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Effects of Novel Synthetic Nucleosides as Anti-Tumor Agents on Human Acute Promyelocytic Leukemia Cell Line (HL-60)

Salma Awad Merghani Mahmoud

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By
Salma Awad Merghani Mahmoud

A thesis
Submitted to

United Arab Emirates University
In partial fulfillment of the requirements
For the Degree of M.Sc. in Environmental Sciences

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Supervisors

<table>
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<tr>
<th>Dr. Ahmed Al-Marzouqi</th>
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2005
The Thesis of Salma Awad Merghani Mahmoud for the Degree of Master of Science in Environmental is approved.

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United Arab Emirates University

2005/2006
Dedications

To my parents, for all their support and guidance throughout my life and for putting up with me during my Master’s years.

And to Suha, Bayan and Mariem my soulmates, my inspiration and my friends.
Acknowledgment

All praise be to Allah, the lord of the worlds, for helping me to complete this work.

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Thanks to my... may I say soulmates Suha Al-Araimi, Bayan Abdulrahman and Mariem Abdulrahman, whose advice affected me more than they'll ever know.
Abstract

Nucleosides and their analogs are considered a clinically proven class of therapeutic agents possessing anticancer and antiviral activity. Several trifluoromethyl-substituted pyrazole N-nucleosides (coded NIA, NIIA and NIIIA) and their nucleobases were synthesized and tested for the ability to induce apoptosis in acute human promyelocytic cell line (HL-60). The growth and proliferation of HL-60 was more effectively inhibited by NIA, NIIA and their nucleobases compared to NIIIA and its nucleobase. In addition, DNA fragmentation was detected in a concentration-dependent manner as a result of nucleosides treatment. A caspase-3-dependent apoptosis was observed based on the Western blot analysis of poly-ADP-ribose polymerase (PARP) and caspase-3. Nucleosides and nucleobases also trigger the release of cytochrome-c from the mitochondria by disruption of mitochondrial membrane potential and ROS formation. Furthermore, the use of zDEVD-fmk (caspase-3 inhibitor) and zLEHD-fmk (caspase-9 inhibitor) resulted in an inhibition of the activity of caspase-3 and 9, accompanied with no change in the activity of caspase-8 after the use of zIETD-fmk (caspase-8 inhibitor). These findings implicate the involvement of the caspase-9-dependent mitochondrial pathway. Treatment with nucleosides also resulted in a concentration-dependent upregulation and translocation of the proapoptotic molecule Bax, increased expression of other proapoptotic proteins Bad, Bak, decreased expression of antiapoptotic proteins (i.e. Bcl-2 and Bcl-xL), and enhancement of p53 expression. Moreover, treatment with these agents resulted in accumulation of cells in the G0-G1 phase of the cell cycle, indicating the degradation of the cellular DNA. Interestingly, these nucleosides were found to posses anti-histone deacetyl transferase(s) (HDACs) potential, which is an exciting turn.
in cancer therapy. This conclusion was based on the observed decrease in the expressed level of HDAC-1, -2 and -3. Our results suggest that these novel synthetic nucleosides can induce apoptosis via the mitochondrial pathway, and therefore they could be considered as candidate anti-tumor agents.

Key words: Nucleosides, Trifluoromethyl analogue, HL-60, anti-cancer, apoptosis, mitochondria.
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<tr>
<td>AIF</td>
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<td>Apoptotic protease-activating factor-1</td>
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<td>Ara-C</td>
<td>Arabinosylcytosine or cytarabine</td>
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<td>Bid</td>
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<td>Cysteine-dependent aspartate-directed proteases</td>
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<td>2',7'-Dichlorofluorescein diacetate</td>
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<td>Deoxribonucleic acid</td>
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<td>MDR</td>
<td>Multidrug Resistance</td>
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<td>Full Name</td>
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<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
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<tr>
<td>PCD</td>
<td>Programmed cell death (apoptosis)</td>
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<td>PI</td>
<td>Propidium iodide</td>
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<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>TRAIL</td>
<td>TNF-related apoptosis inducing ligand</td>
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<td>Benzyloxy-Asp-Glu-Val-Asp- fluoromethylketone</td>
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Chapter I:

Introduction and literature review
1. Introduction:

In the recent years cancer has become the world's leading health problem. In particular, there has been an increase of cancer incidence in the Gulf countries (1). Figures varying from one region to another depending on the specific type of the cancer (1). Therefore, the growing need for quality cancer treatment through research has led to the establishment of various cancer therapy strategies and research centers in the world.

Finding a cure or a remedy for cancer has become a challenge for all. The established cancer therapies can delay the spread of the cancer, or in some cases destroy it completely. The outcome depends on the detection of this illness in its early stages, and also on the genetic susceptibility of the patients, meaning that some genes may be in regions of chromosomes which are more susceptible to genetic damage/change or may contain sequences which are more likely to be altered by spontaneous mutations. Therefore, it could be stated that cancer is a genetic disease in which an uncontrollable cell division takes place, resulting in the disruption of the homeostasis between cell proliferation and cell death (2).

The term chemotherapy refers to the use of a wide range of drugs that work by killing dividing cells. Since cancer cells have lost most of the regulatory functions present in normal cells, they will continue to divide when other cells do not (2). This feature makes cancer cells more susceptible to cytotoxic agents. The chemotherapeutic agents are either naturally occurring compounds that have been identified in various plants or synthetic chemicals (2). They can cause cell death in a variety of ways, and some of these chemotherapeutic drugs have been briefly described below (2).

1) Antimetabolites: Drugs that interfere with the formation of key biomolecules within the cell such as nucleosides, which are part of the building
blocks of DNA (nucleotides). These drugs ultimately interfere with DNA replication and therefore cell division.

2) Genotoxic Drugs: Drugs that damage DNA. By causing DNA damage, these agents interfere with DNA replication, and normal process of cell division.

3) Spindle Inhibitors: which prevent proper cell division by interfering with the cytoskeletal components that enable one cell to divide into two.

4) Other Chemotherapy Agents: These agents inhibit cell division by mechanisms that are not covered in the three categories listed above.

Nucleosides and their analogues, which belong to the first category, are considered a clinically proven class of chemotherapeutic agents possessing anticancer and antiviral activity (3). The effectiveness of this class of chemotherapeutic agents or their mode of action (toxicity) results from their ability to cause covalent modification of DNA. Thus, they become incorporated into the genetic material during DNA replication and destroy it by preventing the fidelity of repair systems within the DNA (3), which in turn results in the death of the cell (apoptosis). In other words they act as antimetabolites by competing with the natural physiological nucleosides within the body (3).

There are several classes of nucleosides, the most important of which are the fluorinated compounds containing trifluoromethyl group (CF₃). These compounds have several medical applications, as they act as antiviral, antifungal and antiinflammatory agents (4). The presence of trifluoromethyl groups (CF₃) results in alteration of the chemical and pharmaceutical properties, for example increasing their cytotoxic effect within the cells (4), due to the presence of the fluorine.

Apoptosis or programmed cell death (PCD) is a natural mechanism in the body to maintain its integrity and functionality by destroying non-desirable and
harmful cells (2). Most physiological cell death proceeds by apoptosis, which ensure cell removal without affecting neighboring cells or causing an inflammation.
1. Basic medical chemistry of nucleosides:

Chemical analogues of nucleosides have been frequent subjects of research in medicinal chemistry due to their marked activity in several fields of therapy including anticancer and antiviral analogues \((3)\). They also might act as cytotoxic compounds or immunosuppressive drugs \((3)\). The cytotoxic class comprises the anti-cancer nucleosides, which are antimetabolites that inhibit the synthesis of nucleic acids \((3, 5)\). As they are hydrophilic (water-loving) compounds, they require special transporter proteins to enter the cells. Once inside the cells they are activated to become triphosphate derivatives, which can incorporate within the DNA or RNA and damage them by interfering with the enzymes responsible for their synthesis such as the polymerases. This results in damaging of the genetic component of the cell and eventually leading to apoptosis \((3, 5, 6)\).

Natural nucleosides and their synthetic models are considered one of the most important metabolites of the cell \((3)\). The nucleosides found in cells are derivatives of the heterocyclic, highly basic compounds such as purine and pyrimidine. Their chemical basicity (alkalinity) has given them the common term "bases". There are five major nucleobases found within the cells (see Fig.1.1), that primarily function as the building blocks of the genetic material, commonly referred to as DNA and RNA.

Nucleoside analogues are synthetic compounds that are structurally similar to natural nucleosides. Each of these are small molecules that effectively target several enzymes such as kinases and polymerases \((3)\). Naturally occurring nucleosides are modified in cells to generate derivatives, termed nucleotides that are utilized by polymerases as the basic building blocks of DNA and RNA genetic material.

Nucleosides also have other physiological roles in processes such as coronary blood flow, myocardial \(O_2\) balance, inflammation and neurotransmission \((5)\). Nucleosides are either derived from the diet or produced by tissues through \textit{de novo}
biosynthetic pathways \(^{(5,7)}\). Cells deficient in *de novo* biosynthetic pathways are dependent on salvage pathways of nucleosides from the extracellular environment, which includes cells such as erythrocytes, leukocytes, bone marrow cells, some brain cells, primitive progenitor and stem cells \(^{(3)}\).

![Diagram of nucleobases](image)

**Fig.1.1**: Major nucleobases found in the cells: Purine analogues include guanine (G) and adenine (A), Pyrimidine analogues include cytosine (C), thymine (T) (found in DNA), and uracil (U) (found in the RNA).

### 1.1. Chemical structure of the physiological nucleosides:

The main idea behind the concept of synthetic nucleoside analogues is their ability to compete with the natural ones. In order to understand their mechanism of action, a brief overview of the physiologically important nucleosides is discussed below. When a sugar bonds together with a nitrogenous base, which are two of the three components of the building blocks of nucleic acids (nucleotides), a structure
known as a "nucleoside" is generated. There are five nitrogenous bases that are found in DNA and RNA (although uracil is found ONLY in RNA). These five bases are divided into two categories; purine and pyrimidine based upon their molecular structure. The derivatives of purine are called adenine (A) and guanine (G), and the derivatives of pyrimidine are called thymine (T), cytosine (C) and uracil (U) (see Fig. 1.2)\(^{(7)}\).

![Diagram of nucleoside and nucleotide structures](image)

**Fig. 1.2**: Schematic view of the nucleoside structure. Nitrogen bases with either purine or pyrimidine rings that are associated with pentose sugar moieties are called nucleosides. If a phosphate group is attached to the nucleosides it forms a nucleotide.

The pyrimidine ring system is planar, whereas the purine rings have some pucker. The plain bases have low solubility, but their nucleoside form has higher solubility due to the additional polar groups\(^{(7)}\). Purines and pyrimidines can undergo a keto-enol tautomeric shift. The keto tautomer is called a lactam ring, and is the
predominant form at neutral pH (7). The nucleosides are coupled either to D-ribose or to 2'-deoxy-D-ribose through a β-N-glycosidic bond between the anomeric carbon of the ribose and the N9 of a purine or N1 of a pyrimidine. That is why nucleosides are nitrogen bases either with purine or pyrimidine rings that are associated with sugar moieties, while nucleotides are nucleosides that are bound to a phosphate group (7).

1.1.1. Mode of action of nucleosides:

In order to understand the antimetabolitic action of nucleosides, it is necessary to briefly discuss the processes that are targeted by these agents. The term metabolism refers to the complex chemical reactions that take place in the body (8). A metabolite is a general term for the organic compounds that are synthesized, recycled, or broken down in cells (8). The materials that provide the key metabolites enter the body as food. These compounds can be degraded into simpler structures that can be re-used in our cells. Examples of such simple structures include vitamins and amino acids. Metabolites that are the end-products of a process or pathway may be excreted from the body. An example is urea, the end-product of protein metabolism, excreted from the body as a component of urine.

Antimetabolites are structurally similar to metabolites, but they cannot be used by the body in a productive manner (8). In the cell, antimetabolites are not distinguished from the metabolites they resemble, and are processed within the cell in a manner analogous to the normal compounds. The presence of the 'decoy' antimetabolites prevents the cells from carrying out vital functions and the cells are unable to grow and survive (22). Nucleoside analogues therefore are molecules that are chemically modified versions of one of the natural nucleosides. Mimicking the role of natural nucleosides, therapeutic nucleoside drugs are generally incorporated by
endogenous polymerases (3,22), and this event impairs either the synthesis or the functionality of the resultant genome and therefore suppresses replication (8). Many of the antimetabolites used in the treatment of cancer interfere with the production of the nucleic acids, RNA and DNA. If new DNA cannot be made, cells are unable to divide.

