead cells (stained blue) in the 4 large corner squares including upper and left borders were counted to determine cell concentration using the equation: cell # per ml = (total number of cells counted/4) x 2 (dilution factor) x 10^4.

The percentage of viable cells was calculated by dividing the total viable cell number by the total cells counted. The Jurkat cell culture normally doubles in number after overnight cultivation, and its viability remains > 90 % following 72 h.

2.2.4. Treating Jurkat cells with sulforaphane and 5-aza-2'Deoxycitidine:

Treatment of Jurkat cells were conducted with 3 different concentrations of Sulforaphane (10, 20, and 30 µM) and with 3 different concentration of 5-aza-2' Deoxycitidine (1, 2, and 3 µM) for 3 different durations (24h, 48h, and 72h).

I have chosen these ranges of concentrations and durations based on previous studies reporting that these treatment conditions were found effective in inhibiting cell growth, inducing apoptosis, or causing epigenetic changes [42, 68].

Each treatment experiment was repeated 3 times to test for reproducibility. Over a period of 2 to 3 months, 36 T-24 flasks of Jurkat cell cultures were prepared: 9 for SFN treatment (10,20 or 30 µM); 9 for AZA treatment (1,2, or 3µM), and 18 with DMSO (10,20, or 30 µl as controls to SFN and 1,2, or 3 µl as controls I to AZA).

Under sterile environment in the safety cabinet, each flask were added with $5\text{-}5.5 \times 10^6$ Jurkat cells in 10 ml medium, followed by appropriate amounts of SFN, AZA or DMSO. All flasks were cultured at 37°C in 5% CO_2 incubator for 24 or 48 or 72 h.

2.2.5. Analysis of Surface Expression of MHC classes I and II molecules in Jurkat cells:

Surface expression of MHC classes I and II molecules in Jurkat cells, treated with SFN or AZA or DMSO control, were analyzed by flow cytometry after staining the cells with

monoclonal antibodies to MHC class I (W6/32)and to MHC class II (L243), respectively, together with a FITC-conjugated secondary antibody. Antibody-stained and control samples were prepared for flow cytometry analysis consecutively after24h, 48h, and 72h of treatment with each concentration of AZA, SFN or DMSO control. At each time point for each treatment, 12 flasks of Jurkat cultures were processed (triplicates of 3 concentrations for SFN or AZA, as well as untreated and DMSO controls).

The content of each flask was transferred to a 15 ml Falcon conical tube, vortexed, and 100µl from each sample was taken in a 0.5 ml microfuge tube for cell counting.

Each Falcon 15 ml tube was centrifuged at 200G for 5 minutes at 4°C. Supernatants were discarded and cell pellets were resuspended in 6 ml of ice-cooled glutamate enriched RPMI and vortexed well. Then each sample was distributed to 6 labeled 1.5 ml microfuge tubes (each receiving 0.5 ml). The 1.5 ml microfuge tubes were centrifuged, supernatants discarded, and cell pellets were resuspended in 100 µl of medium and centrifuged. Again, supernatants discarded, and cell pellets were resuspended in 100 µl medium, and 50 µl of primary antibody was added and mixed. A typical test would include a negative control tube of 50 µl RPMI medium without primary antibody, a second tube with the negative IE3 antibody, and duplicate tubes with either anti-MHC class I antibody (w6/32, hybridoma sup neat) and duplicate tubes with anti-MHC class II antibody (L243 ascites 1:50 dilution). All tubes were incubated on ice for 45 minutes.

While waiting for incubation, frozen aliquots of the FITC-conjugated secondary antibodies were thawed and a 1:100 dilution was made in enriched RPMI medium.

It was wrapped in aluminum papers and kept in ice in the dark until ready to use.

After incubation, the microfuge tubes were centrifuged, the supernatant discarded, and the pellets resuspended in 100μl of enriched RPMI to wash the cells.

The microfuge tubes were centrifuged and the cells were washed repeatedly one more time. After the final wash, each cell pellet was resuspended in 100μl of enriched RPMI medium and added with100 μl of FITC-secondary antibody conjugate.

After incubation in ice for 45 min. in dark, the tubes were centrifuged, the supernatants discarded, and each cell pellet was resuspended in 100 μl of enriched RPMI to wash the cells. After washing twice and centrifugation, each cell pellet was resuspended in 100 μl of ice-cooled PBS to wash the cell. After washing twice with PBS, each cell pellet was resuspended in 500 μl of ice-cooled PBS. Thus, the crucial cell washing steps include : 2 times initially with RPMI for cell preparation, 3 times after the primary antibody incubation with RPMI, twice after the second antibody incubation with RPMI, and then finally twice with PBS.

The samples were transferred into 12x75mm plastic tubes to be analyzed for the results of fluorescent antibody staining using the flow cytometer system (Model BD FACSCALIBUR) at USM Core Lab, by the application of the CellQuest on Mac-OS X operating system. If the cell samples were not analyzed right after preparation they would be fixed in 10% paraformaldehyde in PBS for storage up to a week before analysis.

3. Results:

3.1. Effects of SFN and AZA on the growth of Jurkat cells:

Both SFN and AZA inhibited the growth of Jurkat cells, in a dose dependent manner and in a time dependent manner but consistent results were observed only for AZA.

Both treatments have shown an inhibition on cell growth. This was confirmed by cell counting and comparing with the initial 5×10^6 cell seeding. Using MS-excel 2007 software, I calculated the percentages of growth for each treatment and DMSO control by subtracting the initial 5×10^6 cell seeding from the sample count and dividing it by 5×10^6 and multiplying the result by 100% (see Tables 2 and 5).

I calculated P-values for the differences between treated samples and their corresponding DMSO control for % of growth using chi squares and by the aid of an online calculator [73]. All P-values were less than 0.0001, which means that, as compared to the DMSO controls, SFN and AZA inhibition of cells growth was statistically significant. Then by calculating P-values for difference in doses of each SFN and AZA for each treatment period for effects on % of growth, all P-values were less than 0.05, which means that the effects were statistically significant and dose dependent. P- values for difference in duration of treatment for a specific dose were found to be all less than 0.05 except for SFN at 10 and 30 µM for difference between 72 and 48 h treatments in % of growth. The results were consistent in that overall both SFN and Aza showed inhibition of the growth of Jurkat cells. However, for SFN, the data were not consistent for dose dependent effects between 20 and 30 µM, and for treatment duration dependency between 48 and 72 h, as it is obvious from Figure 1. The degree of growth

inhibition was significant and it was ~50% for 24 h and ~ 150% for 48 and 72 h as compared with the DMSO control.

Figure 1. The effect of SFN on % Growth of Jurkat Cell
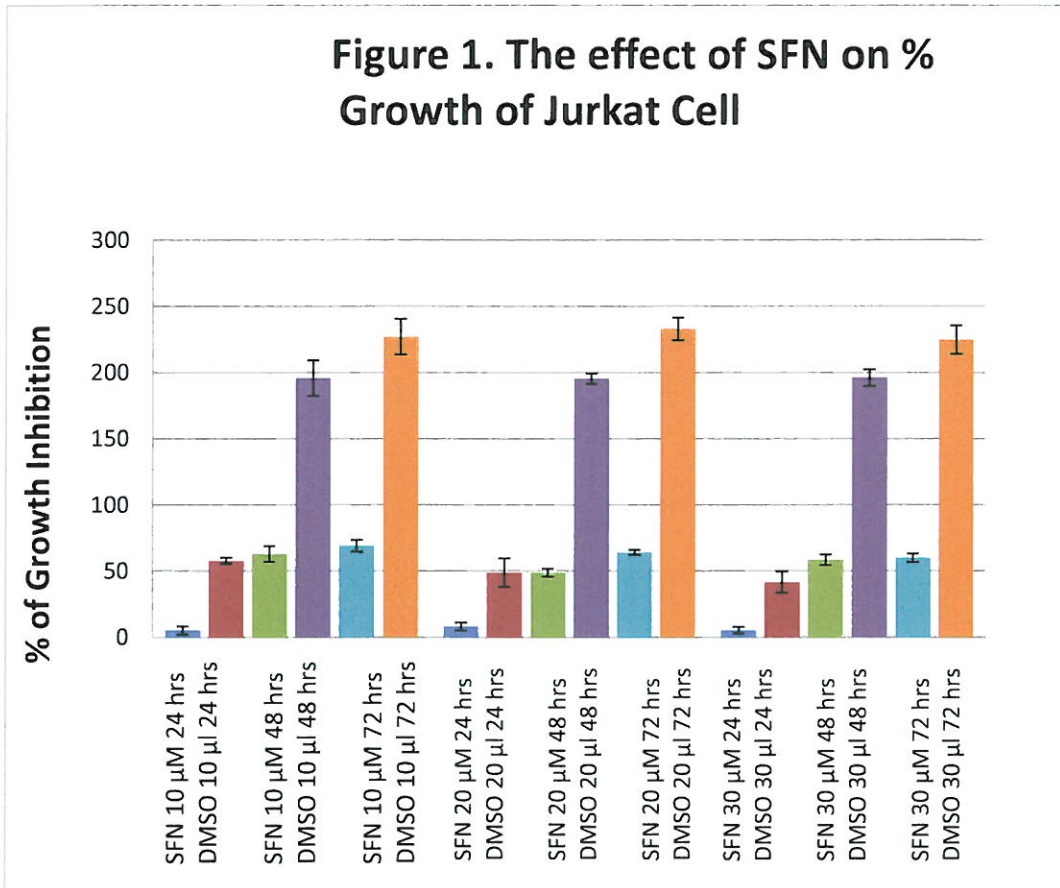
For Aza, from Figure 2, it seems there was no inhibition of growth at 1 μM after 72 h treatment; the degree of inhibition was not consistent with dose and duration of treatment. However, the inhibitory effect was significant with treatments other than 1 μM plus 72 h, and ranged from ~ 25% for 1 μM plus 24 h and ~ 150% for 3 μM plus 48 h.

SFN and AZA are both mostly effective in inhibiting growth after 48 h; 3 μM for Aza and 30 μM SFN are the most effective doses.

Figure 2. Effect of AZA on % Growth of Jurkat Cell
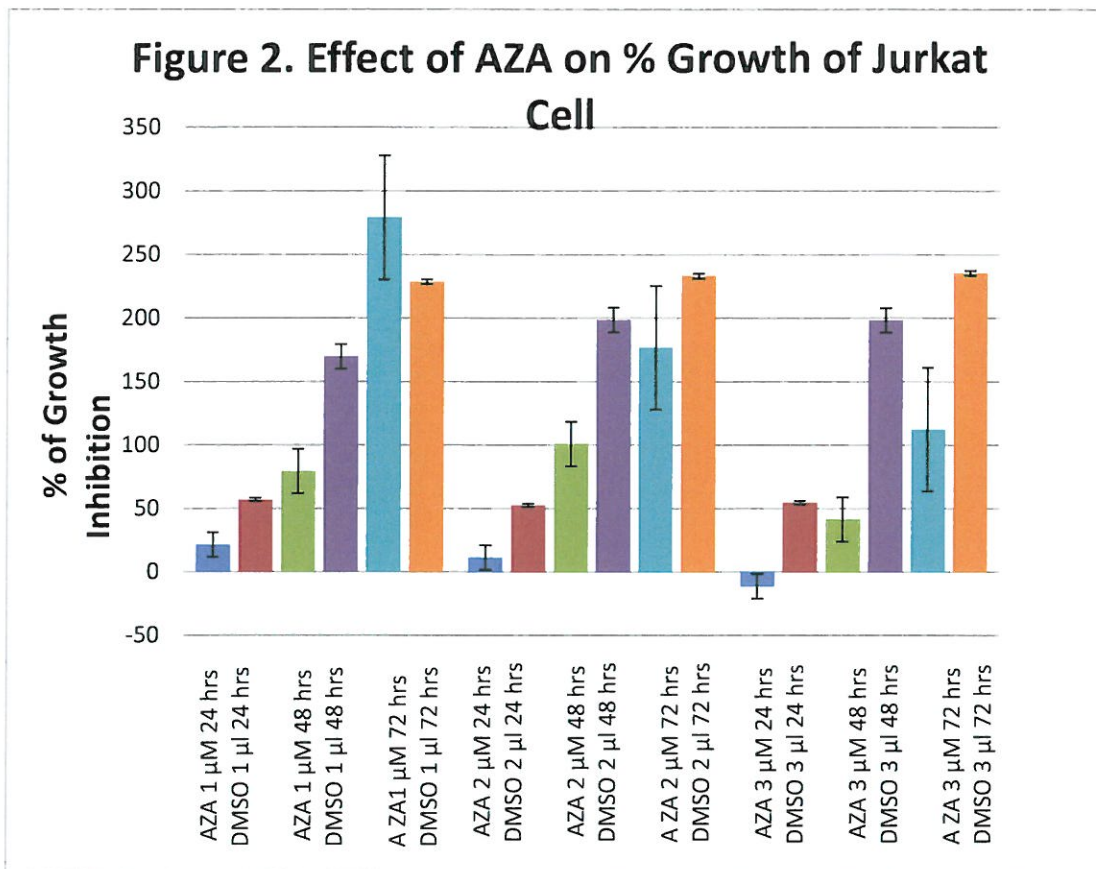
3.2. Effects of SFN and AZA on the viability of Jurkat Cells:

Using MS-excel 2007 software, I calculated the percentages of dead cells for each treatment and DMSO control by dividing the number of dead cells by the total count cells and multiply the result by 100%.

