AMERICAN UNIVERSITY OF BEIRUT

SYNTHESIS OF NOVEL DINUCLEOBASE-HYDRAZIDE TRIAZINE DERIVATIVES AND THEIR ANTI-CANCER ACTIVITY IN COLORECTAL CANCER CELLS

by
SANDY MOUNIR CHOKER

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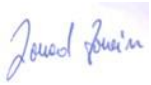

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ABSTRACT
OF THE THESIS OF

Sandy Choker for Master of Science
Major: Pharmacology and Therapeutics

Title: Synthesis of Novel Dinucleobase-Hydrazide Triazine Derivatives and Their Anti- Cancer Activity in Colorectal Cancer Cells.

Background: Colorectal cancer remains a principal cause of cancer-related deaths worldwide in both males and females. Despite significant advances in colorectal cancer therapies, the treatment regimen is still insufficient. Indeed, the anti-metabolite drug, 5-fluorouracil, is highly efficient in treating colorectal cancer treatment; however, its clinical application has extremely declined due to cellular drug resistance. This urges the need for the urgent need of more advanced anti-cancer drugs in order to overcome emerging problems such as multidrug resistance and metastasis. Here, we propose nucleoside analogues as new synthetic agents that act as an effective chemotherapeutic drug in colorectal cancer and suppress their malignant phenotype.

Methods and results: We investigated the anti-cancer activities of nucleoside analogues on the tumor hallmarks such as proliferation, migration, adhesion and angiogenesis. Treatment of human colon cancer cell line, HCT116, with increasing concentrations of nucleoside analogues showed anti-proliferative activity. Additionally, wound healing assay and, respectively, showed that these nucleosides significantly decreased cell migration, while aggregation assay showed an increase in cell aggregation. Furthermore, our analogues inhibited angiogenesis by reducing blood vessel density in CAM assay. These effects were concomitant with the downregulation of focal adhesion kinase and mitogen-activated protein kinases, ERK1/2 and p38.

Conclusion: Taken together, our findings suggest that novel synthetic nucleoside analogues seem to be effective therapeutic drugs against colorectal cancer.

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CHAPTER I

INTRODUCTION

A. Colorectal cancer

1. Epidemiology

Over 190 types of cancers may affect human body cells. Generally, cancer is defined as uncontrolled cell growth and proliferation. In 2018, more than 18 million new cancer cases were reported globally; 5.4 million of in which occurred in Asia making it have the highest occurrence level in the world. Among cancers, colorectal cancer (CRC) is the third most frequent neoplasm in the world. In addition, CRC is one of the most frequent cancer types associated with low survival rate in developed countries [1]. According to the International Agency for Research on Cancer, CRC is among the top five cancer types in Asia. CRC affects both men and women, with a slight increase in its incidence in males [1].

2. Colorectal risk factors

There are many risk factors associated with colorectal cancer. Colorectal cancer significantly rises after the age of 40 years, with 90% of cases diagnosed in people aged fifty and above [2]. Besides age, smoking plays a key role in increasing the risk of colorectal cancer. Contextually, epidemiological evidence showed that the incidence of

colorectal cancer is strongly associated with excessive cigarette and water pipe tobacco smoking [3, 4]. In addition,12 percent of CRC-related deaths are correlated to long-term cigarette smoking [5]. It has been shown that the carcinogens present in cigarette smoke promote tumor formation in the colon and rectum [6]. Furthermore, alcohol intake exacerbated smoking induced risk of colorectal cancer [7]. In fact, DNA mutations induced by smoking are less efficiently repaired in the presence of alcohol. Some researchers suggested that moderate alcohol intake may reduce the risk of CRC while consumption of higher alcohol amounts greatly increases the risk of this cancer [8]. Indeed, a pool analysis of eight cohort studies confirmed that alcohol consumption increases the risk of colorectal cancer [9]. Moreover, it has been shown that intestinal dysbacteriosis form a risk of colorectal malignancy. For instance, butyrate and hydrogen sulphide, components of gut flora, are involved in the development of bowel neoplasia [10]. In addition, the infection with Fusobacterium nucleatum and Bacteroides fragilis is strongly associated with increased risk of large intestinal cancer [11]. Furthermore, physical activity has a major effect on the incidence of colorectal cancer. Studies reported that patients who practice sports on a regular basis have a lower risk of being diagnosed with colorectal cancer as compared to those who are physically inactive[12]. The benefits of physical activity include: re-enforcing the immune system, reducing bowel transit period, reducing hyperinsulinemia, diminishing insulin resistance, and increasing weight loss [13]. These effects contribute to reducing the probability of having colorectal malignancy [13]. Over and above, genome-wide association studies of colorectal cancer demonstrated a strong association between single-nucleotide polymorphisms and bowel cancer risk [14]. Finally,

people who have families with a positive history of adenomatous polyps or bowel cancer in one or more first-degree relatives are at higher risk of being diagnosed with the disease.