1.1.2. Transportation and bioavailability of nucleosides:

Nucleosides are involved in signaling in a number of physiologic systems (9,10). Plasma membrane transport of nucleosides is mediated by equilibrative and concentrative nucleoside transporters, which may have specificity for purines or pyrimidines (11). The two families, equilibrative and concentrative, differ in their mode of transport, substrate selectivity, and sensitivity to inhibitors (9-11). Equilibrative transporters mediate the influx and efflux of both purine and pyrimidine nucleosides down a concentration gradient (10). The second family of nucleoside transporters mediates the sodium-dependent, concentrative uptake of nucleosides (10). These transporters are driven by an electrochemical ion gradient and under physiological conditions mediate only the influx of nucleoside analogues (10). In mammalian cells, nucleosides are transported through the membrane by both equilibrative and Na+-dependent (concentrative) nucleoside transporters. Successful transport of the nucleosides through the membrane direct the cellular uptake of many therapeutic nucleosides used in the treatment of cancer, viral infections and cardiac arrhythmias (9-11). This means that the efficient transportation and delivery of the nucleoside into the cell contribute indirectly to its cytotoxicity and therefore its therapeutic potential, which indicates that the better the transportation the more potential the nucleoside may exert. Nucleoside transportation is also vital for nucleotide synthesis by the
salvage pathways (nucleotide synthesis by other cells such as brain cells). Several human nucleoside transporters have been identified, cloned, and characterized (3). There is emerging evidence that the abundance and tissue distribution of nucleoside transport proteins contributes, in part, to the cellular specificity of and sensitivity to nucleoside analogues (9,10). However, each of these compounds also possesses unique drug-target interactions that help explain their differences in activity in various diseases. For example, the cytotoxic effects of the purine analogue Fludarabine (a chemotherapeutic agent) on quiescent cells may be explained by interaction with targets involving DNA repair rather than replication, combined with direct or indirect effects on mitochondria (10,11). The biologically active form of most purine or pyrimidine analogues is the nucleoside 5'-mono, di- or triphosphate (phosphorylated form) (10). However, the nucleoside (unphosphorylated form) is administered most often, because of the ease with which it penetrates cells by facilitated transport, specially in the case of cancer and HIV treatments (12,13). Tumor cells and virally infected cells are often more permeable to nucleosides and their analogues than normal cells, which could provide a therapeutic advantage (3,11,13,14). A drawback of this is that many nucleoside derivatives fail to exhibit significant antiviral or antitumor activity because they are not phosphorylated by cellular enzymes to the active nucleotide form (3,10).

Thus modulating nucleoside transport processes has been proposed as a means of enhancing the efficacy of the nucleoside analogues (9,10). Pharmacologic inhibitors of transporters are available, such as hENT1 and hENT2, and they are capable of protecting bone marrow progenitors from the cytotoxic effects of nucleoside analogues (disruption of replication and cell cycle processes) (11). However, the degree to which transport inhibitors might also protect malignant cells is unknown, although
inhibitor-insensitive nucleoside transporters have been identified in cultured cancer cells \(^{(11)}\). The cellular regulation of nucleoside transport is now an area of intensive study, and has the potential to allow selective upregulation of nucleosides analogues uptake in malignant clones. For example; in acute promyelocytic cell line (HL-60) the pathways involved in the regulation of nucleoside transporters depend on differentiation status (e.g. granulocytic or monocytic), and this may provide insights into new approaches to modulate the uptake of anticancer nucleosides and increase the efficacy of these drugs in leukemias \(^{(3,10,11)}\). There could be considerable therapeutic potential for nucleoside analogues that can penetrate the tumor cell membranes and that are resistant to enzymatic hydrolysis and can be incorporated into DNA or RNA.

1.2. Purine and pyrimidine analogues:

There are several different cellular targets for antimetabolites. Some common classes of antimetabolites are:

- Pyrimidine Antagonists
- Purine Antagonists

The pyrimidine and purine antagonists act to block the synthesis of pyrimidine and purine containing nucleotides. The drugs used to block the construction of these nucleotides have structures that are similar to the natural compounds. They may exert their effects at different steps in a pathway and may directly inhibit crucial enzymes. These antagonists function by inhibiting DNA synthesis in two different ways: 1) They can compete with the production of the natural pyrimidine and purine containing nucleosides \(^{(11,16,17)}\). If a cell does not have sufficient amounts of them, the DNA
synthesis is halted and the cell cannot divide \(^\text{10,11}\). 2) The antimetabolites may be incorporated into a growing DNA chain and lead to termination of the chain elongation process \(^\text{3,8,15}\). Thus the presence of the inhibitor (nucleoside analogue) is thought to interfere with further cell division \(^\text{10,11,14,18}\). Example of pyrimidine nucleosides are Cytarabin (ara-C), and Gemcitabine, whereas the most common purine nucleosides are Fludrabine and Cladribine (see Fig.1.3) \(^\text{3,8}\).

![Chemical structures of pyrimidine and purine nucleosides](image)

**Fig.1.3:** Pyrimidine and purine analogues used in chemotherapy. Cytarabin (ara-C) and Gemcitabine are pyrimidine analogues, whereas Fludrabine and Cladribine are purine analogues.

1.2.1. **Therapeutic roles of purine and pyrimidine analogues:**

The cellular specificity of and sensitivity to the nucleosides depends on the abundance of their distribution in the tissues \(^\text{3,5}\), and their use in treatment is due to the unique drug-target interactions \(^\text{3}\). For example, purine analogues can be used in the treatment of neurotoxicity, vomiting, septicemia (blood poisoning), and HIV therapy, whereas pyrimidine analogues are used as a cure for diarrhea, hand-foot syndrome, and some leukemia cases \(^\text{3}\).
Cytarabine or Arabinosylcytosine (Cytosar-U®, ara-C) is an antimetabolite that acts as a pyrimidine antagonist \(^{(3,19,20)}\). It is thought that its activity is primarily by interrupting DNA synthesis. Malignancies for which Cytarabine is used include acute non-lymphocytic leukemia, acute lymphocytic leukemia and chronic myelocytic leukemia. Cytarabine is administered as an infusion or as an injection under the skin. Common side effects include bone marrow suppression, anorexia, and oral/anal inflammation or ulceration \(^{(3,21)}\). Because cytarabine is a suppressor of bone marrow activity, it is important to perform blood tests to monitor blood cell and platelet counts during the treatment \(^{(3,18)}\). On the other hand, Gemcitabine is effective against a wide range of solid tumors and it is a potent radiation sensitizer \(^{(3,15)}\), as well as a good substrate for nucleoside transporters \(^{(3)}\). Common side effects include: bone marrow suppression, weakness, fever, nausea and vomiting. It is suggested that the toxic effect of this analogue (radiosensitization) depends on perturbing nucleotide pools producing misrepair after radiation \(^{(22)}\). Previous studies tried to determine whether Gemcitabine-mediated radiosensitization induced apoptosis, and they have found that apoptosis in Gemcitabine-mediated radiosensitization is dependent upon the cell line used and the dose administered \(^{(22)}\). However their investigation was limited to the late apoptotic events (caspase-3 activation and morphological changes) \(^{(22)}\).

Fludarabine leads to termination of the chain elongation process mediated by DNA polymerase by incorporating into the DNA \(^{(3,21)}\). It also can impair DNA replication by inhibiting ribonucleotide reductase \(^{(23)}\). It is suggested that this analogue can induce apoptosis via activation of caspase-3 and -9 \(^{(21)}\). Fludarabine is used to treat chronic B-cell lymphocytic leukemia and is administered as an injection. Adverse effects are similar to Gemcitabine. Nucleoside analogues in general can cause several side effects. Frequent complaints include fatigue, headache and a
variety of gastrointestinal problems such as abdominal discomfort, nausea, vomiting and diarrhea \(^{3}\). They are eliminated mainly by renal excretion and do not interact with drugs that are metabolized by hepatic enzymes, therefore they will not be destroyed before reaching their targets by the detoxifying action of the liver \(^{3}\).

1.3. Synthesis of trifluoromethyl-nucleosides:

A wide range of trifluoromethyl-therapeutic agents exhibit a variety of biochemical properties \(^{3}\). The introduction of fluorine in general and trifluoromethyl groups into organic compounds allows modification of their chemical, physical, and pharmaceutical properties \(^{4,24}\). The preparation of fluorine heterocycles is well documented. There are two general protocols \(^{4,24}\): 1) fluorination of an existing heterocyclic ring including functional group transformation, for example transformation of a carboxy moiety to a trifluoromethyl group, and 2) trifluoromethylation of heterocycles by means of trifluoroacetonitrileoxide or trifluoroacylketenes. The disadvantage of these methods is that they might be dangerous during the preparation, because the reagents used are highly toxic. Therefore new approaches have been developed for the synthesis of trifluoromethylated \((\text{CF}_3)\) heterocyclic compounds \(^{4}\). However the search for new reagents and methods for the synthesis of CF\(_3\)-containing compounds is still a quest in organic synthesis. One simple and useful approach to the syntheses of trifluoromethyl-containing heterocycles is a synthesis based on the utilization of unsaturated ketones with a trifluoromethyl group \(^{24}\). Trifluoromethyl-1,3-dicarbonyl compounds have been proven to be versatile building blocks for a wide variety of trifluoromethyl-substituted heterocycles. These include pyrazoles, isoxazoles thiazoles, pyridines, pyrimidines and pyrones \(^{4}\).
1.3.1. The potential of trifluoromethyl-nucleosides in medicine:

Fluorinated molecules particularly fluorinated heterocycles are the focus of much interest in modern medicinal chemistry, because they exhibit a wide range of biological effects \(^{(4)}\). For example, trifluoromethyl-substituted compounds have been reported to possess biological activities such as herbicides, fungicides, and inhibitors of the platelet aggregation \(^{(4)}\). Recently, much attention has been focused on pyrazoles as antiviral and anticancer agents after the discovery of the pyrazole C-glycoside pyrazofurin, since they were used as tools for studying conformational requirements of oligonucleotides to enhance the understanding of biochemical pathways/processes and the development of therapeutic agents (e.g. antisense and antiviral) \(^{(4)}\). Nucleosides are becoming an increasingly important class of agents in the development, scale-up, and manufacture of new biotherapeutics.

2. Nucleosides and cancer:

Despite promising leads in the search for new chemotherapeutic agents, there remains an urgent need to develop more effective and less toxic drugs. Nucleosides and their corresponding nucleobases are the fundamental building blocks of many biological systems and as a result, synthetic analogues have been investigated due to their inherent structural resemblance to the naturally occurring nucleosides and nucleobases. Due to this intertwined relationship between physiologic and synthetic nucleosides, they (the synthetic analogues) could be used as a chemotherapeutic approach to treating cancer. Most chemotherapeutic nucleosides have been used in leukemia treatment \(^{(3,5)}\).
2.1. Nature of leukemia and possible diagnosis:

Leukemia is a blood cancer in which blood cells mature abnormally or incompletely \(^{(25)}\). These abnormal or immature cells accumulate in the body, and are unable to carry out the functions of normal blood cells \(^{(25, 26)}\). Since leukemic cells are abnormal, they cannot function properly; for example, they cannot help the body fight infections \(^{(25)}\). For this reason, people with leukemia often get infections and have fevers. Also, people with leukemia often have less than the normal amount of healthy red blood cells and platelets. As a result, there are not enough red blood cells to carry oxygen through the body. With this condition, called anemia, patients may look pale and feel weak and tired. When there are not enough platelets, patients bleed and bruise easily \(^{(26, 27)}\). Normal blood contains three major groups of cells: white blood cells, red blood cells, and platelets. All three types develop from one immature cell type called stem cells in a process called hematopoiesis \(^{(27)}\). Leukemia is classified by how quickly it progresses. Acute leukemia is fast-growing and can over run the body within a few weeks or months \(^{(26)}\). By contrast, chronic leukemia is slow-growing and progressively worsens over years \(^{(25)}\). The blood-forming (hematopoietic) cells of acute leukemia remain in an immature state, so they reproduce and accumulate very rapidly \(^{(25-27)}\). Therefore, acute leukemia needs to be treated immediately, otherwise the disease may be fatal within a few months \(^{(26, 27)}\). On the other hand, in chronic leukemia, the blood-forming cells eventually mature, or differentiate, but they are not normal, as they remain in the bloodstream much longer than normal white blood cells, and they are unable to combat infection well \(^{(2-27)}\). Moreover leukemia also is classified according to the type of white blood cell that is multiplying - that is, lymphocytes (immune system cells), granulocytes (bacteria-destroying cells), or monocytes (macrophage-forming cells) \(^{(27)}\). If the abnormal white blood cells are
primarily granulocytes or monocytes, the leukemia is categorized as myelogenous, or myeloid, leukemia \(^{(26,27)}\). On the other hand, if the abnormal blood cells arise from bone marrow lymphocytes, the cancer is called lymphocytic leukemia \(^{(25)}\). There are over a dozen different types of leukemia, but four types occur most frequently. These classifications are based upon whether the leukemia is acute versus chronic and myelogenous versus lymphocytic, that is \(^{(25-27)}\):

- **Acute lymphocytic leukemia (ALL):** ALL - also known as acute lymphoblastic leukemia - is a malignant disease caused by the abnormal growth and development of early nongranular white blood cells, or lymphocytes \(^{(25)}\).

- **Chronic lymphocytic leukemia (CLL):** CLL is thought to result from the gradual accumulation of mature, long-lived lymphocytes \(^{(25)}\). Therefore, this cancer is caused not so much by overgrowth as it is by the extreme longevity and build-up of malignant cells \(^{(25,26)}\). Although the rate of accumulation varies among individuals, the extensive tumor burden eventually causes complications in all CLL patients \(^{(27)}\).

- **Acute myelogenous (granulocytic) leukemia (AML):** AML begins with abnormalities in the bone marrow blast cells that develop to form granulocytes, the white blood cells that contain small particles, or granules \(^{(25-27)}\). The AML blasts do not mature, and they become too numerous in the blood and bone marrow \(^{(25)}\).