I calculated the P-values to compare the % of dead cells between treated and untreated cells, all P-values were below 0.05 except three treatments; 24 h plus both Aza 2 μM (P= 0.1948) and SFN 20 μM (P= 0.4974), as well as 48 h plus Aza 1 μM (P= 0.0557). To see if there are dose and time dependent statistically significant

differences between treatments, I also calculated P-values to compare different treatments (by dose or time) for their effects on % of dead cells.

Dose dependent effects were statistically significant for 24 h plus Aza 2-3 μM treatment, for 48 h plus Aza 2-3 μM treatment or plus SFN 20-30 μM treatment, as well as all treatments for 72 h. The statistically significant time dependent effects were found in all treatments except the treatment with Aza 1 μM for 48-72 h (P = 0.8383).

As shown in Figure 3, effects of Aza are not consistent on cell survival, and the results are not significant (all less than ~25% decrease from the DMSO controls).

But for SFN (Figure 4), the cytotoxic effect were consistently significant, ranging from ~10% for SFN at 10 and 20 μM after 24 h to 65% for SFN at 30 μM after 72 h). The effect is time dependent but not consistent with dose for all doses after 24 h, and for 10 and 20 μM after 48 h.

For both inhibitory effect on growth and increasing dead cell percentage the highest doses (3 μM of Aza and 30 μM of SFN) and the longest duration (72 h) were the most effective, showing significance statistically, and with consistency.

Figure 3. Effects of AZA on % Viability of Jurkat Cells
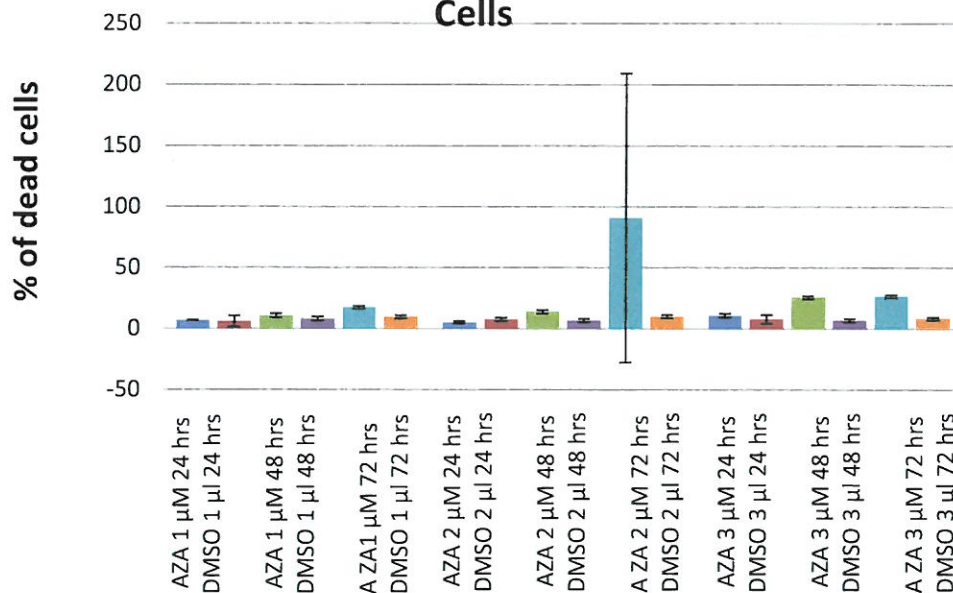
Figure 4. Effects of SFN on % Survival of Jurkat cells
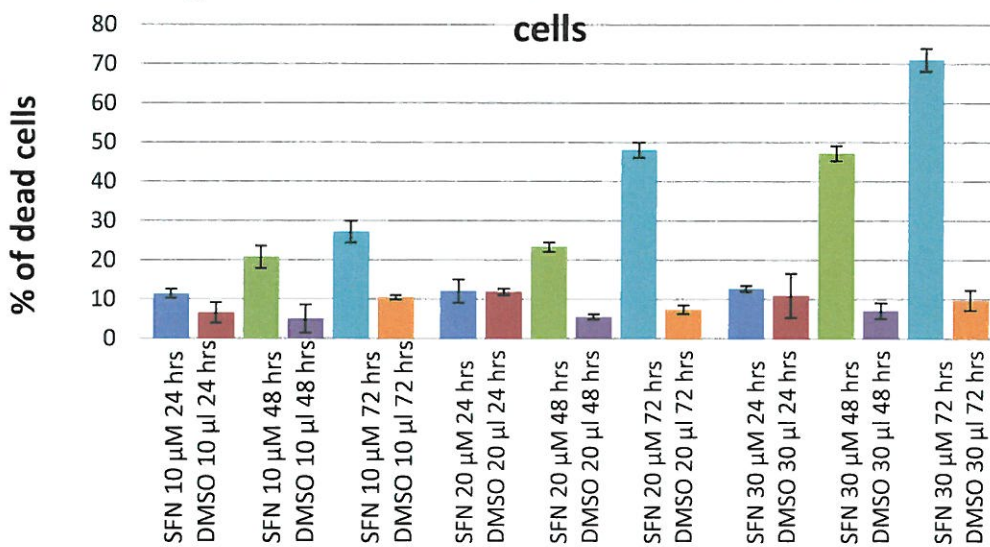
With few exceptions the results show that both SFN and Aza in general appear to have more significant and consistent inhibitory effects on the growth of Jurkat cells, in comparison to their effects on the viability of these cells.

3.3.   Effect of SFN and AZA on MHC Expression by Jurkat Cells:

I used WinMDI v.2.9 software to analyze data from flow cytometry. For every reading I used the geometric means calculated by the software (see Tables 8 and 9). Every drug treatment experiment with a related DMSO control had 5 readings from antibody stained Jurkat cells (1 negative antibody control, 2 anti MHC class I, and 2 anti MHC class II).

I first generated corrected values by subtracting the geometric means of the negative antibody control from each of the test antibody values for each treatment and its DMSO control. Then I took the averages of the duplicates of anti-MHC class I and anti-MHC class II. Then I calculated P values from chi squares by online calculator [73] comparing the MHC expression between drug treatments and their DMSO controls.

3.3.1. Effect of SFN and AZA on MHC class I expression by Jurkat cells:

All P- values for effects of SFN and AZA on MHC class I expression were less than 0.0001 suggesting that all treatments induced statistically significant changes on MHC class I expression. Then I tested differences in treatment time and dose response for statistically significance by P-values determination. All treatments show statistically significant time and dose dependent effects except 1 and 2 µM of Aza for 72 h (P = 0.467), 48/72 h for 10 µM SFN (P= 0.23), and 24/48 h for both 30 (P=0.2837) and 20 (P= 0.0667) µM SFN.

As shown in Figure 5, depicting the effects of SFN on MHC class I, all treatments (except 10 µM for 24 h) reduced the expression of MHC class I, with consistent dose and time

34

dependency. The degree of effects ranged from ~25 geometric mean units for 10 µM/24 h treatment to 100 geometric mean units for 30 µM/72 h treatment.

Figure 5. Effect of SFN on MHC class-I Expression by Jurkat Cells
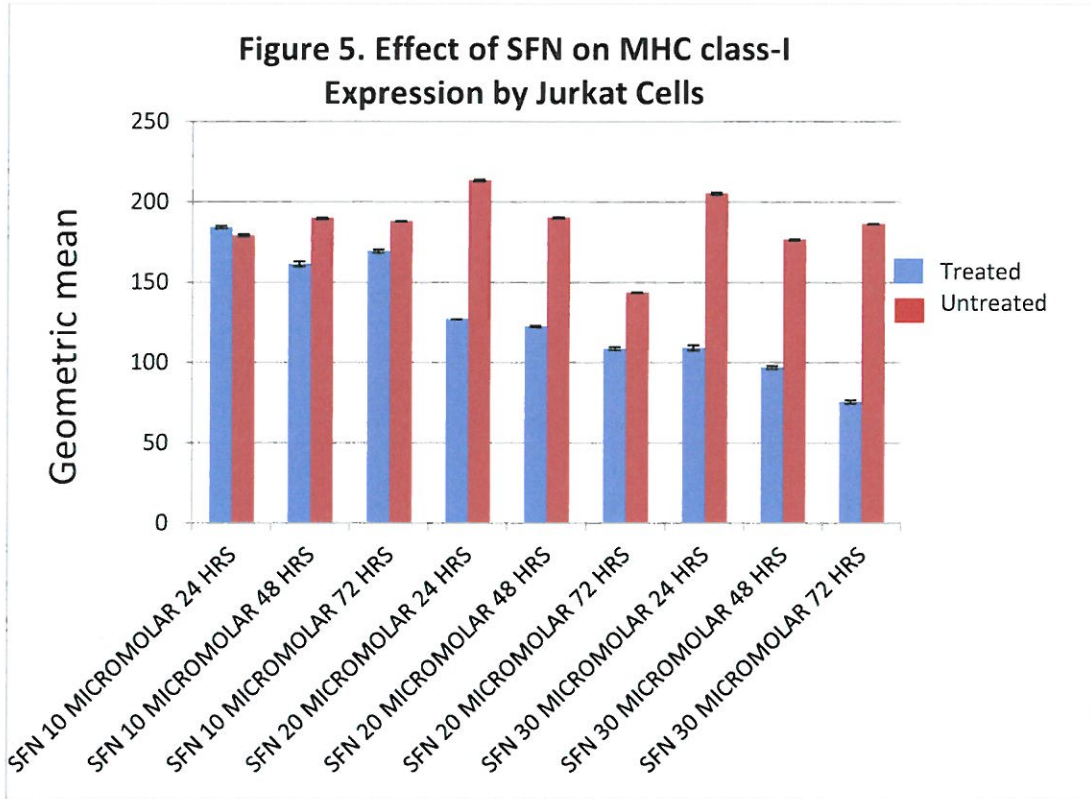
Figure 6 demonstrates the effect of Aza on MHC class I expression by Jurkat cells. Aza in all treatment combinations could up-regulate MHC class I expression, showing consistent results with dose dependent effect but not time dependency. The expression values range from 50 geometric mean units for 1 µM treatment to 100 geometric mean units for 3 µM treatments.

Figure 6. Effect of AZA on MHC class-I Expression by Jurkat Cells
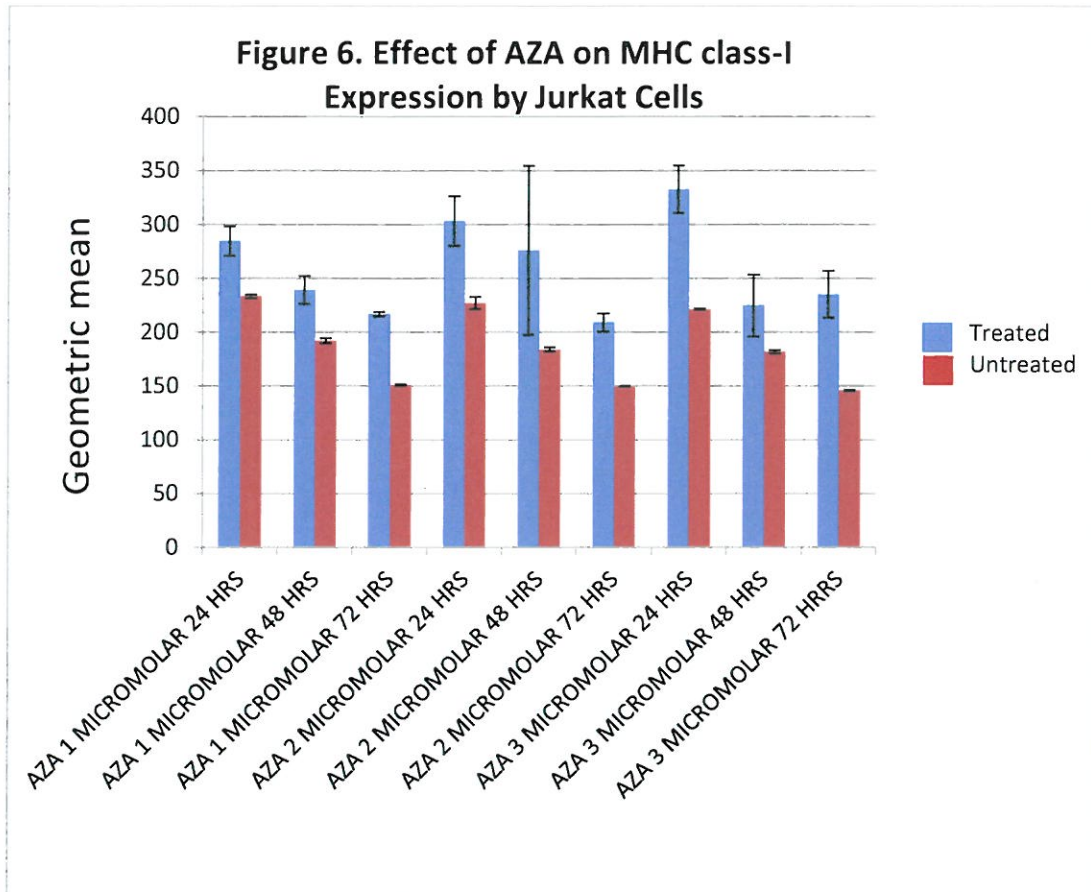
3.3.2. Effect of SFN and AZA on MHC class- II expression by Jurkat Cells:

In contrast to their effect on MHC class I expression, Aza and SFN show neither consistent nor significant effects on MHC class II expression by Jurkat cells. Only few treatments, in comparison with DMSO control, yielded P-values less than 0.05, such as 2 μM Aza for 72 h treatment (P= 0.0188), 72 h treatment with all the 3 doses of SFN (P= 0.0204 for 10 μM, 0.0014 for 20 μM, and less than 0,0001 for 30 μM), and 30 μM SFN for 48 h treatment (P=0.0002).I calculated P-values for results from experiment using 3 concentrations of SFN with 72 h treatments, and all were higher than 0.05 (P= 0.6872 for 10 and20 μM ; and P= 0.6137 for 20 and30 μM). Similarly,

36

for the treatment time between 48 and 72 h with 30 μM SFN, the P-value was more than 0.05 (P=0.8746).