3. Colorectal cancer stages

Colorectal cancer commonly originates within the large intestine, an organ of the digestive tract [15]. It results from the progressive evolvement of luminal lining epithelial cells of the bowel into neoplastic cells [16]. Colorectal cancer starts as small and nonthreatening polyps in the mucosa of the colon [15]. In pathophysiological conditions, these polyps become cancerous due to genetic mutations causing colorectal adenoma. In turn, colorectal adenoma cells grow and spread to the whole parts of the body affecting other organs in a process known as metastasis. Generally, colorectal cancer is classified in several stages. In stage 0, the abnormal cells are restricted in the colon inner layer known as mucosa [18]. This stage is also termed intramucosal carcinoma [17]. Throughout stage I, cancer cells do not remain in the thin mucosa muscle layer, but rather invade the colon submucosa without spreading to lymph nodes or adjacent organs [18]. Stage II is further categorized into three subtypes according to tumor growth. First, stage II A, is when the cancer tumor has reached the outmost colon layer and it has not spread to lymph nodes or other parts of the body [18]. Second, stage II B is when the cancer tumor has grown and reached the inner abdominal layer [18]. Third, stage II C is when cancerous cells have spread through the colon and have grown into neighboring tissues without reaching lymph nodes [18]. Stage III is also divided into three subtypes. First, stage III A describes the

growth of tumor into the muscle layers of the intestines while reaching into at least one to three lymph nodes with no spread to nearby organs [18]. Second, stage III B defines the spreading of cancer tumor into neighboring organs and into one to three lymph nodes [18]. Third, stage III C designates that the tumor has reached more than three lymph nodes without affecting distant organs [18]. Stage IV is divided into two subcategories: stage IV A and stage IV B. Stage IV A indicates that the tumor has reached one distant organ such as the liver or the lungs [18]. . Stage IV B refers to a cancer that has reached more than one distant body organ [18]. Due to the staging of colorectal cancer, treatment selection becomes crucial. Doctors usually start to determine colorectal cancer stages in order to identify cancer severity and select the first line of cancer treatment [17]. It is much easier to treat when it is diagnosed in early stages, I and II.

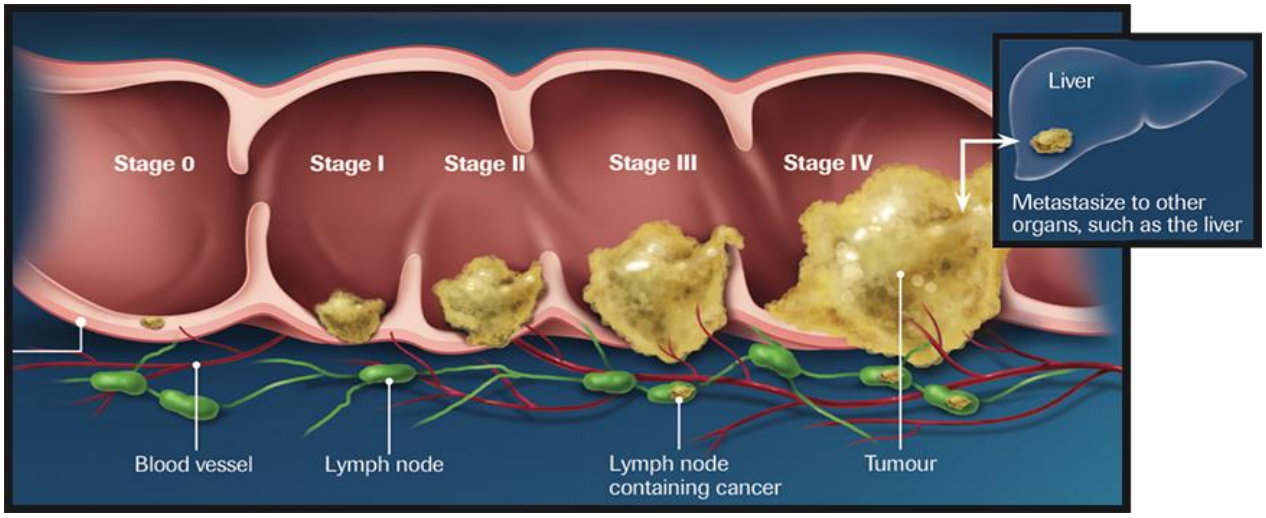

Figure 1: Stages of colorectal cancer. From A guide to colorectal cancer[19]