- **Chronic myelogenous (granulocytic) leukemia (CML):** CML is known as a myeloproliferative disorder - that is, it is a disease in which bone marrow cells proliferate (multiply) outside of the bone marrow tissue \(^{(26,27)}\).
2.1.1. Anti-leukemic analogues:

Treatment of leukemia continues to advance. Improved understanding of the basic disease process as well as the availability of better drugs and supportive care are leading to more remissions. Treatment is divided into two categories: 1) Supportive, including blood transfusions to relieve the anemia, platelet transfusions to help prevent bleeding, and antibiotics to control infections \(^{15, 25-27}\). 2) Chemotherapy treatment with chemical drugs \(^{17, 25-27}\) such as Ara-C, which is one of the oldest chemotherapy drugs that has been commonly used in treatment of acute myeloid leukemia, chronic myeloid leukemia, acute lymphoid leukemia, and lymphomas \(^{1,23}\). As drugs, nucleoside analogues generally offer high selectivity, excellent potency, long duration of action and potential for relatively straightforward scale-up and manufacture \(^3\). As a result, nucleoside and nucleoside analogues are particularly well-suited for the extended treatment of such maladies.

3. Mechanism(s) of anti-cancer effect of synthetic nucleosides:

Several decades of genetic and molecular study have revealed enormous insights into the mechanistic underpinnings of cancer \(^{2,18}\). From the identification of dominantly acting oncogenes to the signaling pathways which modulate the cell cycle, our understanding of the machinery of cell cycle progression as well as the regulatory circuits which control it have never been so detailed \(^{12,18}\). However the translation of these discoveries into improved therapeutic approaches has been slow. Therefore understanding the mechanism of action of the anti-tumorigenic effects of these analogues is vital for the prospect of unraveling therapeutic strategies for cancer. The current understanding of how nucleoside analogues exert their anti-cancer potential is
that, if they destroy cancerous cells, they are achieving this either by the process of programmed cell death or the disruption of cell cycle progression.

3.1. Nucleosides and apoptosis:

Recent investigations indicate that widely used chemotherapeutic agents induce apoptosis in susceptible cells \(^{(28,29)}\). For example, treatment with the topoisomerase II inhibitor, Etoposide, results in morphological and biochemical evidence of apoptosis in a variety of cell types \(^{(28)}\). The events occurring between stabilization of topoisomerase II-DNA complexes and initiation of apoptosis are unclear, but subsequent events in all of these cell types include cleavage of DNA\(^{(29-31)}\) and activation of a family of proteins known as cysteine-dependent aspartate-directed proteases \(^{(32)}\) called caspases.

3.1.1. Definition of apoptosis:

Apoptosis, or programmed cell death (PCD), is a normal process in the development and health of multicellular organisms \(^{(2,32,33)}\). It occurs in response to a variety of stimuli \(^{(33)}\). Apoptosis is distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells followed by inflammatory responses that can potentially lead to serious health problems \(^{(33)}\). During apoptosis caspases are activated in a cascade-fashion, eventually leading to the activation of the "effector caspases", such as caspase-3 and caspase-6. These caspases are responsible for the destruction of the key cellular proteins such as DNA repair enzymes that leads to the typical morphological changes observed in cells undergoing apoptosis\(^{(33,34)}\).
### 3.1.2. Apoptotic pathways:

There are several mechanisms through which apoptosis can be induced (see Fig.1.4). The preference of one mechanism over another depends on a number of factors such as the type and the strength of the stimulus, the expression of pro- and anti-apoptotic proteins (e.g. the Bcl-2 proteins or the Inhibitor of Apoptosis Proteins (IAPs)), and the stage of the cell cycle (35-37).

Fig.1.4: Schematic representation of the apoptotic pathways, featuring the main markers involved in each pathway: Intrinsic pathway (mitochondrial pathway), and extrinsic pathway (death-receptor pathway).
In some cases apoptosis is initiated following intrinsic signals that are produced in response to cellular stress (34, 38). Cellular stress may occur from exposure to radiation or chemicals, or to viral infections. In other cases, the apoptotic stimuli comprise extrinsic signals such as the binding of death inducing ligands to cell surface receptors (39). The latter occurs for example when T-cells recognize damaged or virus infected cells and initiate apoptosis in order to prevent damaged cells from becoming neoplastic (cancerous) or virus-infected cells from spreading the infection (39).

3.1.2.1. Mitochondrial pathway:

Mitochondria have the ability to promote apoptosis through the release of cytochrome c (Cyt c), which together with apoptotic protease-activating factor-1 (Apaf-1) and ATP forms a complex with pro-caspase-9 (40). Formation of this complex leads to the activation of caspase-9 and subsequent induction of apoptosis (40). The factors responsible for the release of Cyt c from the mitochondria are still completely not understood. Anti-apoptotic members of the Bcl-2 family of proteins, such as Bcl-2 and Bcl-xl, are located in the outer mitochondrial membrane and act to promote cell survival (34, 40). On the other hand, pro-apoptotic members of the Bcl-2 family, such as Bad and Bax, also mediate their effects though the mitochondria, either by interacting with Bcl-2 and Bcl-xl, or through direct interactions with the mitochondrial membrane (34, 40, 41). It has been suggested that Bax can form a transmembrane pore across the outer mitochondrial membrane, leading to loss of membrane potential and efflux of Cyt c and apoptosis inducing factor (AIF) (34, 40, 41). It is thought that Bcl-2 and Bcl-xl act to prevent this pore formation and that heterodimerization of Bax or Bad with Bcl-2 or Bcl-xl inhibits their protective effects (40, 42).
3.1.2.2. Receptor mediated pathway:

Death receptors are cell surface receptors that induce apoptosis triggered by the binding of specific ligands (39). Death receptors belong to the tumor necrosis factor (TNF) gene superfamily and generally can have several functions other than initiating apoptosis (39). The best characterized of the death receptors are CD95 (Fas), TNFR1 (TNF receptor-1) and the TRAIL (TNF-related apoptosis inducing ligand) receptors DR4 and DR5 (34,39). These receptors can mediate apoptosis by activation of caspase-8, which in turn activates caspase-3, and leads to apoptosis (39) (Fig. 1.4). However, apoptotic programs cannot simply be described as two parallel programs converging on a common caspase machinery. First, genetic studies with caspase-deficient mice demonstrate that the requirement for different death effector molecules during apoptosis is highly variable, being cell-type and stimulus specific (43,44). Second, a large degree of 'cross-talk' exists between pathways. For example, p53 can transactivate genes encoding death receptors (44). Deficiencies in regulation of apoptosis contribute to well-known pathologies, such as autoimmune diseases, cancer, and viral infections (45).

3.1.3. Apoptosis and cancer therapy:

Several anticancer agents such as daunorubicin (DAU), may initiate apoptosis at low concentrations to kill tumor cells (46,47). Those cells become damaged irreversibly and exhibit biochemical and morphological changes, including mitochondrial alteration, cytoplasmic and nuclear condensation, and DNA degradation into oligonucleosomal fragments (46). The various apoptotic signals induced by different anticancer drugs through different mechanisms may converge on mitochondria resulting in the release of apoptogenic factors and activate caspase
cascade that is involved in the release of Cyt c from mitochondria \(^{34, 47}\). Effector caspases, including caspase-3, may be activated via mitochondria-dependent or -independent pathways \(^{34, 48}\). One of the current theories exploring the release of Cyt c suggests the involvement of pores within the mitochondrial outer membrane formed by proapoptotic members of the Bcl-2 family (e.g. Bid, Bax, and Bad) \(^{34}\). These mechanisms mediate the passage of unbound Cyt c through the mitochondrial outer membrane. However, the mechanism by which Cyt c dissociates from the inner membrane is less understood \(^{34}\).

The process by which cancer spreads from a primary to a secondary site (metastasis) is the major cause of concern in cancer. This process is induced when very few cells leave the primary tumor to give rise to secondary tumors \(^{49}\). Metastatic cells are subjected to various apoptotic stimuli, and in order for the process to succeed, these cells which undergo various genetic changes, must decrease their sensitivity to apoptotic stimuli \(^{49}\). Therefore, if the resistance to apoptotic stimuli could be abolished, or in other words the apoptotic sensitivity could be increased, and the effective weapon against metastatic cancer would be unleashed.

3.2. Nucleosides, the cell cycle and chromatin modification; a quest for a connection:

Proliferation and programmed cell death (apoptosis) exert a concerted action in modeling the organism during normal development and in maintaining tissue homeostasis \(^{2,12,21}\). Both cell cycle progression and apoptosis biochemistry and molecular biology have been widely studied and characterized during the last ten years. Now, it is evident that each cell is able to integrate both extra- and intracellular survival and death signals thereby controlling its own growth rate or, when harmful
signals prevail, inducing its self-destruction \(^{12,21}\). This is achieved mainly because of multiple interactions with the p53 pathway, which guards against genomic instability by inducing both arrest of the cell cycle and apoptosis \(^{21,30}\). The p53 tumor suppressor is also activated by phosphorylation and acetylation and interacts physically with histone deacetyltransferases (HDACs) \(^{50}\). A growing body of literature has established the role of HDACs in chromatin remodeling and gene expression. Furthermore, HDAC inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects. Thus, inhibition of HDACs can be considered a new strategy for anticancer treatment.
Chapter II:

Aims and objectives
II. Aims and objectives:

1. Thesis objectives:

The need for the development of new anticancer drugs is evident and of great importance for the future. Today cancer chemotherapy depends on a rather small panel of compounds. With the enormous body of knowledge which has been accumulated in the last few years in chemistry, cellular and molecular biology, it has become possible to develop novel molecules which, for example, exert their cytotoxic actions on cell membranes and on signal transduction pathways. Thus, synthetic nucleosides which possess anti-tumor activity are gaining increased importance as potential therapeutic agents.

The aim of the present study was to find compound(s) that can destroy cancerous cells with minute or no effect on the normal cells, utilizing natural mechanisms such as apoptosis. Thus the effects of four different synthetic nucleosides (coded: NIA, NIIA, NIIA and NIB) and their nucleobases (N-base1, N-base2 and N-base3) were evaluated. The structures of these new nucleosides were established using the most advanced spectroscopic techniques to insure that all nucleosides had the correct structure with high purity.

To address their cytotoxic effects, we concentrated on studying the ability of these analogues to induce apoptosis in human acute promyelocytic leukemia cell line (HL-60). In addition, some blood samples (normal and leukemic) obtained from leukemia Tawam Hospital were analyzed to observe the effect of these nucleosides on them. Moreover, further investigation was carried out to determine the apoptotic pathway orchestrating this action. Another focus area was cell cycle regulation, chromatin modification and structure activity relationship (SAR) for the biological enhancement of these compounds.
1.1. Study model:

The HL-60 cell line was established in 1977 from a patient with acute myeloid leukemia \(^{(51)}\). The cells largely resemble promyelocytes but can be induced to differentiate \textit{in vitro}. Some reagents cause HL-60 cells to differentiate to granulocyte-like cells, others to monocyte/macrophage-like cells \(^{(51)}\).

Resistance of cancerous cells to cytotoxic agents is one of the major challenges in the face of successful chemotherapy \(^{(52,53)}\). Multidrug Resistance (MDR) is a relevant mechanism of primary or acquired resistance in human leukemias and solid tumors \(^{(52,32)}\). Hence we have chosen HL-60 because it is considered a good model to study anti-tumor agents \(^{(54)}\), and other HL-60 strains that have Multi-Drug Resistance (MDR) characteristic are available, which will open a door for further investigation about whether our novel nucleosides contain improved bioactivity to potentiate the action of antimetabolites and circumvent MDR. Another reason for choosing HL-60 cell line is the annual increase of new cases of cancer, particularly leukemia and lymphoma, in the Gulf area \(^{(1)}\).

1.2. Expected impacts of the work:

There is an annual increase of new cases of cancer in the UAE varying from one region to another depending on the specific type of the cancer. The total number of malignant cases reported to Gulf Center for Cancer Registration (GCCR) \(^{(1)}\) from UAE in 1998 was 225. Males accounted for 127 (49.8%) of the cases and females accounted for 128 (50.2%). In 1998 the crude incidence rate (CIR) of all malignancies in the Emirates population was 38.8/100,000 for males and 39.8/100,000 for females \(^{(1)}\), compared to other countries in the area cancer incidence in UAE was the lowest. For example, the total number of malignant cases reported to GCCR from Bahrain in
1998 was 343 (1). Males accounted for 181 (52.8%) of the cases and females accounted for 162 (47.2%) (1). In 1998 the crude incidence rate (CIR) of all malignancies in the Bahraini population was 91.6/100,000 for males and 83.9/100,000 for females (1). It was also reported that in Bahrain and UAE in 1998, 4.7% and 8.6% of all malignancies occurred before the age of 15 respectively (1). While the total number of cancer cases reported to GCCR from Saudi Arabia in 1998 was 6227 (1). Males accounted for 3243 (52.1%) of the cases and females accounted for 2984 (47.9%) of the cases (1). In 1998 the crude incidence rate (CIR) of all cancers among the Saudi population was 42.5/100,000 for males and 39.7/100,000 for females (1). It is estimated that the Ministry of Health spends more than 160 million Dhs. on chemotherapy for treating UAE patients (1). Moreover, a study by the National Cancer Institute (NCI) expected that a total of 1,368,030 new cancer cases and 563,700 deaths in the United States in 2004 (56). World Health organization declared that most commonly occurring cancers found in children (0 to 14 years) are leukemia (particularly acute lymphocytic leukemia) (57). The work described in this study aims to provide a potential cost effective chemotherapeutic agent to treat cancer patients.
Chapter III:

Materials and methods
III. Materials and methods:

1. Nucleosides and Nucleobases:

Synthetic analogues that have been used in this study were supplied from Chemistry department, faculty of Sciences at the UAE University from Dr. Ibrahim Abdou’s laboratory (organic chemistry division). They were synthesized according to similar procedure published in *Molecules* 2004 by Dr. Abdou and his colleagues. The chemical structures, code names and R-groups of the analogues synthesized for testing are shown in Fig. III.1, III.2, and Table. 1.