As shown in Figure 7, demonstrating effects of SFN on surface MHC class II expression by Jurkat cells, the SD values are too large and the data are not consistent except for 30 μM SFN treatment for 48 and 72 h (an increase in geometric mean units that ranges from ~0.1 to ~2.9 for 48 h treatment; and ~0.4 to ~2.9 geometric mean units for 72 h treatment). However, these effects are insignificant.

Figure 7. Effect of SFN on MHC class-II Expression by Jurkat Cells
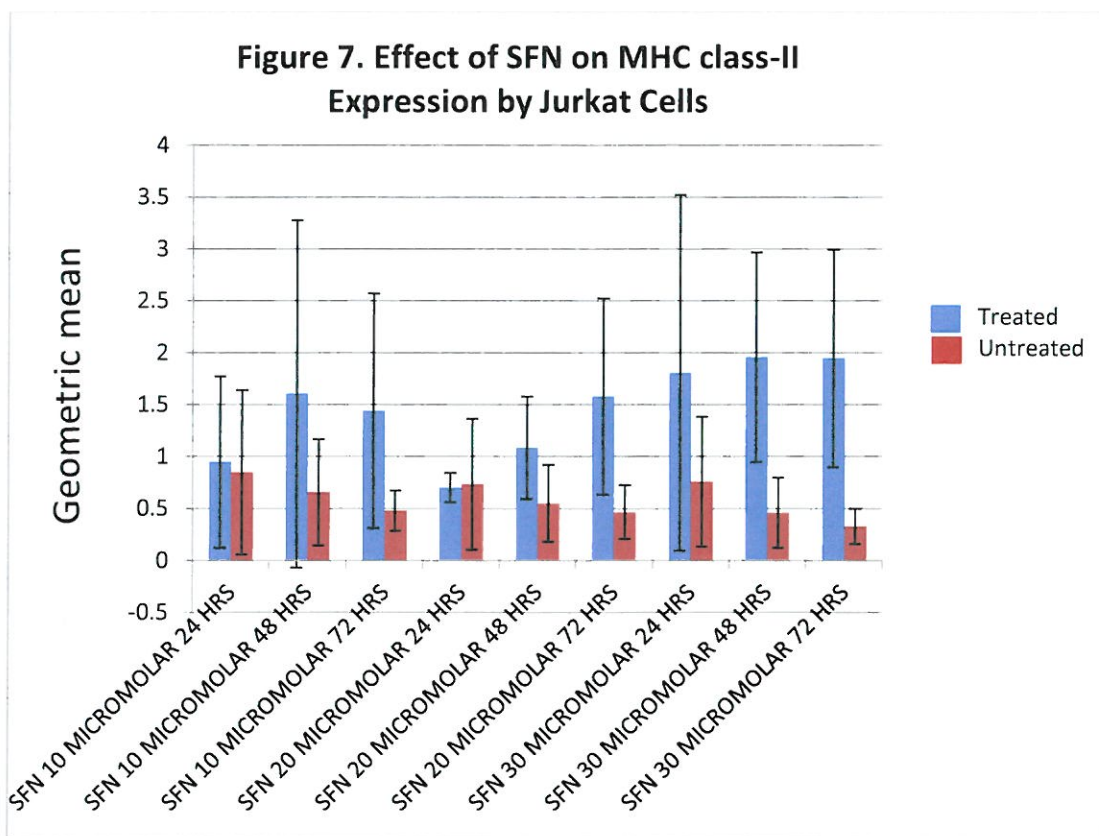
As demonstrated in Figure 8 (showing effects of Aza on surface MHC class II expression), the data are consistent for all 72 h treatment with Aza. There is a slight increase in MHC class II expression by ~0.5 geometric mean unit for 1 μM Aza treatment, 1.2geometric

mean unit for 2 μM Aza treatment, as well as 0.7 geometric mean units for 3 μM Aza treatments. Similarly, 48 h treatment with 2 and 3 μM Aza show an increase in MHC class II expression by ~0.4 geometric mean units for both treatments. Again these effects are insignificant.

Figure 8. Effect of AZA on MHC class-II Expression by Jurkat Cells
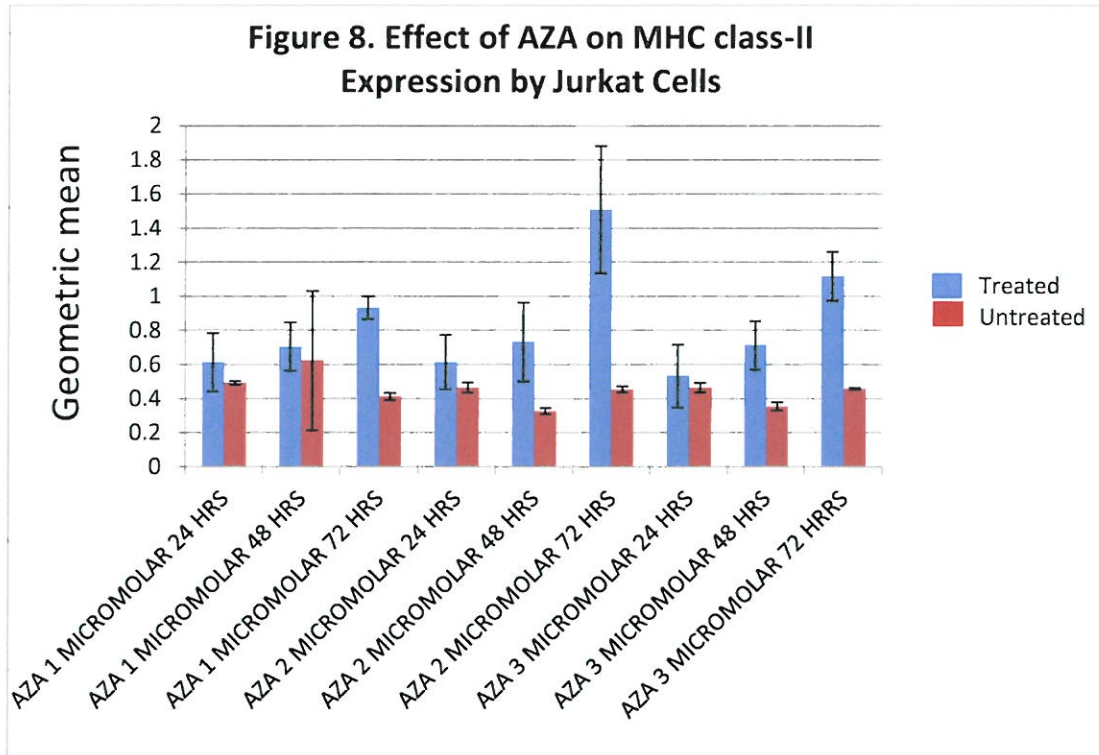
4. Discussion:

As previous studies have shown, both Aza and SFN treatment of cell cultures could increase dead cells in the samples and inhibited growth of cells. The explanation for such effects can be the same as suggested in previous studies with similar observation, i.e. DNA methylation inhibition of relevant genes and the production of cell-damaging ROS.

For growth inhibition both SFN and Aza have approximately equal effects, but for the effects on cell viability expressed as dead cells percentages, SFN (maximum change ~60%) was more toxic than Aza (maximum change ~20%). This is surprising because SFN inhibits NF-kB while Aza activates its function, and NF-kB can induce apoptosis [74].

It is possible that SFN produces more cell-damaging ROS than Aza. In addition, it might involve other cellular mechanism induced by SFN but not by Aza, such as inhibition of phase I enzymes, induction of phase II enzymes, or activation of MAPK pathways.

For the effect on MHC class I expression, this preliminary study shows that SFN down-regulates MHC class I expression by Jurkat cells while Aza up-regulates it. SFN`s ability to down-regulate MHC class I expression could be due to some of its known cellular activities such as MAPK pathway activation and NF-kB inhibition. Aza could up-regulate MHC class I expression and there could be various possible explanations. In addition to up-regulation of NF-kB, it might be able to up-regulate MHC class I transactivator genes such as CIITA, as shown in a previous study [42].

In previous studies, both SFN and Aza were found to induce ROS production which in turn could up-regulate MHC expression [53-57]. It is conceivable that ROS production could also be induced by Aza treatment in Jurkat cells, contributing to the up-regulation of MHC class I. However, this suggested ROS-mediated effect on MHC class I expression is not observed in Jurkat cells following SFN treatment.

Both SFN and Aza could not up-regulate MHC class II. The only study on the effect of DNA methylation inhibitor e.g. 5-aza-2'Deoxycytidine, on MHC class II expression [42] has only shown up-regulation of HLA-DR and CIITA expression at mRNA levels, with no further information on the post transcriptional effect.

It is possible that even if DNA methylation inhibition occurs, leading to up-regulation of CIITA and MHC class-II mRNAs, there might be other posttranscriptional defects that affect the MHC class II protein expression. Alternatively, there might be defects in the posttranslational pathway towards the expression and the presentation of the MHC class II molecules on the cell surface. Also, in the case of SFN, the downstream effect of DNA methylation inhibition might not occur at the MHC and CIITA genes.

There is a possibility that the CIITA was up-regulated in Aza-treated Jurkat cells but the defects were in transcription of MHC class II gene or subsequent protein translation. This assumption is made in consideration of the observation that Aza could up-regulate MHC class I, possibly through up-regulating CIITA.

To my knowledge my study is the first on the effects of an isothiocyanate, SFN, on MHC expression. It is also the first on investigating Aza effect on MHC class I expression and the surface expression of MHC class II in human T leukemia-derived Jurkat cells.

The immune response against cancer involves presentation of processed cancer antigens, loaded on MHC molecules, to T lymphocytes, and it thus depends on the surface expression of MHC classes I and II molecules by the antigen presenting cell including the cancer cell. Cancer cells can escape immune surveillance by different mechanisms including down-regulating expression of surface molecules that are needed to elicit immune response, such as the MHC molecules. The genetic mechanisms by which cancer cells down-regulate MHC expression are either directly genome-based or epigenetic. Because epigenetic mechanisms are reversible, they were widely studied to find ways for manipulating epigenetic changes. One mechanism of epigenetic mechanisms is DNA methylaion mediated by enzymes called DNA methyltransferase (DNMT) [26].

DNMT inhibitors have been used to treat cancer cells to study their effects on MHC expression. In general, there are two types of DNMT inhibitors, nucleoside analogues and non-nucleoside analogues. 5-Aza-2'Deoxycytidine is the most widely used nucleoside analogue DNMT inhibitor, and have been shown to up-regulate expression of MHC class I or II or both in different cancer cells.

Isothiocyanates (ITCs) including SFN, are known anti-cancer chemicals and DNMT inhibitors, and have anticancer activities by inducing apoptosis and/or inhibiting growth

41

in different cancer cells. Consumption of ITGS' natural sources (e.g. cruciferous vegetables) has been shown in epidemiological studies to reduce risk of different cancer types. In this study with Jurkat cells, SFN was found able to down-regulate MHC class I expression, and this effect might facilitate cancer cell's escape from immune recognition.

To gain better insight, more study with Jurkat cells should be conducted to investigate the effect of SFN on its known molecular targets such as ROS production, NF-kB inhibition, and MAPK pathways activation. Further investigations might include exploring the effects of SFN and other isothiocyanates on MHC expression in different cancer cell types, in comparison to normal cells. Also, more studies may be performed to test the effect of MHC class I expression down-regulation on the susceptibility of SFN-treated cancer cells to CTLs' recognition and killing.