4. Screening

Earlier diagnosis of colorectal cancer through several screening tests is correlated with better health outcomes and enhanced survival rate. The percentage of the CRC-related mortality was reduced by 51% from1976 to 2014 due to screening intervention [20]. Screening is recommended at the age of fifty for individuals who have average risk of developing CRC. However, individuals at higher CRC risk, due to family history for example, should consider screening at an earlier age [21] [22]. Thus, screening is crucial at an earlier age to find cancer at its early stages and achieve a lower overall number of deaths. There are numerous screening exams that could be used to detect colorectal cancer. These include colonoscopy, computed tomography (CT) colonography, sigmoidoscopy, fecal occult blood test (FOBT) and fecal immunochemical test (FIT), double contrast barium enema (DCBE), and stool DNA tests [21]. The most common way to screen bowel cancer is colonoscopy, a general technique, whereby a doctor uses thin and stretchy metal, a colonoscope, with a camera to check adenomatous polyps or early cancer in the whole colon with the aim of examining these polyps and eradicating them [21]. This process usually requires sedation. However, computed tomography (CT) colonography does not require a sedation process and needs a trained radiologist to obtain the greatest screening data [21]. Unlike colonoscopy, sigmoidoscopy is a screening method that is used to observe the sigmoid part of the colon without going through cecum, right, transverse or left colons [21]. This procedure is used only to detect polyps without the possibility of removing them. Another screening test is used to detect blood in the stool which might reveal the presence of large bowel cancer [21]. This test is known as fecal occult blood test

(FOBT). Recently, the new method used in this domain is DNA stool test by which cancer can be detected by the presence of abnormal DNA in feces[21].

B. Treatment approaches in colorectal cancer

1. Generality

The cancer staging system, type of cancer, as well as the general health status play a crucial part in determining the treatment approaches [23]. There are several approaches including surgery, radiation therapy, chemotherapy, and targeted therapy. For colorectal cancer in stage 0 or I, surgery is the first line of treatment for the exclusion of adenomatous polyps [24]. Starting with stage II and III, surgery alone is not enough to cure this type of cancer [24]. Besides surgery, patients at these cancer stages also need adjuvant chemotherapy to fight against malignancy and prevent the recurrence of cancer [24]. There are several adjuvant chemotherapies with several mechanisms of actions. For instance, evidence suggests that 5- fluorouracil is the most important treatment for around 2 million cancer patients every year. It is an essential agent of fluoropyrimidine class used against bowel carcinoma and associated with cancer-related death reduction. This compound belongs to the antimetabolite family that fights cancer malignancy by inhibiting thymidylate synthase, an enzyme needed for thymine synthesis by the conversion of deoxyuridylate into thymidylate. The mechanism of action occurs through the transformation of 5-fluorouracil into 5-fluoro-2-deoxyuridine-5-monophosphate that grabs thymidylate synthase enzyme and impedes its function. As a result, there is inhibition of thymine production. This inhibition occurs in the presence of N5, N10-Methylene-

tetrahydrofolate cofactor and results in the reduction of DNA synthesis efficacy and induction of cancer cell apoptosis. Multiple problems are associated with 5-fluorouracil treatment such as headache, nausea, scratchy skin, deficiency in white blood cells, and loss of hair [25]. Furthermore, it can cause hand-foot syndrome in patients treated with 5-fluorouracil injection therapy [26]. Accumulating evidence indicates that patients who received fluorouracil in association with other chemotherapy drugs have a higher chance of recovering from lethal cancer than those who received fluorouracil alone. Unfortunately, 5-fluorouracil alone is not sufficiently effective in patients with advanced cancer stages (stage III and stage IV), thus other chemotherapeutic drugs might be used in correlation such as oxaliplatin. Oxaliplatin is often part of the adjuvant chemotherapy as well. Oxaliplatin refers to alkylating antineoplastic agents that works by cross linking both strands of DNA which prevents DNA replication resulting in cell death [27]. Most common side effects of oxaliplatin include neuropathy induction [28].