![Chemical Structures](image)

**Fig. III.1:** Structure and code names of nucleosides analogues; NIA, NIIA, NIIIA, and NIB
Fig. III.2: General nucleoside structure

Table I: Nucleosides R and R1 groups

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>R1</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1A</td>
<td>(p-CF_3)</td>
<td>Ac</td>
</tr>
<tr>
<td>N2IA</td>
<td>(m-F)</td>
<td>Ac</td>
</tr>
<tr>
<td>N31IA</td>
<td>(p-F)</td>
<td>Ac</td>
</tr>
<tr>
<td>N4IB</td>
<td>(p-CF_3)</td>
<td>H</td>
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</tbody>
</table>
2. Biological activity:

2.1. Reagents:

Nucleoside analogues were synthesized and purified, and then dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich Chemie GmbH Steinheim, Germany) to prepare 0.1M solution and stored at -20°C. Protease inhibitors (PMSF, leupeptin, aprotinin, and peptatin A), N-acetyl-L-cysteine (NAC) and 2', 7'-Dichlorofluorescein diacetate (DCFH-DA) were acquired from Sigma Chemical Company (Sigma Aldrich, Germany). zDEVD-fmk (Caspase-3 inhibitor), zIETD-fmk (caspase-8 inhibitor), z-LEHD-fmk (caspase-9 inhibitor), and zVAD-fmk (a general inhibitor of caspases) were obtained from Alexis Corporation (Switzerland). Polyclonal and monoclonal antibodies were obtained from sources listed as follows: anti-Cyt c, anti-VDAC, anti-ANT, anti-Bax, anti-Bak, anti-Bad, anti-Bcl-2, anti-Bcl-xl, anti-p53 and anti-PARP antibodies from Santa Cruz Biotechnology (CA, USA), anti-caspase-3, anti-Bid antibodies from Cell Signaling Technology (New England Biolabs, USA), anti HDAC-1, -2, -3 and anti-Brg-1 from Upstate (Upstate, USA). All the antibodies are polyclonal except Bax, which is monoclonal.

2.2. Tissue culture:

Human promyelocytic leukemia HL-60 cell line (ATCC, USA) was grown in DMEM medium (GIBCO-BRL) supplemented with 20% fetal calf serum (GIBCO-BRL), 100 units/ml penicillin-streptomycin (GIBCO-BRL). The cells were maintained at 37°C in 5% CO₂ incubator. After reaching confluency, the cells were subcultured into 6-, 12-, 24-, or 96-wells culture plates, allowed to grow for 1-3 days, and treated with various concentrations of nucleosides and nucleobases, and other reagents. Following treatment, cells were harvested by spinning at 1000 rpm for 10
minutes, washed with phosphate buffer saline (PBS), and used for several assays and protocols.

Leukocytes from peripheral blood were harvested from normal individuals and leukemia patients' blood samples that have been prescreened for contagious pathologies, as follow: to each volume of blood three volumes of Histopaque reagent (Sigma, Germany) were added, and the mixture was spunspinned for 30 minutes at 2000 rpm at 25°C. Then the leukocyte layer was separated from serum and red blood cells layers. The leukocyte fraction was washed twice with RPMI-1940 media (GIBCO-BRL) supplemented with 10% Fetal Calf Serum (GIBCO-BRL) and 100 units/ml penicillin-streptomycin (GIBCO-BRL) by spinning at 1000 rpm at 4°C for 10 minutes each, and the partially purified leukocytes were cultured for testing. Cells were treated with various concentrations of nucleosides and nucleobases, as well as other reagents.

2.3. MTT cell proliferation assay:

Cells were plated in 96-well plates at a density $4 \times 10^3$ cells/well/100 µL of the appropriate culture medium and treated with the compounds at concentrations of 12.5 to 100 µM for different time intervals (2-48 hrs) to determine optimum time and concentration. In parallel, the cells were treated with 0.1% of DMSO as a control. A MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Alexis, USA) was performed later according to the manufacturer instructions to examine the cytotoxic effect of these nucleosides. After adding the MTT reagent the plate was further incubated for 1-4 hrs, followed by measuring the absorbance. This assay is based on the cellular cleavage of the tetrazolium salt, MTT, into formazan that is soluble in cell culture medium and is measured at 450 nm directly in 96-well assay
plates. The amount of the formazan dye (absorbance) generated by the activity of dehydrogenases in cells by the intact mitochondria is directly proportional to the number of living cells in culture. All nucleoside compounds tested were dissolved in DMSO (0.1M stock solution) and subsequently diluted in the culture medium to obtain the final concentrations that are going to be tested (12.5 to 100 µM) before treatment of the cultured cells. After this assay we noticed that DMSO did not have any effect on the viability of the control cells, so untreated cells (without DMSO) were chosen as negative control (viable).

2.4. Flow cytometry:

The translocation of the membrane phospholipid phosphatidyl serine from the inner part to the outer part of the plasma membrane (an early apoptotic marker), was detected with an Annexin V-FITC staining kit (BD Biosciences, USA) on HL-60 cells treated with various concentration of nucleosides. Propidium iodide (PI) was used to differentiate apoptotic cells with preserved membrane integrity (Annexin +, PI-) from necrotic cells that had lost membrane integrity (Annexin (-), PI(-)). After staining, the percentage of apoptotic cells under various treatments was analyzed by flow cytometry (FACSCaliber, BectonDickinson, USA).

2.5. DNA fragmentation:

To detect formation of internucleosomal fragments, DNA was extracted from HL-60 cells exposed to nucleosides and nucleobases for 16 hrs. After harvesting, washing with PBS, and fixation in 70% cold ethanol, degraded oligonucleosomal DNA was isolated with 40 µl of 0.2 M phosphate-citrate buffer, pH 7.8. This was followed by treatment of the extracts with RNase (1mg/ml), nonidet NP-40 (0.025%).
and proteinase K (1 mg/ml) for 1 hr at 37°C. The DNA was purified with phenol-chloroform (1:1) followed by precipitation in 100% ethanol and resuspension in TE buffer (10 mM, pH 7.5). DNA samples were resolved by electrophoresis at 40 V for 4 hrs on 1.5% agarose, and the laddering pattern was visualized by ethidium bromide staining with a UV transiluminator (Biodoc analyzer, Biometra, Germany).

2.6. Detection of DNA cleavage by formamide:

Chromatin condensation was detected by use of formamide with an APOSTAIN ELISA kit (Alexis, USA) according to the manufacturer’s instructions. Cells were cultured in 96-well plates at a density 4×10^3 cells/well in 100 μL of the appropriate culture medium and treated with the compounds at various concentrations (12.5, 25, 50 and 100 μM) for 16 hrs. DNA was denatured by heating in the presence of formamide, and the denatured DNA was stained with monoclonal antibody (MAb) F7-26 that is specific to single stranded DNA (ssDNA) in the ELISA format.

2.7. Western blot analysis:

Cells treated with nucleosides were harvested, washed once with PBS, and resuspended in a lysis buffer containing 100 mM Hepes, pH 7.5, 10% sucrose, 10 mM DTT, 0.1% CHAPS, 150 mM NaCl, and protease inhibitors (1 mM PMSF and 1 μg/ml each leupeptin, aprotinin, and pepstatin A). For the experiment with caspase inhibitors, the cells were pre-incubated 10-30 μM of zIETD-fmk (caspase-8 inhibitor), zLEHD-fmk (caspase-9 inhibitor), or zDEVD-fmk (caspase-3 inhibitor), or with 50 μM of zVADfmk (the general caspases inhibitor) for 4 hrs. Next, the cells were exposed to nucleosides and their nucleobases for 8, 16, or 24 hrs, except for the control cells, which were untreated (without DMSO). The harvested cells then were
lysed by 5-6 consecutive cycles of freeze and thaw, and spun at 14000 rpm for 30
minutes at 4°C. The supernatant was separated from the pellet and used immediately
or stored at -80°C. Protein in the cellular extracts was quantified with an assay kit
based on the Bradford colorimetric reaction (BioRad, USA). Cell protein (40 μg per
lane) was analyzed on 8%, 10% or 12% SDS-polyacrylamide gels and electroblotted
onto PVDF membranes (Millipore, USA). After the transfer, the membranes were
blocked with 5% nonfat dry milk and 0.1% Tween-20 for 1 hour. The membranes
were then incubated at 4 °C overnight with the antibodies, diluted as follows:

- 0.1% Tween-20 and PBS for: anti-PARP (1:1000 dilution), anti-
  VDAC (1:1000), anti-ANT (1:1000), anti-Bcl-2 (1:700), anti-Bcl-xL
  (1:1000), anti-Bax (1:500), anti-Bad (1:1000), anti-Bak (1:1000), anti-
  HDAC1 (1:1000), anti-HDAC2 (1:1000), anti-HDAC3 (1:1000), anti-
  Brg1 (1:1000),
- 5% nonfat dry milk in TBS and 0.05% Tween-20 for anti-caspase-3
  (1:1000 dilution),
- In 0.1% Tween-20 and 5% bovine serum albumin for anti-Bid
  (1:1000),
- 2% nonfat dry milk in 0.1% Tween-20 and PBS for both anti-
  cytochrome c (1:2000 dilution) and anti-p53 (1:500).

The blots then were incubated with the appropriate horseradish peroxidase-
conjugated secondary antibodies (1:2000 dilution). The antigen-antibody interaction
on the blots was detected by a SuperSignal chemiluminescence kit (Pierce,
Biotechnology, Rockford, IL, USA) and visualized by autoradiography. To confirm
equal loading of proteins, the blots were immunoprobed with a rabbit polyclonal
antibody against the cytoskeletal protein β-tubulin (1:2500 dilution in 0.1% Tween-
20 and PBS). Western blots were scanned and analyzed quantitatively by measuring band intensities with Biodoc analyzer, Biometra, Germany.

**2.8. Release of cytochrome c:**

To assess the effect of nucleosides on mitochondria, freshly isolated mitochondria extracted from the cells treated with 50μM of nucleosides for 4 and 16 hrs by differential centrifugation and resuspended in H-medium (70 mM sucrose, 220 mM manitol, 25 mM Hepes pH 7.4, 2 mM EDTA pH 8, 1 mM DTT and 0.1 mM PMSF) were incubated at 30°C for 30 minutes in 10 mM Tris-HCl pH 7.4 containing 0.15 M KCl and 5 mM succinate. The incubated samples were centrifuged at 8000 g for 10 minutes at 4°C. Then the supernatant was separated from the pellet (mitochondria) and both mitochondrial (20 μg) and cytosolic (50 μg) fractions were analyzed by Western blot with an anti-cytochrome c antibody.

**2.9. Measurement of mitochondrial membrane potential:**

The decrease in mitochondrial transmembrane potential in cells treated with nucleosides was measured by a MitoCapture™ apoptosis detection kit (Alexis Biochemicals, USA) according to the manufacturer’s instructions. After the treatment with different concentrations of nucleoside analogues for 16 hrs, cells were harvested and incubated in 1 ml of incubation buffer containing Mitocapture™ dye (1 μg/ml) for 20 minutes at 37°C in 5% CO2 incubator. After that the cells were collected by spinning, and the percentage of the green fluorescence was determined by flow cytometry.
2.10. Determination of intracellular reactive oxygen species (ROS) formation:

Measurement of ROS or intracellular peroxides was performed by using 2',7'-dichlorofluorescein diacetate (DCFH-DA), which is de-esterified by endogenous esterases within cells to ionized free acid (2',7'-dichlorofluoresceine; which in turn is oxidized by hydroperoxides to the fluorescent form 2',7'-dichlorofluorescin (DCF)).

10 mM DCFH-DA was prepared in DMSO (dimethylsulfoxide) as a stock solution. HL-60 cells (1x10^6) were cultured overnight in 6-well culture plates. After treatment with nucleosides for different time intervals (4, 8, 16, 24 hrs), cells were further incubated for 15 minutes with 10 mM DCFH-DA at 37°C. They were then washed with pre-warmed medium followed by ice-cold PBS, harvested by spinning at 1000 rpm for 5 minutes and resuspended in PBS containing 10 mM EDTA. The formation of ROS was measured with FACSCallibre flow cytometer. To study whether the inhibition of ROS could prevent apoptosis induced by nucleosides, cells were pretreated with 2, 5, 10 mM N-acetyl-L- cysteine (NAC).