Aza (Decitabine 5-aza-2'Deoxycytidine), a current treatment for myelodysplasia syndrome (MDS) [30], could up-regulate MHC class I in Jurkat cells, and possible explanations could include up-regulation of CIITA-like molecules, NF-kB activity, or the induction of ROS production. These regulation mechanisms warrant further research in view of their potential effect on the immune control over cancer in patients receiving Aza treatment. While up-regulation of MHC I in cancer cells might enhance CTL action, it would inhibit attack by natural killer (NK) cell. For MHC class II, both SFN and Aza could not up-regulate its surface expression in Jurkat cells. It is highly feasible there are additional molecular mechanisms involved other than DNA methylation of CIITA and MHC class II genes, at translational or transcriptional level. Also, the possibility that

42

experimental conditions in this study are not optimal could not be ruled out. I would suggest furthering this study with extended treatment time or increased SFN and Aza doses with the consideration of their ability in cell growth inhibition and apoptosis induction, which will make experimental design and results interpretation more relevant. RT-PCR assays should be conducted to investigate if MHC II genes are activated and transcribed in Aza-treated Jurkat cells. In addition, other DNA methylation inhibitors could be tested for their effects on MHC II expression in Jurkat cells. The additive effect of another molecule such as interferon could also be pursued. This preliminary study with encouraging results have paved the way for future research in our lab to explore the role of epigenetics in immune response against cancer with a focus on MHC expression and its interaction with immune cells. It would be of great interest to explore the effect of SFN and Aza on expression of MHC class I related antigens A and B (MICA/B) ligands for NKG2D activating receptor of NK cell- in cancer cells, beginning with Jurkat cells which are relatively NK resistant. According to my knowledge, there is no study about the effect of SFN or any other isothiocyanate on the expression of MIC/B expression by any kind of cancer cells, except few studies which demonstrate that Aza can up-regulate MICA expression in some cancer cell lines other than Jurkat cells [75]. However, MICA expression by Jurkat cells could be induced after treatment with HDACi (histone deacetylase inhibitors) [76].

In a previous study, NF-kB inhibitor sulfasalazine (Sz) demonstrated a dose-dependent inhibition of MICA expression by Hela cells [77]. MAPK inhibitors could also inhibit MICA expression by Hela cells, as found in another study [78]. As long as SFN activates MAPK

and inhibits both NF-kB and HDAC, it is difficult to expect the effect of SFN on MICA expression by cancer cells. But for Aza, it may be able to up-regulate MICA expression by cancer cells through induction of NF-kB. Only experiments could confirm or disprove these predictions.

Summary and conclusions:

- Both 5-Aza-2' Deoxycitidine (Aza) and Sulforaphane (SFN) could inhibit growth and reduce viability of Jurkat cells, in agreement with previous studies by others on Jurkat cells and other cultured cancer cells.

- Both Aza and SFN were not found to induce cell surface expression of MHC class II in Jukat cells, under the experimental conditions used.

- SFN could down-regulate the cell surface expression of MHC class I in Jurkat cells. This could be due to its reported effects on ROS production, MAPK pathway activation, and NF-kB inhibition.

- Aza could up-regulate the cell surface expression of MHC class I in Jurkat cells. This might result from its known enhancing effect on CIITA expression and NF-kB activity.

REFERENCES

1. Abul K. Abbas et al. (2012). *Cellular and Molecular Immunology*, **7th edition. ISBN: 1-4377-1528-1; 1-4377-3573-8.**

2. The Nobel Prize in Physiology or Medicine 1980". *Nobelprize.org.* Nobel Media AB 2014. Web. 2 Oct 2014.

   <http://www.nobelprize.org/nobel_prizes/medicine/laureates/1980/>

3. Kobayashi (2012). NLRC5/CITA: A Novel Regulator of Class I Major Histocompatibility Complex Genes. *J ImmunodeficDisor* **1:1000e102.**

4. Nabila Jabrane-Ferrat, Nada Nekrep, Giovanna Tosi, et al. (2002). Major Histocompatibility Complex Class II Transcriptional Platform: Assembly of Nuclear Factor Y and Regulatory Factor X (RFX) on DNA Requires RFX5 Dimers. *Mol Cell Biol.* **22: 5616–5625.**

5. Peter J. van den Elsen, Koichi S. Kobayashi, Amy Li, et al. (2012). MHC Class I Gene Expression Transcription Factor Complex To Induce NLRC5 Cooperates with the RFX. *J Immunol 188:4951*-**4958.**

6. Michael Campoli and SoldanoFerrone (2008). HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene* **27: 5869-5885.**

7. Jose Maria Romero, Pilar Jimenez, Teresa Cabrera, et al (2005). Coordinated down regulation of the antigen presentation machinery and HLA class I/β2-microglobulin complex is responsible for HLA-ABC loss in bladder cancer. *Int. J. Cancer* **113:605– 610.**

8. Korkolopoulou P, Kaklamanis L, Pezzella F, et al (1996). Loss of antigen-presenting molecules (MHC class I and TAP-1) in lung cancer. *Br J Cancer* **73:148-153.**

9. Sanda MG, Restifo NP, Walsh JC, et al (1995). Molecular characterization of defective antigen processing in human prostate cancer. *J Natl Cancer Inst.* **87:280-285.**

10. Ritz U, Momburg F, Pilch H, et al (2001). Deficient expression of components of the MHC class I antigen processing machinery in human cervical carcinoma. *Int J Oncol.* **19:1211-1220.**

11. Demanet C, Mulder A, Deneys V, et al (2004). Down-regulation of HLA-A and HLA-Bw6, but not HLA-Bw4, allospecificities in leukemic cells: an escape mechanism from CTL and NK attack? *Blood.* **103:3122-3130.**

12. Masucci MG and Klein E (1991). Cell phenotype dependent expression of MHC class I antigens in Burkitt's lymphoma cell lines. *Semin Cancer Biol.* **2:63-71.**

13. Masucci MG, Stam NJ, Torsteinsdottir S, et al (1989). Allele-specific down-regulation of MHC class I antigens in Burkitt lymphoma lines. *Cell Immunol.* **120:396-400.**

14. Annette Paschen, Rosa Maria MENDEZ, PilarJIMENEZ, et al (2003). Complete loss of HLA class I antigen expression on melanoma cells: a result of successive mutational events. *Int. J. Cancer* **103:759 -767.**

15. Barbara Seliger, Ursula Wollscheid, Frank Momburg, et al (2001). Characterization of the Major Histocompatibility Complex Class I Deficiencies in B16 Melanoma Cells. *Cancer Res* **61:1095-1099.**

16. Yoshii M, Tanaka H, Ohira M, et al (2013). Association of MHC class I expression and lymph node metastasis of gastric carcinoma. *Hepatogastroenterology* **60:611-615.**

17. Claudia B.M. Bijen, Enja J. Bantema-Joppe, Renske A. de Jong, et al (2010). The prognostic role of classical and neoclassical MHC class I expression in endometrial cancer. *International Journal of Cancer* **126: 1417–1427.**

18. Vitale M, Pelusi G, Taroni B, et al (2005). HLA class I antigen down-regulation in primary ovary carcinoma lesions: association with disease stage. *Clin Cancer Res.* **1:67-72.**

19. Koichi Kaneko, SumiyaIshigami, Yuko Kijima, et al (2011). Clinical implication of HLA class I expression in breast cancer. *BMC Cancer* **11:454.**

20. McDougall, Sing Shang Ngoi, Ira S. Goldman, et al (1990). Reduced Expression of HLA Class I and II Antigens in Colon Cancer. *Carl J. Cancer Res* **50:8023-8027.**

21. Chang CC, Campoli M, and Ferrone S (2003). HLA class I defects in malignant lesions: what have we learned? *Keio J Med.* **52:220-229.**

22. Michael Campoli and SoldanoFerrone (2008). HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene.* **27: 5869–5885.**

23. Garson D, Dokhélar MC, Wakasugi H, et al (1985). HLA class-I and class-II antigen expression by human leukemic K562 cells and by Burkitt-K562 hybrids: modulation by differentiation inducers and interferon. *ExpHematol* **13:885-890.**

24. V. Procházka, M. Jarošová, Z. Prouzová, et al. (2012). Immune Escape Mechanisms in Diffuse Large B-Cell Lymphoma. *ISRN Immunology.***2012: article ID 208903. doi:10.5402/2012/208903.**

25. Momburg F, Herrmann B, Moldenhauer G, and Möller P (1987). B-cell lymphomas of high-grade malignancy frequently lack HLA-DR, -DP and -DQ antigens and associated invariant chain. *Int J Cancer.* **40:598-603.**

26. Xiaodong Cheng et al. (2010). Mechanisms of DNA Methylation, Methyl-CpG Recognition, and Demethylation in Mammals. In *Handbook of Epigenetics. Oxford: Academic Press* p 9-24. **ISBN: 978-0-12-375709-8.**

27. Jody C. Chuang, Christine B. Yoo, Jennifer M. Kwan, et al. (2005). Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2'-deoxycytidine.*Mol Cancer Ther* **4:1515-1520.**

28. ManelEsteller (2005). DNA Methylation, In *Epigenetics and Metastasis.* **P 243-244. ISBN 978-1-4020-3642-2.**

29. J. Goffin and E. Eisenhauer (2002). DNA methyltransferase inhibitors-state of the art. *Ann Oncol* **13:1699-1716.**

30. MetinKarahoca and Richard L Momparler (2013). Pharmacokinetic and pharmacodynamic analysis of 5-aza-2-deoxycytidine (decitabine) in the design of its dose-schedule for cancer therapy. *Clinical Epigenetics* **5:3.Doi: 10.1186/1868-7083-5-3.**

31. Oscar Alcazar, Susan Achberger, Wayne Aldrich, et al. (2012). Epigenetic regulation by decitabine of melanoma differentiation in vitro and in vivo. *International Journal of Cancer* **131: 18–29.**

32. Serrano A, Tanzarella S, Lionello I, et al. (2001). Rexpression of HLA class I antigens and restoration of antigen-specific CTL response in melanoma cells following 5-aza-2'-deoxycytidine treatment. *Int J Cancer.* **94:243-251.**

33. Li Y, Hu W, Shen DY, et al. (2009). Azacitidine enhances sensitivity of platinum-resistant ovarian cancer cells to carboplatin through induction of apoptosis. *American Journal of Obstetrics & Gynecology.***200:177.e1 - 177.e9**

34. Sara J Adair and Kevin T Hogan (2009). Treatment of ovarian cancer cell lines with 5-aza-2'-deoxycytidine upregulates the expression of cancer-testis antigens and class I major histocompatibility complex-encoded molecules. *Cancer ImmunolImmunother.* **58:589-601.**

35. Xiao-Wen Hao, Sheng-Tao Zhu, Yuan-Long He, et al. (2012). Epigenetic inactivation of secreted frizzled-related protein 2 in esophageal squamous cell carcinoma. *World J Gastroenterol* **18: 532-540.**

36. Nie Y, Yang G, Song Y, et al. (2001). DNA hypermethylation is a mechanism for loss of expression of the HLA class I genes in human esophageal squamous cell carcinomas. *Carcinogenesis.* **22:1615-1623.**

37. Ma J, Yu X, Guo L, and Lu SH (2013). DUSP6, a tumor suppressor, is involved in differentiation and apoptosis in esophageal squamous cell carcinoma. *OncolLett.* **6:1624-1630.**

38. Shawn P. Murphy, Renae Holtz, Nicole Lewandowski, et al. (2002). DNA Alkylating Agents Alleviate Silencing of Class II Transactivator Gene Expression in L1210 Lymphoma Cells. *The Journal of Immunology.* **169:3085-3093.**

39. Hassler MR, Klisaroska A, Kollmann K, et al. (2012). Antineoplastic activity of the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine in anaplastic large cell lymphoma. *Biochimie.* **94:2297-2307.**

40. Jason A. Dubovsky, Douglas G. McNeel, John J. Powers, et al. Treatment of Chronic Lymphocytic Leukemia with a Hypomethylating Agent Induces Expression of NXF2, an Immunogenic Cancer Testis Antigen. *Clin Cancer Res* **15:3406-3415.**

41. EMILY CHEN, ROBERT W. KARR, JOAN P. FROST, et al. (1986). Gamma Interferon and 5-Azacytidine Cause Transcriptional Elevation of Class I Major Histocompatibility Complex Gene Expression in K562 Leukemia Cells in the Absence of Differentiation. *MOLECULAR AND CELLULAR BIOLOGY* **6:1698-1705.**

42. Tjadine M Holling, Erik Schooten, Anton W Langerak and Peter J van den Elsen (2004). Regulation of MHC class II expression in human T-cell malignancies. *Blood.* **103:1438-1444.**

43. Qin, Taichun, Youssef, Emile M., et al. (2005). Global genomic DNA methylation pattern and combination effects of 1-beta-D-arabinofuranosylcytosine and 5-aza-2'-deoxycytidine in human leukemic cells. *AACR Meeting Abstracts* **2005: 425-d-426.**

44. Hardy TM, and Tollefsbol TO (2011). Epigenetic diet: impact on the epigenome and cancer. *Epigenomics.* **3:503-518.**

45. National Cancer Institute at the National Institute of Health Fact Sheet, "Cruciferous Vegetables and Cancer Prevention", *Cancer.gov*, June 7 2012, Web Sep. 22 2014.