Additionally, there are some adjuvant chemotherapies that act on a protein called vascular endothelial growth factor (VEGF) and others that target epidermal growth factor receptor (EGFR). VEGF is defined as a protein that is yielded by cancer cells in order to make novel blood vessels. However, EGFR is a protein that is found on tumor cell membranes causing tumor development [29]. Bevacizumab is a VEGF blocker that tightly binds to VEGF and thereby inhibits its binding to its receptor. This action plays a pivotal role in inhibiting the angiogenesis process. VEGF blockers (anti-VEGF) have several adverse effects including hypertension, leukopenia (decrease in leukocytes count), bleeding, anorexia (poor appetite), diarrhea and headaches [29]. EGF stimulates epithelial

mesenchymal transition which in turn was associated with tumorigenesis [30]. During epithelial mesenchymal transition, mesenchymal N-cadherin proteins are overexpressed while epithelial E-cadherin proteins are downregulated, both of which contribute to tumor development by increasing motility capacity and migration [31]. The most effective EGFR-inhibiting agents used against colorectal cancer are monoclonal antibodies (mAbs) such as cetuximab and tyrosine kinase inhibitors (TKIs) such as gefitinib [32]. In contrast to monoclonal antibodies that target the extracellular region of EGFR, tyrosine kinase inhibitors target receptor tyrosine kinases [32]. Treatment with monoclonal antibodies may cause unwanted side effects including large amounts of protein in urine (proteinuria), high blood pressure, diarrhea, and toxic hepatitis. It can also induce severe allergy (skin rash) and underactive thyroid [33].

2. Nucleoside analogues

Nucleotides are the core constituents of nucleic acids formed from sugar, phosphate and nitrogenous base. This base could be either pyrimidine such as thymine in DNA, and uracil in RNA or purine such as adenine and guanine. In the absence of a phosphate group, the nucleotide compound is recognized as nucleoside. These two intrinsic residues, nucleotide and nucleoside, play an important role in nucleic acid building, catabolism, anabolism and signal transduction. Nucleoside analogs mimic the genuine nucleosides by several processes including their entry into cells; their phosphorylation metabolic pathways and eventually, their incorporation into DNA chain instead of endogenous nucleoside [34].

Consequently, DNA chain elongation and nucleic acid synthesis are stopped leading to inhibition of cellular proliferation. Nowadays, the synthetic nucleosides are being applied in treatment of neurological disorders, gouts, and viral infections but mainly, they are used in cancer treatment. Concerning anti-neoplastic nucleosides analogs, they are classified into two groups: purine and pyrimidine analogs. Both synthetic compounds are structurally similar to endogenous nucleosides and undergo the same mechanism of action. There are several purines and pyrimidines analogs discovered till now. Based on a study, 6-mercaptopurine and 6- thioguanine were among the discovered purine antagonists showing efficiency in cancer treatment [35]. Currently, recent synthetic purines, such as cladribine and fludarabine, have been discovered to demonstrate high anti-cancer activity, in particular, against neoplastic blood disease [36-40]. Concerning cytotoxic pyrimidine antagonist class, cytarabine and gemcitabine exhibited apoptotic actions respectively in blood and breast carcinoma [41-43]. All these compounds are meant to substitute the endogenous nucleotide, a DNA precursor, with an exogenous nucleoside analogue by several mechanisms of action to potentiate cytotoxicity and lead to cancer cell death.

Researchers' findings show that nucleoside and nucleotide analogues belong to antimetabolite drugs that act by several processes [44]. These processes might be interfering with the synthesis of nucleic acids leading to the prevention of mitosis and cessation of tumor development. It may also cause the inhibition of enzymes like thymidylate synthase leading to nucleic acid destruction[45].