2.11. Cell cycle analysis:

The proportion of cells in G0-G1, S, and G2-M was determined by flow cytometric analysis of DNA content. Cell cycle distribution in HL-60 cells was measured after 16 hrs of treatment with nucleosides. Briefly, 1x10^6 cells were harvested, washed twice with cold PBS, then they were fixed overnight in 10 ml of 70% (-20°C) ethanol at 4°C. Subsequently, cells were washed twice with cold PBS, incubated with RNase (0.25 mg/ml) at 37°C for 1hr, and stained with PI (50 µg/ml in PBS) for 30 minutes at 4°C in the dark followed by flow cytometry.
2.12. **Statistical analysis:**

All experiments were repeated at least three times. Data were generally expressed as means ± standard deviation (SD) from three independent experiments. Statistics were calculated and plotted by Excel software. For the MTT assay each concentration was tested in triplicate and the whole experiment was repeated thrice. To measure the percentage of viability the value of the negative control (cells treated with 0.1% DMSO or untreated cells) was subtracted from the positive control (cells treated with 0.1% SDS) after dividing both numbers over 100 (volume used), then the absorption of each trial was multiplied with this value, then the data were blotted by Excel after measuring the standard deviation. The FACS experiments were repeated also three times. The mean and the standard deviation values were calculated. For the Western blot analysis, a representative gel will be shown and the mean band intensity value of target proteins will measured and blotted by Excel after calculating the standard deviation. Below is a time scale of the experiments that have been conducted in this study (Fig. III.3).

![Time scale of experiments](image)

**Fig. III.3:** time-scale representation of the experiments that were conducted in this study. MTT assay was the first to used to determine the optimum time and concentration, followed by Annexin/PI for further confirmation. To determine the events in the process of cell death induced by these nucleosides further assay such as ROS, measurements of mitochondrial membrane potential, Western blots and cell cycle analysis were performed.
Chapter VI:

Results and discussion
IV. Results and discussion:

1. Results:

1.1. Reduction in HL-60 proliferation and integrity by NIA, NIIA and NIIVA:

The MTT cell proliferation assay showed that there was a decrease in the absorbance and therefore the viability of the cells with increased concentrations of the nucleosides. We found that NIA and NIIA are the most effective compounds, even when 12.5 μM was administered, which is below the inhibitory concentration 50 (IC₅₀), required to inhibit the growth of 50 % of any system, and in this case it was 50 μM (see Fig. IV.1a and b). On the other hand, NIB which has the same structure as NIA except that its sugar is non-acetylated, showed no anti-tumor activity. This result indicated that this analogue either protects against apoptosis or has no effect at all. This finding also suggested that acetylated sugar increases the bioavailability (their delivery into the cell) of the nucleoside and therefore its effectiveness. To test if the chemical structure of the base alone has any role in the cytotoxicity, we exposed cells to NIA with sugar presented or removed and tested them using flow Cytometry by Annexin/PI (see section IV.1.2). Test of NIIVA revealed that it has reduced bioactivity compared to the other nucleoside analogues. Interestingly, NIIVA shares the same chemical composition with NIIA, except the position of the fluorine in NIIA is para, while in NIIVA it is meta, which indicates that there was a structure-activity relationship (SAR) determining the cytotoxic effect of these analogues. The initial observations about the apoptotic potential of these drugs in the HL-60 cell line prompted us to further investigate the mechanism of their apoptotic induction. The controls that were used in these experiments if not specified were
untreated cells as negative control and Etoposide (a known anti-cancer agent that stimulates caspase activation) as positive control.
Fig. IV.1: Viability and IC$_{50}$ determination from MTT cell proliferation assay. HL-60 cells were treated with the various compounds listed at concentrations ranging from 12.5 to 100 µM for different time intervals (2-48 hrs) to determine optimum time and concentration. In parallel, control cells were treated with 0.1% DMSO: a) Decrease in the viability of the cells with increased concentrations of the nucleosides, b) IC$_{50}$ value of the nucleoside (NIA). Line plot represents % of viability of different concentration ± SD, n= 3.
1.2. Apoptotic effect of the novel nucleosides on HL-60 cell viability:

To confirm the data obtained with MTT, HL-60 cells were treated with various concentrations of the nucleosides for 2, 4, 8, 16, and 24 hrs. The cell death was measured by Annexin V staining technique. In this assay the loss of plasma membrane asymmetry, which is one of the earliest features of apoptosis, was targeted by the binding of Annexin V to the membrane phospholipids phosphotidylserine (PS) that is translocated from the inner to the outer leaflet of the plasma membrane of apoptotic cells. Because PS translocation also occurs during necrosis, Propidium iodide (PI) is used in conjugation with Annexin V. PI can only penetrate the plasma membrane when membrane integrity is breached, as occurs in the later stages of apoptosis or in necrosis. Cells that are negative for both Annexin V and PI (Annexin (-), PI (-)) are considered viable cells (not apoptotic), while the cells that are stained Annexin V positive and are PI negative (Annexin (+), PI (-)) are considered to be early apoptotic cells. On the other hand, the cells that are positive for both Annexin V and PI (Annexin (+), PI (+)) are late apoptotic cells, and those that are Annexin V negative, PI positive (Annexin (-), PI (+)) are recognized as necrotic cells. The results obtained from this assay (see Fig. IV.2) supported those from MTT cell proliferation assay (see Fig. IV.1), in which that NIA was the most potent analogue as it resulted in more than 50% apoptosis compared to the positive control (Etoposide) followed by NIIA then NIIIA. This assay also indicated that the nucleobases are the cytotoxic part of the nucleosides.
Moreover a time- and concentration-dependent induction of apoptosis was observed in the cells treated with NIA, IIA and IIIA. The concentration-dependent data represent NIA (the most effective analogue), while the time point study was carried with 50 μM since it was the concentration required to induce 50% of the cell death (see Fig(s). IV.3 and IV.4). A gradual increase in the percentage of apoptotic cells was observed at 16 hrs compared with the control cells (untreated cells) (see Fig. IV.4). Increasing the incubation time up to 24 hrs resulted in significantly higher apoptotic rates, reaching 75.45% at 100 μM for NIA (Fig. IV.3). As for NIB, it did not differ from the control, and even at 50 μM the number of viable cells was higher than that of the control (91.14% while control was 89.16%) (data not shown, but not statistically significant, $p>0.05$).
Fig. IV.3: Representative flow cytometric analysis of the time- and-concentration-dependent induction of apoptosis in the HL-60 cells treated with NIA, by use of Annexin-V/PI: a) control (untreated cells), b) 50 μM NIA for 4 hrs, c) 50 μM NIA for 8 hrs, d) 50 μM NIA for 16 hrs e) 50 μM NIA for 24 hrs f) 100 μM NIA for 24 hrs. The arrows point to the % of cells in each quadrant: viable cells (lower left), early apoptotic cells (lower right), late apoptotic cells (upper right) and necrotic cells (upper left), n=3.

Surprisingly, it seems that these nucleoside analogues have a low necrotic index (1.7 in NIA) compared to the Etoposide (4.5%) (Fig. IV.4). Also it seems that the acetylated ribose found in NIA, IIA, IIIA did not play a role in the toxicity of these nucleosides, which again supports the idea that the low-
apoptotic effect of NIII A is not due to the sugar differences, but is rather a result of the structure difference and the chemical composition of the analogues. Furthermore, this confirmed that the importance of the sugar lies in increasing nucleoside availability within the cells, thus indirectly enhancing their cytotoxicity.

Fig. IV.4: Representative flow cytometric analysis of the concentration-dependent induction of apoptosis in the cells treated with NIA, NIIA and NIII A for 16 hrs by use of Annexin-V/PI: a) control, b) 12.5 μM NIA, c) 25 μM NIA, d) 50 μM NIA, e) 12.5 μM NIIA, f) 25 μM NIIA, g) 50 μM NIIA, h) 12.5 μM NIII A, i) 25 μM NIII A, j) 50 μM NIII A, k) 25 μM Etoposide, l) 100 μM acetylated ribose, m) 50 μM N-base I, n=3.
1.3. Effect of NIA, NIIA and NIIIA on normal and leukemia blood samples:

Next, we analyzed the effect of these nucleosides on peripheral blood cells of leukemia patients in the area as well as normal individuals (this experiment was repeated three times and the blood was obtained from four patients representing each case, and from three normal individuals). Leukocytes were isolated from normal and leukemic blood samples obtained from Tawam hospital and treated with different concentrations of nucleosides for 16 hrs (optimum time). Flow cytometric analysis of leukocytes from normal individuals showed that these analogues had only a minute cytotoxic effect even at high concentrations (200 μM) (see Fig. IV.5).

On the other hand 50 μM of the nucleosides significantly affected the white blood cells isolated from the four leukemia patients treated for 16 hrs (Fig. IV.6-8). NIA, IIA and IIIA were tested on three different types of leukemia: pre-B-cell acute lymphoblastic leukemia, acute T-lymphoblastic leukemia and acute

![Image of flow cytometric analysis](attachment:image.png)

Fig. IV.5: Representative flow cytometric analysis of four normal blood samples by Annexin V/PI, showing the effect of these analogues on normal leukocytes; a) control, b) 50 μM NIA, c) 100 μM NIA, d) 200 μM NIA, n=3.
myelogenic leukemia. These nucleosides were effective against lymphoblastic leukemia especially pre-B-cells, reaching 78.34% of apoptosis when 50 μM of NIA was administered for 16 hrs, while in the case of acute T lymphoblastic leukemia it was 72.18% with 50 μM NIA. Treatment of myelogenic leukemia was not effective as indicated with negligible apoptosis percentages (1.26% compared to the control). Interestingly, the necrotic index of these analogues (1.7%) was lower than that of Etoposide (4.5%) (Fig. IV.4). Together, these data indicate that NIA, IIA and IIIA have minimal effect on normal leukocytes as well as myelogenic leukemia with low necrotic rate and significant cytotoxic effect on acute lymphoblastic leukemia.

Fig. IV.6: Representative flow cytometric analysis of four pre-B-cell acute lymphoblastic leukemia sample by use of Annexin V/PI , a) control, b) 50 μM N1A, c) 50 μM NIIA, d) 50 μM NIIIA, n=3.
1.4. Induction of DNA fragmentation and changes in chromatin condensation pattern by NIA, NIIA and NIII A:

DNA fragmentation and chromatin condensation are hallmarks of apoptosis. Therefore, the ability of nucleoside analogues and their corresponding bases to induce internucleosomal DNA degradation and chromatin condensation within 16 hrs were tested on HL-60 cells (see Fig. IV.9). The nucleosides and their corresponding bases were tested at two different concentrations (25 and 50 μM) to monitor a dose response.
Fig. IV.9: DNA fragmentation assay in HL-60 cells treated with nucleosides and nucleobases for 16 hrs. The laddering was detected on 1% agarose gel, and an increase in the intensity of the ladder was observed with increased concentrations. A). Representative agarose gel of DNA fragmentation (n=3); 1) 1 Kb ladder, 2) Control (untreated cells without DMSO), 3) 25 µM NIA, 4) 50 µM NIA, 5) 25 µM N-base 1, 6) 50 µM N-base 1, 7) 25 µM NIIA, 8) 50 µM NIIA, 9) 25 µM N-base 2, 10) 50 µM N-base 2, 11) 25 µM NIIIA, 12) 50 µM NIIIA, 13) 25 µM N-base 3, 14) 50 µM N-base 3. B). Quantitative normalized measurements of fragmented band intensity (means ± SD).
The internucleosomal fragments of DNA were visualized as a ladder on 1% agarose gel. An increase in the intensity of the ladder was observed with increasing concentrations of each agent (lanes 3-6). DNA cleavage products representing internucleosomal fragments were visible in response to 50 μM and even in 25 μM concentrations of the nucleosides NIA and NIIA compared to their bases, and this supports the finding that the base is the toxic part of the nucleoside not the sugar (see Fig. IV.9).

Nucleoside analogues and their bases also induced DNA denaturation in the presence of formamide in a concentration dependent manner (Fig. IV.12). NIA gave the maximum percentage of chromatin condensation followed by NIIA. NIIIA and its base were similar in this induction. These results were consistent with the data obtained previously from FACS and MTT assays (see Fig. IV. 3-10). Whereas histones are responsible for stabilizing DNA structure against thermal denaturation (55), these results suggest that histone digestion may occur during apoptosis stimulated by these nucleosides.
**Fig. IV.10:** Detection of chromatin condensation in a concentration-dependent manner by formamide.

DNA denaturing was examined with an APOSTAIN ELISA kit according to the manufacturer's instructions. Briefly, DNA was first denatured by heating in the presence of formamide. In the second step the denatured DNA was stained with monoclonal antibody (MAb) F7-26 specific to single stranded DNA (ssDNA) in ELISA format. The negative control was untreated cells, while the positive control was single stranded DNA supplied by the manufacturer. Line plot represents % of chromatin condensation ± SD, n= 3.

**1.5. Effects of nucleosides on the expression of key apoptotic proteins:**

During apoptosis a group of proteolytic enzymes called caspases are activated in a cascade-fashion, leading to the activation of the "executioner caspases", such as caspase-3. These executioner caspases are responsible for the destruction of the key cellular proteins such as DNA repair enzymes that leads to the typical morphological changes observed apoptosis\(^{(33, 34)}\). Therefore the effect
of nucleosides on the expression of cleaved caspase-3 was analyzed by Western blotting (Fig. IV.11).