    <http://www.cancer.gov/cancertopics/factsheet/diet/cruciferous-vegetables>

46. Jane V. Higdon, Barbara Delage, David E. Williams, and Roderick H. Dashwood (2007). Cruciferous Vegetables and Human Cancer Risk: Epidemiologic Evidence and Mechanistic Basis. *Pharmacol Res.* **55: 224-236.**

47. Xiang Wu, Qing-hua Zhou, and Ke Xu (2009). Are isothiocyanates potential anti-cancer drugs? *ActaPharmacologicaSinica* **30: 501–512.**

48. Navarro SL, Li F, and Lampe JW (2011). Mechanisms of action of isothiocyanates in cancer chemoprevention: an update. *Food Funct.* **2:579-587.**

49. Myzak MC, Tong P, Dashwood WM, et al. (2007). Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. *ExpBiol Med (Maywood).***232:227-234.**

50. Meeran SM, Ahmed A, and Tollefsbol TO (2010). Epigenetic targets of bioactive dietary components for cancer prevention and therapy. *Clin Epigenetics.* **1:101-116.**

51. Hsu A, Wong CP, Yu Z, et al. (2011). Promoter de-methylation of cyclin D2 by sulforaphane in prostate cancer cells. *Clin Epigenetics.* **3:3.**

52. Clarke JD, Dashwood RH, and Ho E (2008). Multi-targeted prevention of cancer by sulforaphane. **Cancer Lett. 269:291-304.**

53. YipingGu, Chunjie Wang, Chaim M. Roifman and Amos Cohen (2003). Role of MHC Class I in Immune Surveillance of Mitochondrial DNA Integrity. *The Journal of Immunology* **170:3603-3607.**

54. Sheng KC, Pietersz GA, Tang CK, et al. (2010). Reactive oxygen species level defines two functionally distinctive stages of inflammatory dendritic cell development from mouse bone marrow. *J Immunol.* **184:2863-2872.**

55. Garg AD, Krysko DV, Verfaillie T, et al. (2012). A novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death. *EMBO J.* **31:1062-1079.**

56. Harari O and Liao JK (2004). Inhibition of MHC II gene transcription by nitric oxide and antioxidants. *Curr Pharm Des.* **10:893-898.**

57. Kosei Maemura, Qizhi Zheng, Tatehiko Wada, et al. (2005). Reactive oxygen species are essential mediators in antigen presentation by Kupffer cells. *Immunology and Cell Biology* **83:336–343.**

58. Lorenzi S, Forloni M, Cifaldi L, et al. (2012) IRF1 and NF-kB Restore MHC Class I-Restricted Tumor Antigen Processing and Presentation to Cytotoxic T Cells in Aggressive Neuroblastoma. *PLoS ONE* **7:e46928. doi:10.1371/journal.pone.0046928**

59. Pick M, Ronen D, Yanuka O, and Benvenisty N. (2012). Reprogramming of the MHC-I and its regulation by NFκB in human-induced pluripotent stem cells. *Stem Cells.* **30:2700-2708.**

60. Mimura K, Shiraishi K, Mueller A, et al.(2013). The MAPK pathway is a predominant regulator of HLA-A expression in esophageal and gastric cancer. *J Immunol.* **191:6261-6272.**

61. Fandy TE, Jiemjit A, Thakar M, et al. (2014). Decitabine induces delayed reactive oxygen species (ROS) accumulation in leukemia cells and induces the expression of ROS generating enzymes. *Clin Cancer Res.* **20:1249-1258.**

62. Shin DY, Park YS, Yang K, et al. (2012). Decitabine, a DNA methyltransferase inhibitor, induces apoptosis in human leukemia cells through intracellular reactive oxygen species generation. *Int J Oncol.* **41:910-918.**

63. Anna Laurenzana, Luca A. Petruccelli, FilippaPettersson, et al. (2009). Inhibition of DNA Methyltransferase Activates Tumor Necrosis Factor α–Induced Monocytic Differentiation in Acute Myeloid Leukemia Cells. *Cancer Res.* **69:55-64.**

64. Fimognari C, Nüsse M, Cesari R, et al. (2002) . Growth inhibition, cell-cycle arrest and apoptosis in human T-cell leukemia by the isothiocyanate sulforaphane. *Carcinogenesis.* **23:581-586.**

65. Stresemann C, Brueckner B, Musch T, et al. (2006). Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. *Cancer Res.* **66:2794-2800.**

66. Y Morimoto, M Toyota, A Satoh, et al. (2004). Inactivation of class II transactivator by DNA methylation and histone deacetylation associated with absence of HLA-DR induction by interferon-γ in haematopoietictumour cells. *Br J Cancer.* **90: 844–852.**

67. American Type Cell Culture. Jurkat, Clone E6-1 (ATCC TIB-152), *www.atcc.org,* Web. Sep. 22 2014,

   <http://www.atcc.org/products/all/TIB-152.aspx#generalinformation>

68. Sigma Aldrich products sheets 5-Aza-2'-deoxycytidine product#A3656, D,L Sulforaphane product# S4441, and DMSO product#D2560. *www.sigmaaldrich.com*, Web. Sep 22 2014.

5-Aza-2'-deoxycytidine

<http://www.sigmaaldrich.com/catalog/search?interface

=All&term=A3656&N=0&mode=match%20partialmax&focus=product&lang=en

&region=US>

Sulforaphane<http://www.sigmaaldrich.com/catalog/search?interface=All&ter

m=S4441&N=0&mode=match%20partialmax&focus=product&lang=en&region

=US>

DMSO<http://www.sigmaaldrich.com/catalog/product/sigma/d2650?lang=en

&region=US>

69. Barnstable CJ, Bodmer WF, Brown G, et al. (1978). Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. *Cell.* **14: 9-20.**

70. Lampson LA, and Levy R (1980). Two populations of Ia-like molecules on a human B cell line. *J. Immunol.* **125: 293-299.**

71. Carmody MB, Ault KA, Mitchell JG, et al. (1990). Production of monoclonal antibodies specific for platelet activation antigens and their use in evaluating platelet function. *Hybridoma* **9:631-641.**

72. Jackson ImmunoReasearch Laboratories Inc. , Fluorescein (FITC) AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG, Fcγ Fragment Specific, *www.jacksonimmuno.com*, Web. Sep. 22 2014, **<https://www.jacksonimmuno.com/catalog/products/115-096-071>**

73. GraphPad SoftwareInc. (2014). P Value calculator. *Graphpad.com.***<graphpad.com/quickcalcs/PValue1.cfm>.**

74. Yumi Yamamoto and Richard B. Gaynor (2001). Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer .*J Clin Invest.* **107:135–142.**

75. Wu JF, Zeng GL, Shen W, et al. (2009). Up-regulation of major histocompatibility complex class I-related molecules A (MICA) induced by 5-aza-2'-deoxycytidine. *ZhonghuaGanZang Bing ZaZhi.* **17:675-678.**

76. Andresen L, Jensen H, Pedersen MT, et al. (2007). Molecular regulation of MHC class I chain-related protein A expression after HDAC-inhibitor treatment of Jurkat T cells. *J Immunol.* **179:8235-8242.**

77. Luciana L Molinero, Mercedes B Fuertes, María Victoria Girart, et. Al. (2004). NF-kappa B regulates expression of the MHC class I-related chain A gene in activated T lymphocytes. *J Immunol* **173:5583-5590.**

78. Xu X, Rao G, Gaffud MJ, et al. (2006). Clinicopathological significance of major histocompatibility complex class I-related chain a and B expression in thyroid cancer. *J ClinEndocrinolMetab.* **91:2704-2712.**

79. Becton Dickinson BD Biosciences, BD Facscalibur, *www.bdbiosciences.com*, Web.

Sep. 22 2014, <http://www.bdbiosciences.com/instruments/facscalibur/>.
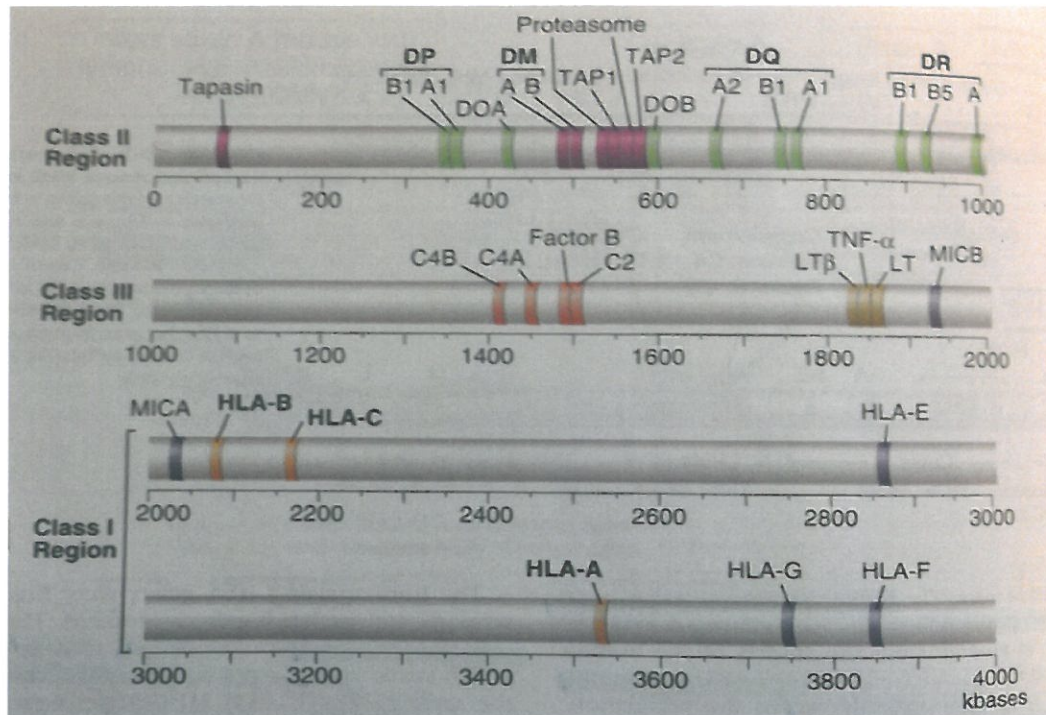
Figure I: MHC gene loci [1]:

MHC gene can be divided into 3 loci (see figure I in Appendix I):

- Class II locus: the first 1000 kilobases (kb) encode for the following genes (with locations indicated by the positions of bases on the MHC gene complex): Tapasin (50-100 kb), DP A1 and B1 (around the 350 kb location), DM A&B (around the 500 kb), genes from location 475 to 600 kb in order (Proteasome genes, TAP1, Proteasome genes, TAP2, and DOB), DQ A2&B2 around 650 kb, DQ A1&B1 around 750 kb, DRB1 at about 825 kb , DRB3-5 at 900 kb, and DRA at 1000 kb.
- Class III locus: the second 1000 kb encodes C4B somewhere between 1400 and 1450 kb, C4A around 1450 kb, factor B and C2 are around 1500 kb, around 1850 kb in order are LT-beta, TNF-alpha, and LT-alpha, and MIC-B between 1900 and 1950 kb.
- Class I locus: the last 2000 kb encodes MIC-A between 2000 and 2050 kb, HLA-B between 2050 and 2100 kb, HLA-C between 2150 and 2200 kb, HLA-E between 2850 and 2900 kb, HLA-A between 2500 and 2550 kb, HLA-G around 2750 kb, and HLA-F around 2850 kb.