a. Mechanism of action of nucleoside analogues

Due to their sturdy water affinity, the ability of nucleoside and nucleotide analogs to cross cell membrane is almost impossible [35]. Thus, the only salvage pathway of therapeutic drugs to prompt cytotoxicity is their passage through the nucleoside membrane transporter [46]. Upon their entrance, there are four ways of mechanisms that can occur. First of all, these transported compounds undergo intracellular modifications known as phosphorylation [47]. The phosphorylation process occurs by a wide variety of specific enzymes. These enzymes include nucleoside kinases, nucleoside monophosphate kinases and nucleoside diphosphate kinases, altogether leading to the formation of the active nucleoside analogue triphosphate [47]. Nucleoside analogue triphosphate is integrated in newly synthesized nucleic acids of cancer cells and induce cancer cell death through DNA synthesis inhibition [47]. Second, other synthetic nucleosides showed an inhibitory effect on the ribonucleotide reductase enzyme [48, 49]. Ribonucleotide reductase is an enzyme responsible for the conversion of ribonucleotide diphosphates into deoxyribonucleotides [50]. Nucleoside analogues inactivate ribonucleotides transformation into deoxyribonucleotides and affect DNA repair mechanism by increasing its affinity to nucleoside analogues incorporation [35]. Third, once nucleoside analogue constitutes a specific substrate for cytidine deaminase active site, a harmless compound is formed due to rapid synthetic nucleoside catabolism reaction [35]. Fourth, although the nucleotidase kinase catalyzes the phosphorylation process, cytosolic nucleotidase might work against it and promote the elimination of nucleoside analogue phosphate group [35]. This leads to

the deactivation or inhibition of nucleoside analogues triphosphate formation thus altering the pool of deoxyribonucleotide.

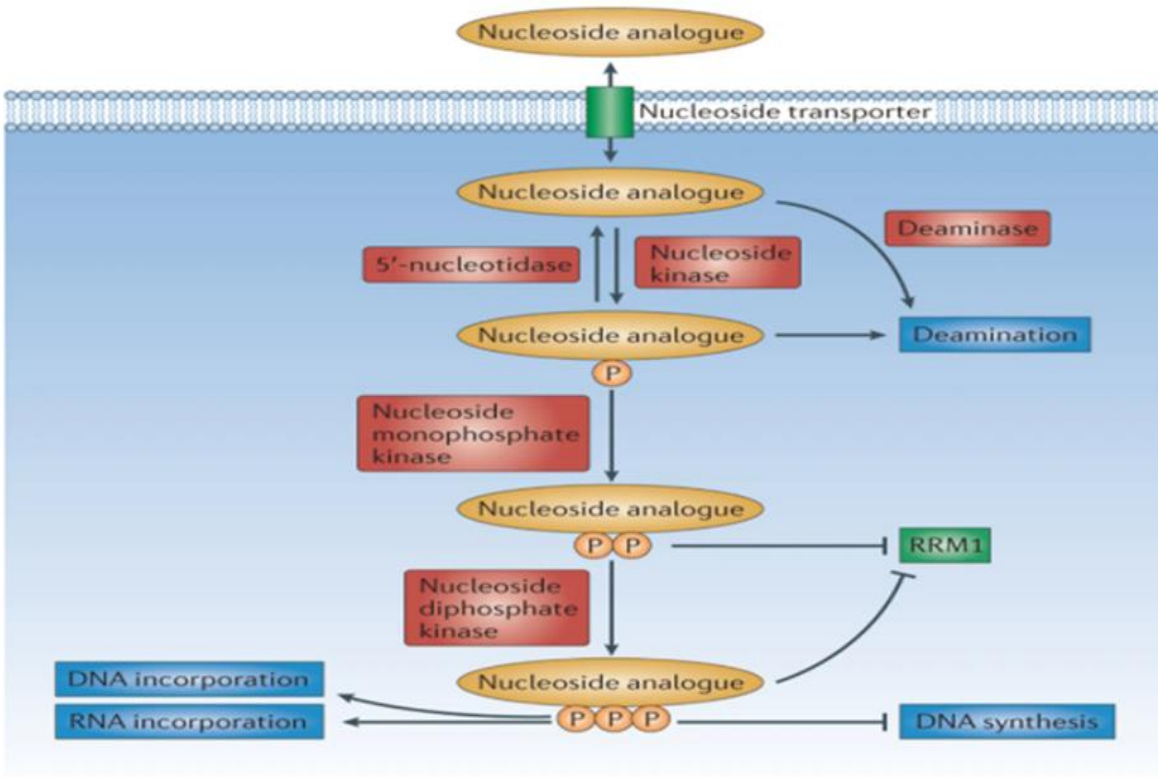

Figure 2:Mechanism of action of nucleoside analogues from: Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases[47]

b. Drug Resistance

Despite the antineoplastic treatment, tumor cells remain dividing and proliferating without cessation. This mechanism is known as drug resistance. Resistance phenomenon is modulated by myriad factors. It can occur in single or multiple steps of physiological modifications of nucleoside analogues resulting in cell death inhibition. Among these factors, there are: 1) the reduction of total amount of nucleoside transporters proteins, 2) the

increase of cytoplasmic nucleotidase dephosphorylation activity, 3) the increase of cytidine deaminase responsible in the deamination activity of cytotoxic agents and 4) the decrease in cytosolic kinases functions [35, 51,54]. All these factors are due to alterations in cancer cell physiology and cause a drop in the abundance of cytosolic therapeutic compounds leading to apoptosis inhibition. There are also other factors including impaired ribonucleotide reductase inhibition or loss of DNA polymerase activity that cause a decrease in the ability of modification of DNA strands and consequently lack of response of neoplastic tumor to cytotoxic drug therapy [35].