The induction of the cleaved form was detected at 25, 50, 100 µM. This activation of the effector (executioner) caspase was assessed at 16 hrs and 24 hrs as well. This concentration-dependent induction resembles the reduction in viability and DNA fragmentation observed previously, in that NIA is the most effective one followed by NIIA (although there was no change in the expression, but the intensity was prominent), then NIII A.
Fig. IV.11: Expression of cleaved caspase-3 (p19) and the full length (upper band) in HL-60 cells treated with various concentrations of nucleosides and nucleobases for 16 hrs. 40 µg of Whole cell extract were loaded on 12% SDS-PAGE and probed with anti-caspase-3. A). Representative Western of three independent experiments. B). Quantification of p19 band intensity of the nucleosides NIA, NIIA and NIIIA. C). Quantification of p19 band intensity of the nucleobases, (means ± SD, n=3).
One of the essential target proteins required for cell viability is the DNA repair enzyme PARP (poly (ADP-ribose) polymerase) (59-61). Cleavage of PARP by caspase-3 leads to DNA fragmentation (62-67). To investigate the activation of PARP, nucleosides treated cells were blotted with PARP polyclonal antibody. The 116 KDa PARP was cleaved into a 89 KDa fragment at 25, 50, 100 µM concentrations of the nucleosides and nucleobases (Fig. IV.12), and this result was consistent with the increased caspase-3 activity observed, in which 50 µM of NIA and NIIA was enough to cause complete loss of the 116 KDa fragment. Moreover, these observations support the finding that NIA is the most effective tested compound, and this is based on comparing the intensity of the 85 KDa band and the disappearance of the 116 KDa fragments.
Fig. IV.12: PARP cleavage in HL-60 cells in the presence of different concentrations of nucleosides and nucleobases administered for 16 hrs. 40 μg of cell extract was loaded on 8% SDS-PAGE, and blotted with anti-PARP. A). Representative Western of three independent experiments. B). Quantification of cleaved PARP band intensity of the nucleosides. C). Quantification of cleaved PARP band intensity of the nucleobases (means ± SD, n=3).
Next, we examined the expression of the Bcl-2 family proteins. The Bcl-2 proteins are a group of proteins involved in the resistance against or enhancement of apoptosis. Some of these proteins (such as Bcl-2 and Bcl-xI) are anti-apoptotic, while others (such as Bad, Bak, Bid or Bax) are pro-apoptotic. A concentration-dependent decrease in the expression of Bcl-2 and Bcl-xI was detected in HL-60 pretreated with nucleoside analogues, especially with the treatment at 50 μM and 100 μM of NIA and IIA (Fig. IV.13), while a consistent increase in Bax, Bad, Bak, and induction of truncated Bid was observed (Fig. IV.13). These results indicate that these nucleosides might induce apoptosis through the mitochondrial pathway, because Bcl-2 family members have been shown to control Cyt c release from the mitochondria (58). The ability of Bax to induce the efflux of Cyt c within minutes after its translocation from the mitochondria have been reported (58). A concentration dependent increase in the expression of the tumor suppressor protein p53 was observed; again this was much clearer in the case of NIA and NIIA (50 μM and 100 μM).
Fig. IV.13: Effects of various concentrations of nucleosides and nucleobases applied for 16 hrs on the expression of the anti-apoptotic proteins, Bcl-2 and Bcl-xL, and pro-apoptotic proteins Bax, Bad, Bak, Bid, and on p53. 40 μg of HL-60 cells extract was loaded on 12% and 8% SDS-PAGE, and probed with the corresponding antibodies. Representative Western of three independent experiments.

1.6. Effect of caspases inhibitors on NIA, NIIA and NIIIA- stimulated activation of caspase-3:

To understand the events proceeding caspase-3 activation and apoptosis induced by nucleoside analogues, we examined the effect of various caspase inhibitors on HL-60 cells post-treated with these agents (see Fig. IV.14).
Fig. IV.14: Determination of apoptotic pathways with different concentrations (10-30 μM) of caspases inhibitors; including z-VAD (general inhibitor), z-IETD (caspase-8 inhibitor), z-LEHD (caspase-9 inhibitor), z-DEVD (caspase-3 inhibitor). These inhibitors were added 4 hrs before adding the nucleosides (50 μM) for 6 hrs, then a Western blot analysis was carried on with anti-caspase3. A). Representative Western blot with anti-caspase3 of three independent experiments. B). Quantification of p19 cleaved caspase3 band on post treatment with NIA, C). Quantification of p19 cleaved caspase3 band on post treatment with NIIA, D). Quantification of p19 cleaved caspase3 band on post treatment with NIIIA, (means ± SD, n=3).
Before incubation of cells with nucleosides (50 μM), they were treated for 4 hrs in the absence and presence of (10-30 μM) of zIETD-fmk (caspase-8 inhibitor), zLEHD-fmk (caspase-9 inhibitor), zDEVD-fmk (caspase-3 inhibitor), or with 50 μM of zVAD-fmk- the general inhibitor of caspases. The result of cleaved caspase-3 immunoblotting showed that the increased concentrations of caspase-8 inhibitor did not significantly change the nucleoside-induced apoptosis, while there was a nearly total inhibition observed at preincubation with increased concentrations (10-30 μM) of caspase-3, -9, and z-VAD. These results demonstrate the critical role of caspase-9 activation in NIA, NIIA and NIIIA-induced apoptosis.

1.7. Effect of NIA, NIIA and NIIIA on the release of Cyt c from mitochondria:

According to the above results, induction of the apoptotic machinery by the nucleoside analogues was caspase-9 dependent. This caspase is part of the apoptosome (caspase-9 and Apaf-1) that triggers the release of Cyt c from the mitochondria into the cytoplasm. Therefore, the cytosolic and mitochondrial fractions were prepared from cells treated with 50 μM of nucleosides and their bases and Cyt c expression was detected by Western blot. Release of Cyt c in the cytosolic fraction was detected after 8 hrs incubation with nucleosides (Fig. IV.15) with a disappearance in the mitochondrial fraction for all the nucleosides with the exception of bases, in which some of Cyt c was detected in the mitochondrial fraction. To study the relation between NIA, IIA, IIIA- induced apoptosis and Bax, we examined the kinetics of Cyt c release and expression/ upregulation of Bax. Cyt c release from the mitochondria was
detected as early as 8 hrs after incubation with nucleosides and nucleobases, but it was clearer after 16 hrs of incubation (which is the optimum time of incubation with these nucleosides) (Fig. IV.15), while increase in Bax expression was detected only after 16 hrs of post-treatment (Fig. IV.13).

Fig. IV.15: Release of cytochrome c after nucleoside administration for 16 hrs, and involvement of Bax in the amplification of Cyt c release. The cytosolic and mitochondrial fractions were prepared from cells treated with 50 μM of nucleosides and their bases. Then 20 μg of mitochondrial and 50 μg of cytosolic fractions were analyzed by Western blot. A). Representative Western of three independent experiments. B). Quantification of Cyt c level in the cytosol after nucleosides treatment, C). Quantification of translocated Bax level into the mitochondria after nucleosides treatment, (means ± SD, n=3).
Interestingly, there was a Bax translocation to the mitochondria at 16 hrs after nucleosides treatment (see Fig. IV.15), which suggests that, i) NIA, IIA and IIIA, and their bases initiated apoptosis through a direct effect on the mitochondria, which resulted in Cyt c release, and ii) the apoptotic signal was further amplified by the translocation of Bax. Up-regulation of Bax is controlled by p53 (Fig. IV.13), which is a tumor suppressor protein induced in response to DNA damage \(^{(35)}\). We showed that DNA fragmentation by nucleoside analogues is dependent on caspase-3 activation; thus induction of p53 follows caspase-3 activation. Furthermore, NIA, IIA and IIIA triggered a caspase-dependent truncation of Bid (see Fig. IV.14), which in addition to p53, promotes translocation of Bax that subsequently enhance the release of Cyt c. The expression levels of VDAC, which serves in combination with the proapoptotic members of Bcl-2 family to form a pathway for Cyt c release from the mitochondria \(^{(34,55)}\), and its regulatory protein ANT were examined. There no change in their expression was detected (Fig. IV.16). These observed changes in the expression of the Bcl-2 family member might indicate that the release of Cyt c may take place also through different channels and mechanisms.
1.8. Alterations of mitochondrial membrane potential of HL-60 cells:

Changing the mitochondria membrane potential is believed to be one of the mechanisms of Cyt c release (58-61). Therefore the mitochondria membrane potential was measured with the MitoCapture™ cationic dye as per manufacturer’s protocol. In healthy cells, MitoCapture™ accumulates in the mitochondria and gives off green fluorescence, while in apoptotic cells, due to the altered mitochondrial membrane potential, the green fluorescence remains in

Fig. IV.16: Effect of different concentrations of nucleosides administered for 16 hrs on cytochrome c releasing gates (VDAC and ANT). 40 µg of HL-60 cells extract was loaded on 12% SDS-PAGE, and probed with the appropriate antibodies. A). Representative Western of three independent experiments. B). Quantification of ANT band intensity after nucleosides treatment, C). Quantification of VDAC level after nucleosides treatment, (means ± SD, n=3).
The nucleoside-induced membrane depolarization could be observed either by the concentration-dependent decrease in the red fluorescence given by intact mitochondria or by the increase in the intensity of the green fluorescence in the cytosol of the treated cells. In this assay, we examined the alteration in mitochondrial membrane potential by the decrease in the intensity of the red fluorescence (decrease in the number of healthy mitochondria). For example, in the control (untreated cells), one peak with maximum red fluorescence intensity should be detected, and a decrease of the fluorescence and formation of another peak (corresponding to damaged mitochondria) should be detected as result of the administration of the nucleosides.

Test of the nucleosides revealed that they induced mitochondrial membrane depolarization in a concentration-dependent manner (see Table 2 and Fig. IV.17). 25 μM of NIA altered the potential of 62.12% of the mitochondria, while 50 μM disrupted 65.36%. On the other hand, 25 μM and 50 μM of NIIA resulted in an alteration of 53.09% and 55.04% respectively, of the membrane potential. NIIIA depolarization values were 54.34% (25 μM) and 54.78% (50 μM). Administration of a base resulted in an alteration of 54.78%. These data collectively agreed with previous findings, in which nucleoside-stimulated apoptosis was via mitochondria as indicated with the alterations the membrane and that NIA is the most effective one among them.
Table 2: Percentages of alterations of mitochondrial membrane potential due to nucleosides administration of three independent experiments, (means ± SD, n=3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>% of membrane potential alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated without DMSO)</td>
<td>3.51% ± 7</td>
</tr>
<tr>
<td>25 μM of NIA</td>
<td>62.12% ± 12</td>
</tr>
<tr>
<td>50 μM of NIA</td>
<td>65.36% ± 9</td>
</tr>
<tr>
<td>25 μM of NIIIA</td>
<td>53.09% ± 13</td>
</tr>
<tr>
<td>50 μM of NIIIA</td>
<td>55.04% ± 20</td>
</tr>
<tr>
<td>25 μM of NIIIIA</td>
<td>54.34% ± 15</td>
</tr>
<tr>
<td>50 μM of NIIIA</td>
<td>54.78% ± 6</td>
</tr>
<tr>
<td>50 μM of N-base 1</td>
<td>54.34% ± 13</td>
</tr>
<tr>
<td>25 μM of Etoposide (positive control)</td>
<td>17.14% ± 9</td>
</tr>
</tbody>
</table>
Fig. IV.17: Alteration of the mitochondrial membrane potential in HL-60 cells treated with various concentrations of nucleosides for 16 hrs. Membrane potential disruption was detected using MitoCapture™ cationic dye as per manufacturer’s protocol: a) control, b) 25 µM NIA, c) 50 µM NIA, d) 25 µM NIIA, e) 50 µM NIIA, f) 25 µM NIIIA, g) 50 µM NIIIA, h) 50 µM N-base1, i) 25 µM Etoposide. Representative FACS histogram of one of three different trials.
1.9. Generation of ROS by NIA, NIIA and NIlla:

One of the earliest apoptotic features that is linked to mitochondrial membrane potential disruption is the induction of Reactive Oxygen Species (ROS) \(^{68-70}\). To investigate the involvement of ROS in the nucleosides-mediated apoptosis, the level of ROS was measured by a fluorometric probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), which is de-esterfied within the cells by endogenous esterases to the ionized free acid 2',7'-dichlorofluorescin. 2',7'-dichlorofluorescin is then oxidized to the fluorescent 2',7' dichlorofluorescin (DCF) by hydroperoxide (a reactive oxygen species: ROS). An increase in ROS generation was detected as early as 4 hrs (1.76\%) by 50 \(\mu\)M of NIA, but the significant induction was observed after 16 hrs (19.1\%) (Fig. IV.18). This was clearly observed with the increase in shifting of the peak to the right (the over-relayed peaks in the green indicate the control [untreated cells]). Generation of ROS was dependent on the concentration of the nucleosides. 50 \(\mu\)M of NIA resulted in formation of 19.1\% of ROS. Interestingly, 50 \(\mu\)M of NIIA was more effective than 50 \(\mu\)M of NIlla in generating ROS (3.56\% and 0.21\% respectively) (Fig. IV.19- IV.21a). ROS formation due to nucleoside treatment was synchronized with the early induction of apoptosis, which was clear with the reduction in the height of one peak and formation of another.