Abul K. Abbas & Andrew Lichman . (2012). ***Cellular and Molecular Immunology, 7th edition. Saunders ISBN: 1-4377-1528-1; 1-4377-3573-8.***
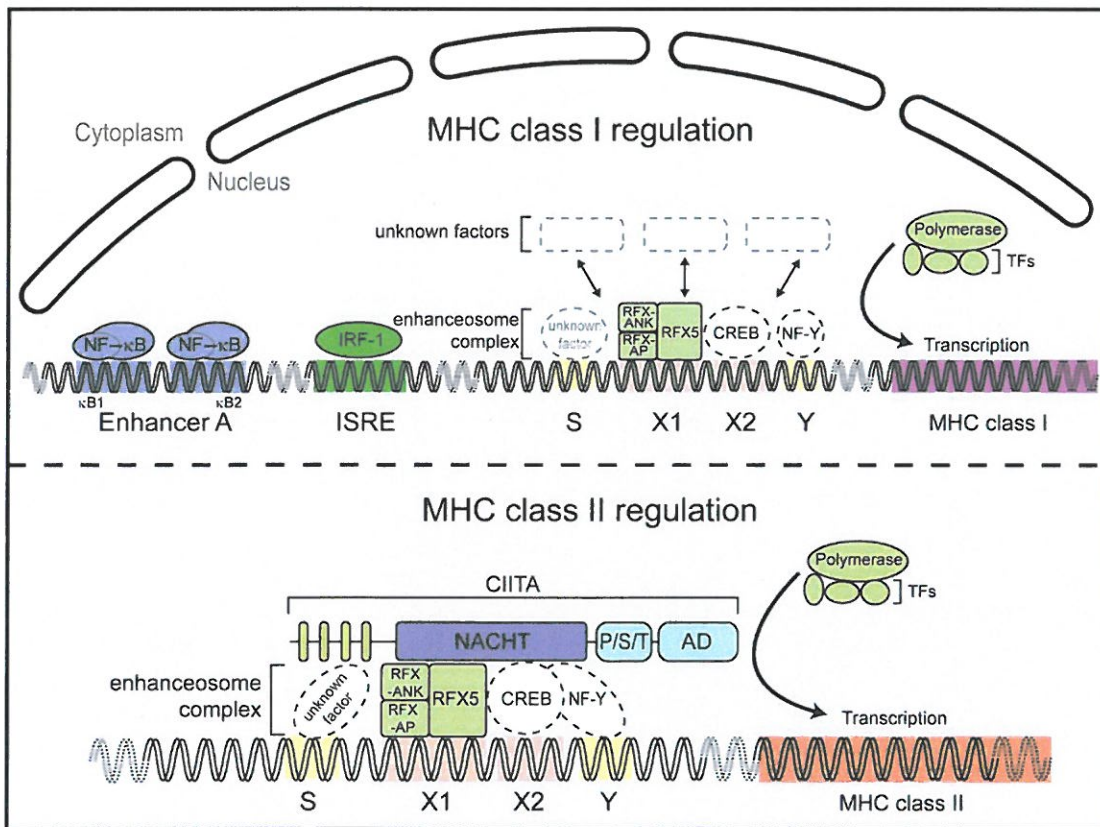
Figure II: Major-histocompatibility complex class I and II promoters: MHC class I genes share similar cis-regulatory elements in their proximal promoters, termed W/S, X1, X2 and Y-box motifs. Class I Transactivator or CITA can associate with the RFX transcription factor complex at the X1 box and can cooperate with ATF1/CREB family transcription factor at the X2 box of MHC class I promoter. NFY transcription factor binds to Y box. MHC class II gene has conserved upstream sequences with cis-regulatory elements: S (binds to RFX), X (binds to RFX, AP1, X2BP, and CREB), and Y (binds to NFY). All form MHC enhanceosome that requires Class II Transactivator or CIITA to be active.

Andreas Neerincx, Wilson Castro, GreetaGuarda, and Thomas Kufer(2013). NLRC5, at the heart of antigen presentation. Front. Immunol., 22.
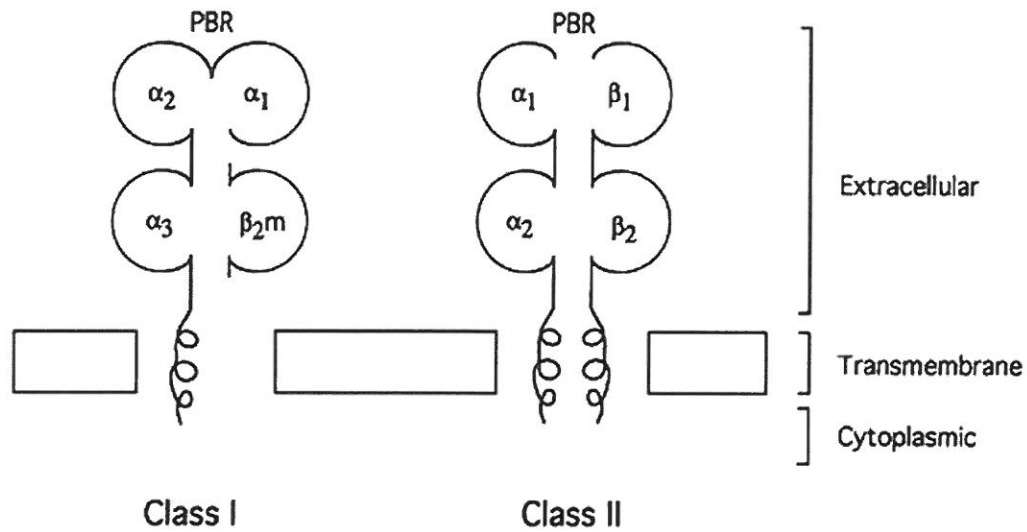
Figure 2. Schematic presentation of the structure of MHC class I and class II molecules. PBR = peptide-binding region. (Reprinted, with permission, from the Annual Review of Genetics, Vol. 32 ©1998 by Annual Reviews, www.annualreviews.org).

Figure III: MHC structure:

Class I: Non covalently linked alpha (1, 2, and 3 segments) and beta-2 microglobulin chains. Peptide binding cleft is 8-11 amino acids long formed by alpha 1 and 2 segments. Alpha 3 and beta-2 microglobulin are conserved (non-polymorphic) and alpha 3 segments are for binding with CD8.

Class II: Non-covalently linked alpha and beta (each is of 2 segments: segment 1 is polymorphic, segment 2 is conserved) chains. Peptide binding cleft is formed by alpha 1 and beta 1 segments. alpha 2 and beta-2 segments are conserved (non-polymorphic) and beta 2 segment is for binding with CD4.

V.S.R. Dukkipati, H.T. Blair, D.J. Garrick and A. Murray (2006). Ovine major histocompatibility complex: structure and gene polymorphisms. Genet. Mol. Res. 5 : 581-608.
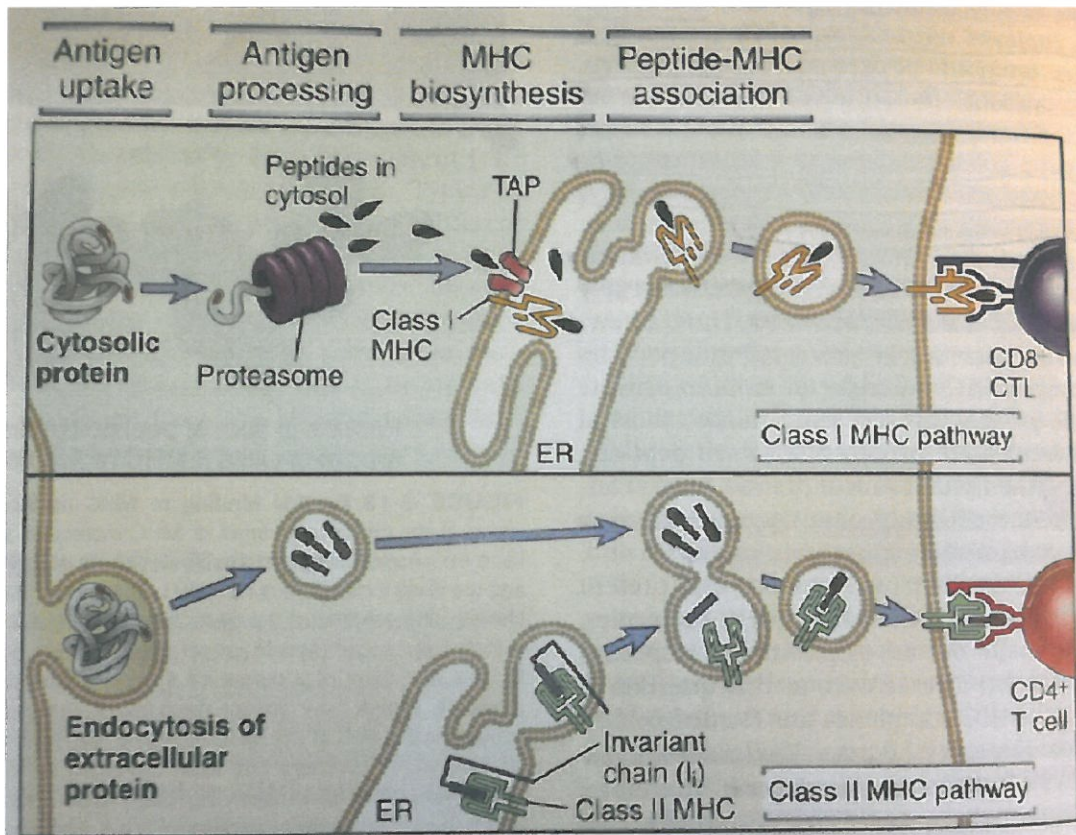
Figure IV: Antigen Processing Machinery: In MHC class I pathway (top panel), protein antigens are processed in the cytosol by proteasomes, and peptides are transported into the ER, where they bind to MHC class I molecules. In MHC class II pathway (bottom panel) extracellular antigens are endocytosed into vesicles where they are processed and bind to MHC class II molecules.

Abul K. Abbas & Andrew Lichman . (2012). **Cellular and Molecular Immunology, 7th edition. Saunders. ISBN: 1-4377-1528-1; 1-4377-3573-8.**
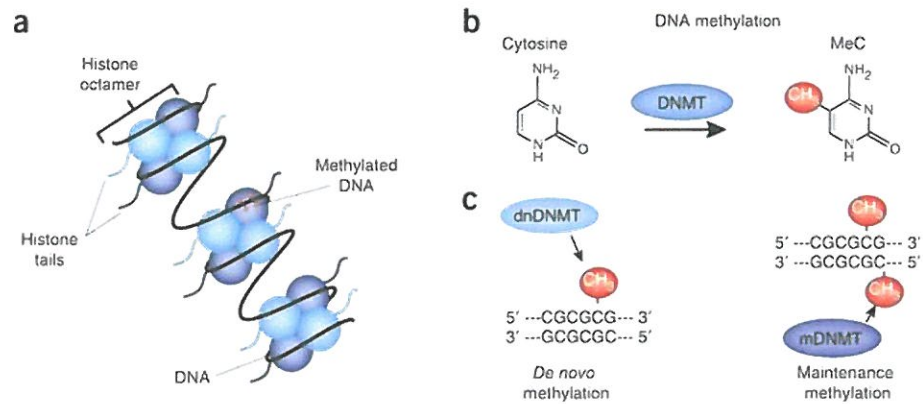
**Figure V: DNA Methylation:**

DNA methylation, an epigenetic mechanism, is mediated by DNA methyl transferases (DNMTs). In mammalian cells, it occurs at the C5 of cytosine (5mC) usually within CpG dinucleotide. The mechanism of DNA methylation starts with DNMT binding to the DNA and eversion of target nucleotide to project out of the DNA helix (base flipping), then the DNMT attacks cytosine C6 and results in transfer of the methyl group from S-adenosyl-l -methionine (AdoMet) to the activated cytosine C5

DNA methylation and memory formation.Jeremy J Day& J David Sweatt (2010).Nature Neuroscience 13,1319–1323.
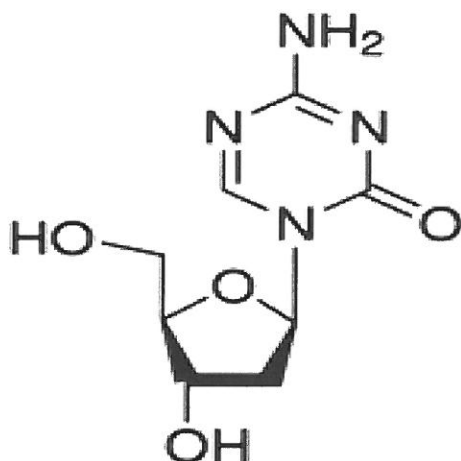
Figure VI: structure of 5-Aza-2'Deoxycitidine [84]
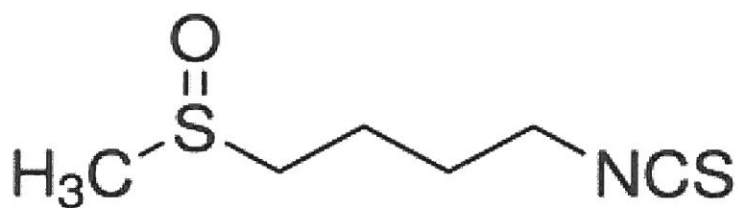
Figure VII: structure of Sulforaphane

Sigma Aldrich products sheets 5-Aza-2'Deoxycytidine product#A3656, D,L and Sulforaphane product# S4441. *www.sigmaaldrich.com*, Web. Sep 22 2014.

5-Aza-2'Deoxycytidine**<http://www.sigmaaldrich.com/catalog/search?interface =All&term=A3656&N=0&mode=match%20partialmax&focus=product&lang=en&regio n=US>**

Sulforaphane**<http://www.sigmaaldrich.com/catalog/search?interface=All&term=S44 41&N=0&mode=match%20partialmax&focus=product&lang=en&region=US>**

APPENDIX II

Raw Data of Flow Cytometry and Cell Counts

Table 1

P-values of effects of SFN and AZA on MHC expression in Jurkat Cells

Treatment	MHC class-I	MHC class-II
SFN 10 µM 24 h	less than 0.0001	0.9619
SFN 10 µM 48 h	less than 0.0001	0.0976
SFN 10 µM 72 h	less than 0.0001	0.0204
SFN 20 µM 24 h	less than 0.0001	0.6243
SFN 20 µM 48 h	less than 0.0001	0.403
SFN 20 µM 72 h	less than 0.0001	0.0014
SFN 30 µM 24 h	less than 0.0001	0.0901
SFN 30 µM 48 h	less than 0.0001	0.0002
SFN 30 µM 72 h	less than 0.0001	less than 0.0001
AZA 1 µM 24 h	less than 0.0001	0.9029
AZA 1 µM 48 h	less than 0.0001	0.7925
AZA 1 µM 72 h	less than 0.0001	0.3645
AZA 2 µM 24 h	less than 0.0001	0.8776
AZA 2 µM 48 h	less than 0.0001	0.3763
AZA 2 µM 72 h	less than 0.0001	0.0188
AZA 3 µM 24 h	less than 0.0001	0.8898
AZA 3 µM 48 h	less than 0.0001	0.5556
AZA 3 µM 72 h	less than 0.0001	0.2288

The Chi squares and P-values were calculated using MS-Excel 2007 software. All treatments had P-values less than 0.0001 for the effect on MHC class I expression by Jurkat cells which means that these treatments have a very statistically significant effect on MHC class I expression by Jurkat cells. The effects on MHC class II expression by Jurkat cells were statistically significant only with SFN for 72 h (P= 0.0204 for 10 µM, P= 0.0014 for 20 µM, and P=0.0002 for 30 µM), SFN 30 µM for 48 h (P= less than 0.0001), and with Aza 2 µM for 72 h (P= 0.0188).