To determine whether ROS inhibition could rescue nucleoside treated cells from apoptosis, we preincubated the cells with 2, 5, 10mM of \(N\)-acetyl-L-cysteine (NAC), which is an antioxidant. Pretreatment of NAC prevented the formation of ROS and therefore decreased apoptosis partially, as indicated by the retreat of the peaks to resemble that of the control (Fig. IV.21b). Therefore, these
results indicate that ROS formation contributes to the ability of the nucleosides to induce apoptosis. Nucleobases were also able to generate ROS at 16 hrs in a concentration dependent manner. Although these bases seemed to be more effective in stimulating cell death scenarios, their ability was not favored because of the high necrotic rate they posses, which was clear from the severe decrease in the height of the peak, in addition to the low solubility due to the absence of the sugar.
Fig. IV.18: Formation of ROS in HL-60 cells subjected to 50 μM NIA for different periods of time. The level of ROS was measured by the ionization of fluorometric probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) into 2',7'-dichlorofluorescin (DCF) by hydroperoxide: a) control, b) 4 hrs, c) 8 hrs, d) 16 hrs, e) 24 hrs, f) 15 μM cisplatin (another anti-tumor agent as positive control) for 16hrs. Representative FACS histogram of one of three different trials.
Fig. IV.19: Formation of ROS in HL-60 cells treated with nucleosides for 16 hrs: a) control, b) 12.5 μM NIA, c) 25 μM NIA, d) 50 μM NIA, e) 50 μM NIIA, f) 25 μM NIIA, g) 50 μM NIIA, h) 12.5 μM NIIA, i) 25 μM NIIIA, j) 50 μM NIIIA. Representative FACS histogram of one of three different trials.
Fig. IV.20: Formation of ROS in HL-60 cells treated with nucleobases for 16 hrs: a) 12.5 μM N-base1, b) 25 μM N-base1, c) 50 μM N-base1, d) 12.5 μM N-base 2, e) 25 μM N-base 2, f) 50 μM N-base 2, g) 12.5 μM N-base 3, h) 25 μM N-base 3, i) 50 μM N-base 3, j) 15 μM cisplatin. Representative FACS histogram of one of three different trials.
Fig. IV.21: Effect of various concentrations of the nucleosides in the presence and absence of NAC on ROS formation. ROS generation was detected 16 hrs in HL-60 cells treated with nucleosides and the effect of preincubation with N-acetyl-L-cysteine (NAC); a) ROS generation was concentration-dependent, b) Administration of NAC inhibited ROS formation. Representative FACS histogram of one of three different trials.
1.10. Effects of nucleosides on the cell cycle distribution of HL-60 cells:

The fundamental process underlying all biological growth and reproduction is the cell cycle \(^{(12)}\). The cell cycle is the sequence of events whereby a living cell duplicates its nucleus and some cytoplasmic components and distributes them between daughter cells capable of carrying out the whole sequence again \(^{(12)}\). It is a very well programmed phenomenon and is grossly divided into 4 phases. These are M-Mitosis phase, G\(_1\)-growth phase 1, S-synthesis phase, and the G\(_2\)-growth phase 2. In order to assess the extent of nucleoside-induced apoptosis and to investigate whether this process was selective to any phase of the cell cycle, the cell cycle distribution of HL-60 cells treated with nucleosides was studied. An accumulation of cells in G\(_0\)-G\(_1\) phase was observed (Fig. IV.22), and the increased concentration of the nucleosides lead to profound changes in the cell cycle profiles (see Fig. IV.22). These changes included a decrease in the G\(_2\)-M phase and S-phase populations accompanying the increase in the G\(_0\)-G\(_1\) population. The most interesting finding was that the lowest concentration of NIA (25 \(\mu\)M) increased the percentage of apoptosis the most (20.89%), compared to 50 \(\mu\)M (3.78%), while there was an increase in apoptosis induction with the highest concentrations of NIIA, and NIIIA. Possible explanations for this observation are as follows, during this assay, samples need to be syringed through a 25-gauge needle to prevent nuclear clumping and to allow further discrimination between G\(_1\), G\(_2\) and S-phase cells, so that the cells would not be mistakenly read as subdiploid cells indicating apoptosis. Another explanation could be that 50 \(\mu\)M of NIA halted the cells in G\(_0\)-G\(_1\) phase, preventing them from proceeding in the cell cycle and eventually the cells committed suicide, affecting several check points and regulatory proteins more than those that are affected by NIIA, and NIIIA.
Fig. IV.22: Flow cytometric analysis of the effect of different nucleosides concentrations on the distribution of HL-60 cells in the cell cycle by PI staining. PI-stained HL-60 cells were analyzed by flow cytometry after addition of different nucleosides concentration (25 μM and 50 μM) for 16 hrs: a) control (untreated cells), b) 25 μM NIA, c) 50 μM NIA, d) 25 μM NIIA, e) 50 μM NIIA, f) 25 μM NIIA, g) 50 μM NIIA, h) 25 μM Etoposide. A). Representative FACS histogram of one of three different trials. B). Quantification of cells distribution in G0-G1 phase after nucleosides treatment, (means ± SD, n=3).
1.11. Effects of nucleosides on Histone Deacetyl-transferases (HDACs), and human brahma-related gene 1 (Brg1):

HDACs have been implicated in the development of cancer with the activity of several tumor suppressors regulated in part by HDACs (72-76). For example, the retinoblastoma tumor suppressor protein, Rb, represses transcription by recruiting HDAC1 (76, 77). The p53-dependent gene activation is specifically inhibited by HDAC1-3 (76, 77). To address the involvement of chromatin modifications in nucleoside-induced apoptosis, the expression of HDAC1, 2, 3, and Brg1 were examined by Western blot analysis. HDACs are a family of enzymes that catalyze the removal of acetyl group from post-translationally acetylated proteins, which include histones, transcription factors, and architectural proteins (76). The human BRG1 (brahma-related gene 1) protein is a component of the SWI/SNF family of the ATP-dependent chromatin remodeling complexes (77).

A concentration-dependent decrease in the expression of the HDACs was observed upon treatment with the nucleosides (see Fig. IV.24). On the other hand, a slight increase in the expression of Brg1 was detected with the increased concentration of NIA, and no change with the administration of NIIA, and NIIIA (Fig IV.23). Accordingly, these data suggest that these nucleosides possess an anti-HDACs ability, and might enhance the functionality of chromatin modifying complexes such as SWI/SNF. As a result, inhibition of HDAC activity by HDAC inhibitor might increase p53 stability. p53-induced growth arrest and apoptosis are mainly mediated through its transcription activity by transactivating or transrepressing a number of down-stream target genes (78). Importantly, the DNA binding and transcriptional activities of p53 require a post-translational modification by acetylation (78). The
acetylation of p53 can be induced by a variety of p53-activating agents, which include agents that induce DNA damage and oxidative stress.

Fig. IV.23: Effects of various nucleosides concentrations on the expression of HDAC-1, -2, -3 and Brg1 proteins. 40μg of nuclear cell extract was resolved on 10% SDS-PAGE and probed with the appropriate antibodies. A). Representative Western blot of one of three different trials. B). Quantification of HDAC-1 level after nucleosides treatment, C). Quantification of HDAC-2 level after nucleosides treatment, D). Quantification of HDAC-3 level after nucleosides treatment, E). Quantification of Brg-1 level after nucleosides treatment, (means ± SD, n=3).
2. Discussion:

In this study an integrated approach has been used to examine the ability of synthetic nucleosides analogues to induce apoptosis and to elucidate the sequence of events involved in the mechanism of nucleoside-stimulated apoptosis.

From the MTT cell proliferation assay and AnnexinV/PI FACS analysis, we showed that three of the four nucleosides, which were NIA, NIIA and NIIIA, had the most powerful antiproliferative effect. Interestingly, NIIIA was less effective, probably because of the meta position of the fluorine, which decreases its solubility and hence renders its uptake by the cells efficiently. In contrast, NIB has either no effect at all or exhibited some anti-apoptotic potential. To verify this conflict, we examined if it can act as apoptosis inhibitor, by treating the cells for 4 hrs followed by treatment with Etoposide (a known anti-tumor agent and apoptosis inducer). We observed that the rate of apoptosis was abolished (data not shown).

Apoptosis was detectable following 16 hrs exposure to 50 μM of NIA, IIA, and IIIA by four distinct criteria (MTT proliferation assay, Annexin V/PI staining, DNA fragmentation, and activation of caspase-3 and PARP cleavage). Caspases, found in mammalian cells as inactive protease precursors, are grouped into upstream initiator caspases (e.g. caspase-9) and downstream effector caspases (e.g. caspase-3). The inactive initiator caspase are first activated in response to apoptotic stimuli and are responsible for processing and activation of the effector caspases. The activated effector caspases, subsequently, execute apoptosis by cleaving various cellular substrates that are vital for cell survival (34). Western blot analysis showed that there is an increase in the activity of caspase-3, and PARP in a concentration-dependent manner due to nucleosides treatment. The activation of caspase-3 (effector enzyme) leads to the cleavage of PARP, which is one of the essential proteins required for cell
integrity and viability \(^{(63,64)}\). PARP cleavage prevented the repair of DNA damage, blocked the depletion of NAD\(^+\) and ATP, resulting in the death of cells via necrosis, while enhancing the role of Ca\(^{2+}\)/Mg\(^{2+}\) - dependent endonucleases \(^{(55)}\). Inactivation of PARP by the proteolytic activity of caspase-3 eventually lead to fragmentation of nuclear DNA (a biochemical hallmark of apoptosis), which can be detected by DNA laddering assay.

The type of apoptotic pathway taken can be deduced from the class of initiator caspase that is activated \(^{(34)}\). In this study, we observed that inhibition of caspase-9 activation by (zLEHD-fmk) caused a reduction in or inhibition of caspase-3 processing. On the other hand, the inhibition of caspase-8 by (zLEHD-fmk) did not cause any change in caspase-3 activity, which indicates that these nucleosides-mediated apoptosis is triggered via activation of caspase-9.

It is known that activation of caspase-9 results in the release of Cyt \(c\) from the mitochondria \(^{(34,65)}\). Indeed, we found that there was a decrease in the amount of Cyt \(c\) in the mitochondrial extracted from the nucleosides treated cells and an increase in its amount in the cytosolic fraction. Thus, these compounds allowed the release of Cyt \(c\), and therefore initiated the apoptotic machinery through the mitochondrial pathway.

The efflux of Cyt \(c\) from mitochondria to the cytosol occurred as early as 8 hrs following treatment with 50 \(\mu\)M of NIA, NIIA and NIIIA, while a decrease in Bcl-2 and Bcl-xl, an increase in the translocation of Bax, cleavage of Bid, induction of Bak and Bad, and over-expression of p53, all took place after 16 hrs post-treatment with nucleosides. These data indicated that there was a leakage of Cyt \(c\), and Bax translocation to the mitochondria depended on the amplification of caspase cascade, and that the induction of p53 in response to DNA degradation triggered the up-
regulation of Bax \(^{40,41,42,66}\). Therefore, this disrupted the balance between the anti-
apoptotic heterodimer (Bcl-2/Bax) to the pro-apoptotic homodimer (Bax/Bax) \(^{42,65}\).

Our study further indicated that NIA, IIA and IIIA were capable of disrupting mitochondrial membrane potential and causing the release of Cyt c, which demonstrated the direct effect of these agents on the mitochondria. Moreover, they caused a decrease in the expression of the anti-apoptotic protein Bcl-xL. Recent studies have shown that Bcl-xL blocks the release of Cyt c \(^{42,55,58,66}\). In addition, our results showed that the interaction was specific for the large subunit of Bcl-xL and not for the small subunit. The large subunit is predominantly localized into the mitochondrial membrane \(^{67}\).

VDAC is an abundant protein in the outer mitochondrial membrane that forms a large voltage-gated pore in the planar lipid bilayer, and seems to serve in combination with the pro-apoptotic members of the Bcl-2 family as a pathway for Cyt c from the mitochondria \(^{40,42}\). However, our data demonstrated no change in the expression of VDAC and its regulatory protein adenine nucleotide translocator (ANT), suggesting that the release of Cyt c might take place through other gates.