Table 2
Total and Dead Cell Count in Millions of Jurkat Cells After Being Treated with SFN and AZA for 24 h

Treatment	Total	Dead	Total	Dead	Total	Dead
SFN10 μM	5.25	0.55	5.1	0.65	5.4	0.6
SFN20 μM	5.4	0.5	5.25	0.8	5.55	0.65
SFN30 μM	5.2	0.65	5.4	0.65	5.15	0.7
DMSO10 μl	7.95	0.35	7.95	0.75	7.75	0.45
DMSO20μl	7.2	0.8	8.05	0.95	7.05	0.9
DMSO30μl	6.6	1.05	7.25	0.35	7.35	0.9
AZA1μM	6.2	0.45	5.95	0.4	6.05	0.4
AZA2 μM	5.5	0.25	5.75	0.35	5.45	0.25
AZA3 μM	4.55	0.55	4.35	0.4	4.4	0.5
DMSO1 μl	7.8	0.3	8.05	0.9	7.7	0.25
DMSO2 μl	7.15	0.65	8.15	0.5	7.55	0.55
DMSO3 μl	8.1	0.95	7.85	0.55	7.25	0.35
untreated	7.75	0.75	7.95	0.45	8.25	0.8

The cell count was performed using hemacytometer under light microscope using Trypan Blue dye to distinguish dead cells from live ones. These cells were treated with different concentration of SFN or AZA for 24 h and with DMSO as a control. The last row is for untreated control sample.

Table 3
Total and Dead Cell Count in Millions of Jurkat Cells After Being Treated with SFN and AZA for 48 h

Treatment	Total	Dead	Total	Dead	Total	Dead
SFN10µM	8.35	1.8	7.8	1.8	8.25	1.45
SFN20 µM	7.6	1.75	7.4	1.65	7.3	1.8
SFN30 µM	7.95	3.8	8.1	3.65	7.7	3.75
DMSO10 µl	14.35	1.3	15.55	0.6	14.45	0.3
DMSO20 µl	14.55	0.7	14.9	0.85	14.85	0.9
DMSO30 µl	15.15	1.2	14.7	1.25	14.55	0.7
AZA1 µM	9.2	0.8	8.8	1.05	8.9	1
AZA2 µM	10.2	1.3	9.9	1.3	10.05	1.55
AZA3 µM	7.05	1.9	7.2	1.75	6.95	1.8
DMSO1 µM	14.65	1	14.35	1.05	11.44	1.15
DMSO2 µM	15.25	0.85	14.75	0.95	14.8	1.25
DMSO3 µM	14.85	1.25	15.05	0.95	14.85	0.8
untreated	15.25	0.95	14.95	1.1	14.2	1.35

The cell count was performed using hemacytometer under light microscope using Trypan Blue dye to distinguish dead cells from live ones. These cells were treated with different concentration of SFN or AZA for 48 h and with DMSO as a control. The last row is for untreated control sample.

Table 4
Total and Dead Cell Count in Millions of Jurkat Cells After Being Treated with SFN and AZA for 72 h

Treatment	Total	Dead	Total	Dead	Total	Dead
SFN10 µM	8.6	2.35	8.2	2	8.55	2.55
SFN20 µM	8.2	4.05	8.1	3.95	8.3	3.8
SFN30 µM	7.8	5.8	8.05	5.55	8.1	5.65
DMSO10 µl	17.1	1.85	16.15	1.75	15.8	1.55
DMSO20 µl	16.6	1.05	16.25	1.4	17.1	1.25
DMSO30 µl	16.85	1.65	15.85	1.95	16	1.15
AZA1 µM	19.53	3.2	18.2	3.1	19.15	3.55
AZA2 µM	13.85	31.5	13.6	2.95	14.05	3.25
AZA3µM	10.8	2.8	10.55	2.75	10.5	2.95
DMSO1 µl	16.4	1.75	16.55	1.35	16.35	1.55
DMSO2 µl	17.05	1.6	16.95	1.6	15.95	1.85
DMSO3 µl	16.45	1.4	17.15	1.65	16.7	1.2
untreated	16.45	1.65	16.1	1.95	17.05	1.15

The cell count was performed using hemacytometer under light microscope using Trypan Blue dye to distinguish dead cells from live ones. These cells were treated with different concentration of SFN or AZA for 72 h and with DMSO as a control. The last row is for untreated control sample.

<p style="text-align:center">Table 5
% Growth of Jurkat Cells After Being Treated with SFN and AZA</p>

Treatment	24 h			48 h			72 h		
SFN10	5	2	8	67	56	65	72	64	71
SFN20	8	5	11	52	48	46	64	62	66
SFN30	4	8	3	59	62	54	56	61	62
DMSO10	59	59	55	187	211	189	242	223	216
DMSO20	44	61	41	191	198	197	232	225	242
DMSO30	32	45	47	203	194	191	237	217	220
AZA1	24	19	21	84	76	78	290.6	264	283
AZA2	10	15	9	104	98	101	177	172	181
AZA3	-9	-13	-12	41	44	39	116	111	110
DMSO1	56	61	54	193	187	128.8	228	231	227
DMSO2	43	63	51	205	195	196	241	239	219
DMSO3	62	57	45	197	201	197	229	243	234
untreated	55	59	65	205	199	184	229	222	241

% Growth of Jurkat cells after treatment with SFN, Aza, or control was calculated by subtracting the initial 5×10^6 cell seeding from the sample count and dividing it by 5×10^6 and multiplying the result by 100% using MS-Excel 2007 software.

Table 6
% of Dead Cells in Cultured Jurkat Cells after Being Treated with SFN and AZA

Treatment	24 h		
SFN10μM	10.4761905	12.745098	11.1111111
SFN20 μM	9.25925926	15.2380952	11.7117117
SFN30 μM	12.5	12.037037	13.592233
DMSO10 μl	4.40251572	9.43396226	5.80645161
DMSO20 μl	11.1111111	11.8012422	12.7659574
DMSO30 μl	15.9090909	4.82758621	12.244898
AZA1 μM	7.25806452	6.72268908	6.61157025
AZA2 μM	4.54545455	6.08695652	4.58715596
AZA3 μM	12.0879121	9.1954023	11.3636364
DMSO1 μl	3.84615385	11.1801242	3.24675325
DMSO2 μl	9.09090909	6.13496933	7.28476821
DMSO3 μl	11.7283951	7.00636943	4.82758621
untreated	9.67741935	5.66037736	9.6969697
Treatment	48 h		
SFN10 μM	21.556886	23.076923	17.575758
SFN20 μM	23.026316	22.297297	24.657534
SFN30 μM	47.798742	45.061728	48.701299
DMSO10 μl	9.0592334	3.8585209	2.0761246
DMSO20 μl	4.8109966	5.704698	6.0606061
DMSO30 μl	7.9207921	8.5034014	4.8109966
AZA1 μM	8.6956522	11.931818	11.235955
AZA2 μM	12.745098	13.131313	15.422886
AZA3 μM	26.950355	24.305556	25.899281
DMSO1 μl	6.8259386	7.3170732	10.052448
DMSO2 μl	5.5737705	6.440678	8.4459459
DMSO3 μl	8.4175084	6.3122924	5.3872054
untreated	6.2295082	7.3578595	9.5070423

Treatment		72 h		
SFN10 μM	SFN10	27.325581	24.390244	29.824561
SFN20 μM	SFN20	49.390244	48.765432	45.783133
SFN30 μM	SFN30	74.358974	68.944099	69.753086
DMSO10 μl	DMSO10	10.818713	10.835913	9.8101266
DMSO20 μl	DMSO20	6.3253012	8.6153846	7.3099415
DMSO30 μl	DMSO30	9.7922849	12.302839	7.1875
AZA1 μM	AZA1	16.385049	17.032967	18.537859
AZA2 μM	AZA2	227.43682	21.691176	23.131673
AZA3 μM	AZA3	25.925926	26.066351	28.095238
DMSO1 μl	DMSO1	10.670732	8.1570997	9.4801223
DMSO2 μl	DMSO2	9.3841642	9.439528	11.598746
DMSO3 μl	DMSO3	8.5106383	9.6209913	7.1856287
untreated	untreated	10.030395	12.111801	6.744868

% of dead cells of Jurkat cells after treatment with SFN, Aza, or control was calculated by dividing the number of dead cells by the total count cells and multiplying the result by 100% using MS-Excel 2007 software.

Table 7

Geometric Means of Fluorescent Intensity Detected by Flow Cytometer From Stained Jurkat Cells with Anti-MHC Antibodies After Being Treated with SFN

	Treated Negative		Treated MHC-I	Treated MHC-II		DMSO control Negative	DMSO control MHC-II		DMSO control MHC-II	
10 µM 24 H	5.15	129.62	178.38	6.23	7.85	6.22	114.2	109.95	7.17	8.79
	2.09	187.92	174.21	2.43	2.47	1.74	205.64	178.34	2.12	2.08
	2.11	228.72	225.24	2.7	2.71	1.67	243.69	242.91	2.08	2.11
10 µM 48 H	4.3	131.52	125.82	6.9	8.76	2.76	196	186.63	3.91	4.09
	2.23	196.77	201.61	2.64	2.85	1.86	186.91	186.9	2.21	2.09
	2.41	168.27	161.73	3.16	3.19	1.63	199.17	195.96	2.08	2.05
10 µM 72 H	6.66	120.63	139.2	10.14	8.1	3.47	219.91	228.65	4.17	4.15
	2.07	189.26	215.57	2.31	2.28	1.57	186.14	182.04	1.9	1.86
	3.28	199.22	175.73	4.91	4.91	1.72	162.4	162.36	2.18	2.14
20 µM 24 H	3.51	101.24	106.82	4.2	4.24	3.57	217.05	238.89	5.07	4.97
	2.21	121.34	120	2.64	2.89	1.78	177.67	184.49	1.98	2.14
	2.2	164.13	164.04	3.06	3.01	1.63	238.43	237.8	2.09	2.1
20 µM 48 H	5.29	105.76	96.11	6.7	7.03	3.05	175.91	179.91	4.1	3.93
	2.58	125.1	133.17	3.25	3.09	1.82	208.01	189.99	2.08	2.08
	2.82	151.5	144.44	3.92	3.9	1.64	198.93	202.39	2.07	2.07
20 µM 72 H	6.92	91.6	105.93	9.93	8.96	3.31	131.51	124.9	3.68	3.72
	3.08	106.5	99.95	3.71	3.72	1.6	199.28	148.06	1.88	1.83
	4.1	137.67	138.7	5.66	5.7	1.72	132.46	138.82	2.45	2.5
30 µM 24 H	5.84	87.98	70.61	11.43	7.81	3.48	198.18	212.35	4.85	5.06
	3.55	100.17	123.54	4.45	4.53	1.85	182.46	168.47	2.22	2.15
	2.32	144.35	151.57	3.03	3.01	1.65	238.79	245.24	2.11	2.12
30 µM 48 H	6.24	94.33	93.51	9.32	9.18	2.92	185.78	219.27	3.82	3.6
	4.34	121.54	111.31	5.16	5.52	1.81	141.35	133.72	1.96	1.89
	5.52	98.46	94.47	7.51	7.25	1.64	197.43	194.51	2.18	2.05
30 µM 72 H	10.49	102	108.78	14.79	12.5	3.71	207.44	215.51	4.05	4.42
	4.81	71.04	70.14	5.84	6.46	1.64	187.55	167.13	1.88	1.86
	4.81	71.04	70.14	5.84	6.46	1.64	187.55	167.13	1.88	1.86

Jurkat cells treated with SFN and DMSO were stained by FITC-anti IgG secondary antibodies after being treated with primary antibodies which are IE3 (a mouse negative control antibody), mouse anti-MHC class I antibody (W6/32), and mouse anti-MHC class II antibody (L243).The geometric means of fluorescent intensity of each sample were calculated as listed in the table.