Taken together, our findings supported the model that the release of Cyt c from the mitochondria is due to potential alterations of other pores (not VDAC and ANT), enhanced by the increase in the levels of Bax, upregulation by p53, and the decrease in Bcl-2 and Bcl-xL. Although the exact mechanism by which these nucleosides affected mitochondria and induced the release of Cyt c remain poorly formation of ROS could be one of the mechanisms that triggered the depolarization and alteration of mitochondrial membrane permeability \(^{60,61,65}\). ROS induced mitochondrial membrane depolarization through the oxidation of certain amino residues in the pore voltage sensor \(^{68-70}\), causing alterations in membrane
permeability that lead to release of apoptogenic agents from the mitochondria. N-acetyl-L-cysteine (NAC), an antioxidant partially inhibited the generation of ROS partially, which suggested that nucleoside-induced apoptosis may involve factors other than stimulation of reactive oxygen species (ROS). Furthermore, we showed that NIA, NIIA and NIIIA-mediated cell suicide involved chromatin alterations as indicated from the denaturation of DNA with formamide. Formamide denatures DNA in apoptotic cells but not in necrotic cells, or in the cells with DNA breaks in the absence of apoptosis. These chromatin changes resulted from histonal degradation, since histones are known to stabilize DNA against thermal modifications. Accordingly, these data suggested that our nucleoside-induced apoptosis might result in the digestion of histones and therefore alter DNA-histone interactions. Thus, chromatin condensation detected by ss-DNA MAb F7-26 triggered apoptosis as evidenced by the DNA fragmentation upregulation of p53, and increase in ROS production.

p53 is a DNA-binding transcription factor that controls distinct programs of gene expression in response to DNA damage and cellular stress. In response to various stress conditions such as DNA damage, p53 is activated to induce growth arrest to allow cells to repair damaged DNA or apoptosis to eliminate damaged cells, if the damage is severe and repair becomes impossible. p53 is thus, a very broad sensor of many stress conditions. Under normal circumstances, the level of p53 is relatively low in the cell. However, if there is damage to the cell’s DNA, this activates the transcription of p53. The process of activation requires changes in the conformation of the molecule and accumulation of p53. Nucleosides treatment resulted in the increase in the expression of p53. It has been reported that gene transcription is controlled in part by the dynamic acetylation and deacetylation of
histone proteins (75-77). In general, histone acetylation catalyzed by histone acetyltransferases increases gene expression by altering nucleosomal conformation and making chromatin templates accessible by transcription factors (76), while, histone deacetylation catalyzed by HDACs reverses this process to shut down gene expression (76). Thus, precise balance between protein acetylation and deacetylation controls a variety of intracellular activities involving gene expression and protein activation, thereby playing an important role in cell growth, differentiation, and apoptosis. Since activation of p53 is enhanced by histone deacetylases (HDACs) (mainly HDACs -1, -2 and -3), this suggested the involvement of chromatin modification in the process of cell death. To further investigate the role of chromatin modifying complexes in the cytotoxicity of these analogues, we examined the expression of (HDACs), and observed that nucleosides treatment (more prominent in the case of NIA) resulted in inhibition of HDAC 1, 2 and 3 expressions. Previous studies indicated that the aberrant transcription due to altered expression or mutation of genes that encode histone acetyltransferases (HATs), HDACs or their binding partners, is a key event in the onset and progression of cancer (75, 76,79). HDAC inhibitors can reactivate gene expression and inhibit the growth and survival of tumor cells. The remarkable tumor specificity of these compounds, and their potency in vitro and in vivo, underscore the potential of HDAC inhibitors as exciting new agents for the treatment of cancer (78).

Another finding is that these nucleosides affect the expression of Brg1, which is the a subunit of the human SWI/SNF (77). SWI/SNF is a large multi-subunit protein complex that has the general property of disrupting the structure of nucleosomes (77). This interesting complex exists in biochemically distinct forms and specific subunits are required for normal development and tumor suppression (78). We have
demonstrated that the nucleosides analogues tested, specially NIA, decreased the expression of Brgl indicating there might be an enhanced role of SWI/SNF in gene regulation and that SWI/SNF gene might be altered in this type of leukemia, repressing the expression of other genes, which could be the anti-apoptotic genes since a decrease in their expression was detected earlier. By understanding how chromatin structure and function is normally modulated by diverse enzymatic complexes, we hope to gain insight into how these complexes are mistargeted during tumorigenesis. In summary, these nucleosides analogues acted as HDACs inhibitors through the decreasing of the expression levels of HDACs -1, -2 and -3 and indirectly increased the activity of HATs, therefore increasing the activation of p53. This in turn induced cell cycle arrest and allowed the DNA to repair or, if the damage is too severe and can not be contained, p53 induced apoptosis to prevent further cell growth.

Nucleosides also induced a concentration-dependent increase in cells in G0-G1 with a corresponding decrease in cells in S-phase of the cell cycle. G0-G1 is a crucial phase of cell growth because the decision to begin another mitotic cycle is made during this period. Occurrence of DNA damage in G1 poses a particular challenge, because replication of damaged DNA can be deleterious and, since no sister chromatid is present to provide a template for recombinational repair (80-82). It has been proposed that DNA damage is the exclusive signal that triggers the arrest response (80-81). We found that these nucleosides caused a G0 or early G1 arrest. Taken together, this observation, the increase expression of p53 and DNA fragmentation allowed us to propose that p53 can serve as a metabolite sensor activated by these analogues. Accordingly, p53 may play a role in inducing a quiescence-like arrest state in response to nutrient challenge and a senescence-like arrest state in response (apoptosis) to DNA damage. These results have important implications for the
mechanisms by which p53 prevents the emergence of genetic variants and for
developing more effective approaches to chemotherapy based on genotype.
It must be noted that the signaling pathways for arrest and apoptosis, although linked,
can function independently, because the cellular biomacromolecules involved in each
pathway are different. However, the two pathways do have intersections and one such
focus is the p53, although the exact mechanism by which p53 accumulation selects
either arrest or apoptosis is not known. The p53 pathway is probably the most
important pathway to cell cycle arrest and apoptosis. It is also a major target for
mutations in many cancers. Modeling this pathway might shed light on aspects of the
pathway not commonly observed in experiments. This may lead to breakthroughs in
cancer therapy by identifying target molecules and genes that may help manipulate
cell cycles and identify and eliminate cancerous cells.

Moreover, we observed that NIA, NIIA and NIIIA had minimal effects on
normal leukocytes and with a low necrotic rate. As for leukemic samples, these
nucleosides were effective against lymphoblastic leukemia, especially acute B-cell
leukemia and acute T-cell and not against myelogenic leukemia. This might be
because lymphocytes and the cell types such as those in the bone marrow and in the
lining of the intestine are normally rapidly dividing. Therefore, they are more
permeable to such agents, allowing them to exert their cytotoxic effect. Also, the
carcinogenicity of lymphoid leukemia usually result from over expression of Bcl-2
protein which could be reversed by the activation of p53. On the other hand myeloid
leukemia arise from the presence of Philadelphia chromosome, which results from a
reciprocal translocation between chromosomes 9 and 22, and their consequences can
not be corrected by p53 activation.
Taken together, these findings indicated that these nucleosides affected the proliferation of HL-60 cell line, as well as certain types of leukemia, through apoptosis induction, cell cycle arrest and chromatin modification (Fig. IV.24). Therefore, exhibiting a promising anticancer activity.
Conclusions and recommendations

Fig. IV.24: Proposed mechanism of action of the synthetic nucleosides. NIA, IIA and IIIA induce apoptosis via mitochondrial pathway through the activation of caspase-3 and -9, and the release of Cyt c., they also cause accumulation of the cells at the G_0-G_1 phase of the cell cycle, inhibits HDACs -1, -2 and -3, facilitating the role of chromatin modifying complexes (e.g. SWI/SNF) and therefore enhancing the induction of p53, which either induces cell cycle arrest and allows the DNA to repair or induce apoptosis if the damage is too severe and can not be contained.
Chapter V:

Conclusions and recommendations
V. Conclusion(s) and recommendations:

1. Conclusion(s):

Nucleoside analogues are either purine analogues or pyrimidine analogues designed to inhibit DNA/RNA synthesis. In either case, the structures of the drugs closely resemble their natural counterparts. There are several classes of synthetic nucleosides, one of the most important ones being the trifluoromethyl nucleosides. They are of special importance because they exhibit a wide range of biological properties, including anti-tumorigenicity. In order for a drug to be considered an anticancer agent it has to be able to destroy the cancerous cells and spare most normal cells, ideally through the mechanism of apoptosis.

The synthetic trifluoromethyl substituted nucleosides investigated (exclusively NIA) in this study induce apoptosis in time and concentration-dependent manners. This stimulation occurs via the mitochondrial pathway, as observed from the activation of caspase-3, cleavage of PARP and attenuation of apoptosis after pretreatment with caspase-3, -9, and z-VAD inhibitors. Furthermore, treatment with these analogues resulted in Cyt c release in a time-dependent manner. Investigating the mechanisms of Cyt c efflux revealed the disruption of mitochondrial membrane potential, ROS formation, decreases in Bcl-2/ Bcl-XL anti-apoptotic proteins, and no change in Cyt c releasing gates VDAC and ANT, the later suggesting the involvement of other pores. In addition, these agents resulted in accumulation of the cells in the G0-G1 phase of the cell cycle, indicating the degradation of the DNA. Nucleosides also have been found to possess anti-HDACs potential, a relatively new and exciting direction in cancer therapy. These data indicate that these nucleosides could be considered as effective anticancer agents that may lower the toxic risk of chemotherapeutic agents, because they are potent apoptosis inducers with low
necrotic rates in both normal and cancerous cells, even at concentrations as high as 100 μM.
2. Recommendations and future directions:

From the currently known pharmacological data, it can be postulated that nucleosides possess a very high potential to retain its strong cytotoxic activity in soft tumors (e.g. leukemias) and possibly, due to its different cellular uptake and the solubility properties, also in multidrug resistant (MDR) tumors. Nucleosides analogues are taken up by tumor cells by a nucleoside transporter mechanism, and due to its lipophilic properties, their bioavailability is facilitated by passive membrane diffusion. These analogues seem to influence certain membranal transduction mechanisms as well. Therefore, determining which transporter is involved in their delivery into the cells might be a direction for future research. The fact that our nucleosides are highly cytotoxic to a leukemic cell lines and different lymphoblastic leukemia samples through the induction of apoptosis allows us to assume that other, unknown mechanisms of action are responsible for its excellent cytotoxic activity. Testing these agents *in vivo* to determine the best route of administration and the lethal dose and therapeutic index of theses compounds might help to further justify their promising clinical anti-tumor activity. Another approach would be to investigate the pharmacokinetics of these nucleosides (how they are absorbed and metabolized within the body).

Data presented in this thesis shed a light on the promising effects of these nucleoside analogues as potential anticancer agents and open new areas of research into the design and development of better anti-cancer agents.
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الخلاصة

النيوكليوزيدات ونظائرها البنائية تعتبر أحد أنواع التقنيات العلاجية التي تمتلك خاصية مقاومة الأمراض الفيروسبائية والسرطانية. في هذه الدراسة تم تصنيع العديد من النيوكليوزيدات ثلاثية الفلوروميثيل (Trifluoromethyl) (Pyrazole) واحتبارها لإحداث الموت المبرمج للخلايا (apoptosis) في خلايا سرطان الدم الحاد في خلايا المنشأ الأولية للإنسان 60 (Leukemia) من خلال هذه الدراسة وجدنا أن نمو وانتشار خلايا HL-60 قد تم تثبيته نفاذية أكبر لدى استخدام النيوكليوزيدات NIA و NIIA مقارنة بالنظير الآخر NIIA هذا بالإضافة إلى ذلك، فإن ظاهرة تنشيطية (تكسير) الحمض النووي، وهي إحدى العلامات البارزة للموت البرمائي قد تم ملاحظتها ووجد أن شدتها تزداد زيادة التركيز. كما وثبتت الدراسة أن معالجة الخلايا بهذه النيوكليوزيدات أدت إلى نشوب إنزيم ADP المستجيب لإنزيمات الzell (PARP) إلى السائل (mitochondria) (cytochrome c ) بواسطة إزالة البروتينات الحرة (reactive oxygen) (cytoplasm) عن طريق الاحترام الدافع جدار العصبي وتكوين الشوارد الحرة بالإضافة إلى ذلك فإن تعرض الخلايا للكليوزيدات أدت إلى زيادة معدل البروتينات المحفرة (species: ROS) لعملية الموت البرمائي وتشبيط معدل البروتينات المعنوية لهذا العملية (anti-apoptotic) مما أدت النتيجة (cell cycle) من دورة الخلية G1-G1-0، كما أن هذه النيوكليوزيدات أدت إلى تراكم الخلايا في مرحلة DNA الخلوية، أيضاً أثبتت هذه الدراسة أن تلك النيوكليوزيدات لها خاصية مضادة للإنزيمات المضادة لانتقال مجموعة الأستيل (HDACs)، وهذه النتيجة تعتبر من النقط المحورية المستجدة في علاج السرطان. كل هذه النتائج مجتمعة تؤكد أن هذه النيوكليوزيدات قادرة على إحداث الموت المبرمج للخلايا عن طريق الإدخال بالمكثيات بالإضافة إلى قدرتها على تدمير العضيات المنهجية لعملية دورة الخلية، مما يدل على أن هذه النيوكليوزيدات يمكن اعتبارها كيويل ناجع لعلاج السرطان.

الكلمات المفتاحية: النيوكليوزيدات، ثلاثية الفلوروميثيل، 60، مضادات السرطان، الموت المبرمج للخلايا، متغيرات
دراسة تأثير النيوكليوزيدات الجديدة كمضادات للأورام السرطانية على خلايا سرطان الدم الحاد لخلايا المنشأ البشرية

HL-60

رسالة مقدمة من:
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مقدمة إلى:
جامعة الإمارات العربية المتحدة
استكمالاً لمتطلبات الحصول على درجة الماجستير في علوم البناء

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