Table 8
Geometric Means of Fluorescent Intensity Detected by Flow Cytometer From Stained Jurkat

Cells with Anti-MHC Antibodies After Being Treated with Aza

	Treated Negative	Treated MHC1		Treated MHC2		Untreated Negative	Untreated MHC1		Untreated MHC2	
1 µM 24 h	1.72	300.74	302.74	2.12	2.18	1.64	234.12	234.38	2.13	2.14
	1.67	276.96	274.9	2.24	2.64	1.62	233.69	239.58	2.11	2.13
	1.71	320.89	241.12	2.36	2.33	1.63	234.32	232.8	2.13	2.09
1 µM 48 h	2.05	226.19	226.26	2.67	2.97	1.67	194.95	195.28	2.15	2.03
	1.94	251.42	242.05	2.69	2.79	1.65	198.33	192.24	2.69	2.79
	2.02	252.82	247.27	2.44	2.68	1.66	189.35	192.41	2.01	2.02
1 µM 72 h	2.28	226.36	209.15	3.3	3.27	1.76	154.58	152.04	2.16	2.17
	2.31	230.14	212.84	3.27	3.18	1.74	151.21	153.13	2.12	2.15
	2.28	220.53	214.81	3.1	3.21	1.71	153.19	152.71	2.15	2.14
2 µM 24 h	1.69	311.09	302.2	2.23	2.26	1.63	235.68	233.88	2.06	2.06
	1.72	283.59	277.74	2.49	2.54	1.62	223.09	223.82	2.09	2.09
	1.69	344.36	309.28	2.21	2.15	1.64	224.99	230.29	2.12	2.14
2 µM 48 h	2.03	206.32	207.55	2.51	2.58	1.65	183.1	186.27	2	1.99
	1.98	269.06	260.5	2.67	2.7	1.68	188.3	187.48	2	2.01
	2.07	376.36	347.32	2.92	3.17	1.71	180.85	188.06	2.01	2.03
2 µM 72 h	2.56	217.07	223.29	3.83	3.79	1.72	152.22	151.16	2.16	2.15
	2.5	211.66	210.5	4.91	3.96	1.71	150.36	151.66	2.16	2.17
	2.45	202.91	203.58	3.9	3.68	1.67	150.06	152.25	2.15	2.13
3 µM 24 h	1.73	351.21	333.89	2.16	2.16	1.62	222.3	223.03	2.09	2.13
	1.69	312.84	305.25	2.41	2.46	1.64	225.39	219.98	2.08	2.07
	1.72	356.35	345.78	2.13	2.15	1.63	225.12	221.96	2.08	2.12
3 µM 48 h	2.08	206.09	222.76	2.74	2.71	1.67	186.71	178.41	2	1.99
	2.16	263.4	255.64	3.03	3.04	1.64	183.85	186.56	2	2.02
	2.06	197.45	214.93	2.69	2.66	1.65	182.65	183.68	2.02	2.01
3 µM 72 h	2.62	252.1	243.14	3.71	3.74	1.7	148.29	146.33	2.14	2.16
	2.37	219.38	205.74	3.63	3.64	1.68	147.36	147.11	2.14	2.14
	2.52	252.23	252.59	3.51	3.49	1.71	146.69	149.88	2.17	2.17

Jurkat cells treated with Aza and DMSO were stained by FITC-anti IgG secondary antibodies after being treated with primary antibodies which are IE3 (a mouse negative control antibody), mouse anti-MHC class-I antibody (W6/32), and mouse anti-MHC class-II antibody (L243). The geometric means of fluorescent intensity of each sample were calculated as listed in the table.

Equipments and Materials

- Nuaire NU-425-400 Biological Safety Cabinet, Class II Type A/B3, 110v/60hz.

- VWR international, model 2325Waterjacket CO_2 Incubator with microprocessor controls. CO_2 is kept 5% andMili-Q/Millipore dH_2O tray is putat the bottom to keep humidity ~90%. Temperature is set on 37°C.

- Water bath: VWR Scientific Inc. 1230 is set on 37°C.

- Centrifuges: Thermo Electron Corporation IEC CL30R and Eppendorf 5415 C.

- Cell counting bench supplied with 0.4% Trypan blue (Sigma Cell Culture Reagents, Sigma chemical company St. Louis, MO 63178 USA lot#31H-4617), Microscope (MicrosOptics, IV900 series), and hemacytometer (HausserScientific,USA, Brightline hemacytometer, Improved neubauer, 0.1 μM deep).

- Flow cytometer [79]: BD FACSCALIBUR, with air-cooled argon-ion laser (15 mill watt, 488 nm). It is supplied with up to four high-performance high dynamic range photomultipliers with band pass filters: 530 nm (FITC), 585 nm (PE/PI),>670 nm (PerCP) with base unit, and optional 661 nm (APC). It has three selectable flow rates of 60, 35, and 12 μL/min.

- Others: 4°C refrigerator, -20°C refrigerator, -70°C Freezer, Liquid nitrogen tank, Ice maker, tubes [Flacon conical tubes 15 ml (352097) and 50 ml (352098), Eppendorf 1.5 ml (B146774H2209) tubes, 0.5 ml microcentrifuge tubes USA Scientific, Inc. 1605-0000, 1 ml cryovials (NaunC 375353), and 12x75 mm 5ml VWR 211-0048 culture tubes for flow cytometer], Vortex Genie-2 VWR Scientific Inc., cell culture flasks [BD Falcon with blue vented cap 24T (353108) and 75T (353136)], pipetters (Thermo electron corporation and Labsystems 0.5-10, 10-100, and 200-1000 μl), pipette aids (Drummond scientific Inc. D113289), serological pipettes (VWR), pipette tips (TipOne and Axygen), and aluminum papers.

- 5-Aza-2'Deoxycytidine [68]: Sigma Aldrich product#A3656, 5 mg. Synonym: 2'-Deoxy-5-azacytidine, 4-Amino-1-(2-deoxy-β-D-ribofuranosyl)-1,3,5-triazin-2(1H)-one, and Decitabine. Molecular Formula $C_8H_{12}N_4O_4$. Molecular Weight 228.21. Soluble in 50 mg/ml in 50% acetic acid, 50 mg/ml in DMSO, or 0.25 mg/ml in water.

WARNING: It is toxic, mutagenic, and carcinogenic and should always be handled with no skin exposed, with facial masks, and with eyes protection.

GHS07: Acute toxicity (oral, dermal, inhalation) category4, skin irritation category2, eye irritation category2, skin sensitization category1, and specific target organ toxicity-single exposure category3.

GHS08: respiratory sensitization category1, germ cell mutagenicity categories1A,1B,2, carcinogenicity categories1A,1B,2, reproductive toxicity categories1A,1B,2, specific target organ toxicity-single exposure, categories1,2, specific target organ toxicity-repeated exposure, categories1,2, and aspiration hazard, category1.

Cell culture procedure

The cell culture protective cabinet was prepared and disinfected first; the water bath was turned on and adjusted to 37°C. RPMI 1640 1x 500ml medium bottle with frozen aliquots of FBS, 200 mM glutamate, and antibiotics (penicillin/streptomycin) were placed in the water bath to be warmed to 37°C. Cells were taken from the cells stock/USM tank of liquid nitrogen. The sample was harvested to the lab on ice. The cells were thawed for less than a minute in the water bath and taken with RPMI medium, FBS, 200 mM glutamate, and antibiotics to the cabinet after being sprayed and wiped by 70% ethanol. Medium was first prepared by adding 5 ml of each FBS, glutamate, and antibiotics to a 500 ml RPMI bottle.

To wash the cells from the DMSO used for cryopreservation (which is toxic), in a 5 ml falcon tube, 5 ml of the enriched RPMI medium was added with cells. Cells were then centrifuged by 200G for 5 minutes at 4°C. The falcon tube were taken back to the cabinet after being sprayed and wiped by 70% ethanol, then the supernatant was discarded into the waste container and the cells pellet was resuspended in 10 ml of RPMI medium. 1 ml was transferred to an Eppendorf 1.5 ml tube for counting and the rest 9 ml was taken to a labeled date and name 24T cell culture flask. The cells were incubated in the incubator with 37°C temperature, 5% carbon dioxide (CO_2), and with proper humidity level. The medium was taken back to the 4°C refrigerator and the 1 ml sample of cells was taken to be counted.

Cells counting procedure

20 µl of cell suspension after being mixed by pipetting were added to 20 µl trypan blue.

The mixture was mixed well by pipetting and 20 µl was taken and loaded into a haemacytometer chamber. I counted live and dead cells in the 4 large corner squares including upper and left borders. I used the equation:

Cell concentration per ml = total number of cells counted/4 squares x 2 (dilution factor) x 10^4.

The percentage of dead cells was calculated by dividing the total dead cells number by the total cells counted.

The initial cell count showed total number of cells in 9 ml = 640,000 cells with 14% dead cells.

Replacing medium and sub culturing:

According to the American Type Cell Culture (ATCC), for Jurkat cells, it is recommended that cell concentration to be kept between 1×10^5 and 3×10^6 viable cells/ml [67].

Before taking cells out of the flask, I took a look under the microscope to the cells in the flask looking for congruency and for the presence of contaminations.

To replace medium, cells were transferred from the flasks to falcon tubes and centrifuged by 200G for 5 minutes at 4°C. The supernatant was discarded into wastes and cells pellet was resuspended by the proper volume for the target concentrations.

After six days from the initial culturing, medium was replaced and cells counted ~2.5 million with ~ 85% viability. They were cultured in 10 ml medium.

After 72h, cells counted 16 million. Here, when I resuspended the cells pellet in 16 ml medium, took 5 ml (5 million) aside to be frozen in liquid nitrogen and 3 ml (3 million) to test anti-MHC antibodies and flow cytometry protocol, and the rest 8 million were divided and cultured into four 24T flask with medium volume completed to be 10 ml (2 million cells in each) .

The 5 ml (5 million) cells were centrifuged, supernatant discarded, and cells pellet was resuspended in 2 ml of a prepared 5% w/v DMSO in culture medium (freezing medium: 250 µl DMSO to 4750 ml medium). Then 1 ml (2.5 million cells) was added to each of two labeled 1 ml-cryovials and were kept overnight in a -70°C freezer. The next day the cryovials were taken to the liquid nitrogen tank to the same place from which the original sample was taken.

After 6 days, I had four 24T flasks with ~ 10 million in each. I transferred cells from each flask to a 75T flask with 30 ml medium. After another 6 days I had ~ 100 million in each 75T flask.

Because I needed ~ 200 million for every experiment, I used the whole contents of two 75T flasks for the treatment experiments and the rest two flasks were divided into 4 flasks each of which will contain at least ~100 million viable cells every 72h to repeat the treatment experiments.

Flow cytometry

Application of CellQuest on Mac-OS X operating system

A) Instrument setting: go to Acquire>connect to cytometer, go to

cytometer>Detectors /Amps and make adjustment:

Parameter	Detector	voltage	AmpGain	Mode
P1	FSC-H	E-1	7.21	Lin
P2	SCC-H	457	1.00	Lin
P3	FL1-H (FITC)	461	1.00	Log
P4	FL2-H	550	1.00	Lin
P5	FL3-H	650	1.00	Lin
P6	FL2-A		1.00	Lin
P7	FL3-W		1.00	Lin
Leave four color box unchecked , make DDM Param= FL2				

Go to cytometer>threshold: Select Primary Param FCS and make it value=52 to make the instrument neglect particles with sizes smaller than lymphocytes (like debris or even bacteria if present). Secondary Param = none.

Then save setting by cytometer>instrument setting>save. Every time I use the flow cytometer, I open the saved setting by choosing "open" instead of "save" in the same command.

B) Document setting:

1) Draw a dot plot: from the inspector make it Acq (acquisition) plot type, and make X parameter = FSC-H 1024 and Y = SSC-H 1024.

2) Draw a histogram: from the inspector check "Log Y scale".

3)   From "File" choose "save" to save these documents and open them every next time by File>open.

C)   **Acquisition:** go to Acquire>"connect to cytometer", then uncheck setup box in the window appears. Then windows>"show browser", choose Acquisition, and make P1,P2, and P3 as mentioned before (FSC-Height, SSC-Height, and FITC respectively). Choose the director where to save you data and edit the name of the files.

Next, go to Acquire>"Acquisition & Storage", then in the appearing window appears make the following choices: Accept ALL, Acquisition will stop when 5,000 of ALL events are counted, Data file will contain ALL, and Resolution =1024. Finally go to Acquire>Counters, then Cytometer>Status. When the status is "Ready" we can start analyzing samples.

At the beginning, the cytometer's active buttons are Standby and Low. Remember to vortex the sample (500µl volume) before analyzing, move the swing arm aside, enter the sample tube, move back the swing arm below the tube, press buttons RUN and High, and choose Acquire in the software. Repeat these steps for every sample.

D)   **Maintenance:** before every time using the flow cytometer there are two things to do :

1) Check the sheath fluid and waste tanks (when to open the tanks switch the button there to RELEASE from PRESSURIZE and to PRESSURIZE after putting the tanks back) and check for air bubbles after filling them (by closing the valve to shift the fluids to the filter container).

2) Washing the flow cytometer by flowing (RUN-HIGH) 70% bleach for 1 minute with swing arm aside and 5 minutes with swing arm below the tube. Then the same way with the Contrad70 cleaner and finally with dH_2O (Mili-Q) but with 3 and 10 minutes instead of 1 and 5 minutes respectively.