c. Approaches for resistance problem

Nucleoside analogues undergo subsequent steps in order to exert their biological effect. Remarkably, they inhibit the incorporation of endogenous nucleoside precursors by substitution reaction causing the inhibition of DNA strand synthesis. Following a stepwise manner, to overcome the mechanism of cancer cell resistance and allow apoptosis induction, enough concentration of therapeutic agents must be administered to ensure proper targeting the tumor. Eventually, upon their entry inside tumor cells and their conversion into nucleoside analogue triphosphate derivative which is an active form the of the compound that induces cytotoxicity [55, 56]. Another suggested solution would be the synthesis of a non-degradable compound that possesses anti- cytoplasmic nucleotidase and/or anti-cytidine deaminase activity [35]. Thereby, the inhibition of the dephosphorylation of monophosphate derivative and/or the blockage of therapeutic drug deamination is one of the rescue processes to get rid of resistance problems. Researchers

could also utilize a combination of anti-neoplastic drug and cytosolic nucleotidase or cytidine deaminase inhibitors in order to increase the anti-tumor activity and prevent resistant mechanisms [35]. Also, the last proposition suggests synthesizing anticancer compounds that can cause deprivation of endogenous deoxyribonucleotides by inhibiting ribonucleotide reductase action.

3. Colorectal cancer complications

Despite the significant improvements made in treating large bowel cancer and the high accuracy obtained in identifying this cancer type, hepatic metastasis remains a major challenge in prolonging overall survival. In patients suffering from large intestinal cancer, liver occupies the first prevalent site of metastatic spread [57]. Around seventy percent of patients diagnosed with bowel cancer undergo metastatic spread to the liver organ [58]. Moreover, only fifteen percent of these patients might undergo surgical resection [59]. Unfortunately, with no treatment, patients with liver metastasis cannot survive more than eight months [60]. This is a clear indication of the elevated need for novel and promising therapeutic drugs.

C. The progression of Colorectal cancer

1. Hallmarks of Colorectal cancer

a. Proliferation

A wide range of studies has demonstrated that cellular proliferation, differentiation and apoptosis are crucial physiological processes within the organism. These three processes are necessary and beneficial to preserve homeostasis and to assure the development of multicellular organisms. There is convincing evidence suggesting a correlation between proliferation and cell death. Consequently, deregulation of either cellular proliferation or cell death is tightly associated with myriad of diseases including cancer [61]. Studies have shown that there are many signaling pathways involved in proliferation, the first hallmark of intestinal tumorigenesis, . One of the most important pathways is the aberrant activation of mitogen- activated protein kinases that induce proliferation and initiate colorectal carcinogenesis [62]. This may be implicated as a novel chemopreventive target in bowel cancer treatment [63]. Other pathways include the Notch pathway which is highly expressed in bowel cancer cells and play a pivotal role in triggering pathogenesis [64]. Wnt signaling pathway participates in the proliferation of normal cells. , The disruption of this pathway is tightly associated with the development of colorectal malignancy. Therefore, either Notch signaling inhibition or Wnt signaling inhibition form an effective and beneficial therapeutic approach against bowel cancer [65]. Furthermore, TGF-β signaling cascade is implicated in a wide variety of cellular processes such as proliferation, differentiation and apoptosis in healthy cells. In contrast, this pathway is over activated in malignant cells, especially in late stages, inducing the acquisition of

large intestinal cancer through a high production of growth factors [66]. Further studies have reported that the inhibition of TGF-β pathway is a promising targeted therapy and can prevent colorectal malignancies [66] .

b. Migration

Even though intestinal cancer mortality rate decreased throughout recent years, cancer metastasis remains the main cause of death worldwide. One of the prerequisite steps of metastasis is migration. That in turn is considered as a dissemination of metastatic cells from initial tumor site, their invasion through basement membrane to reach blood or lymphatic channels and eventually their migration in order to reach a distant organ [67]. In other terms, migration is another hallmark of advanced intestinal cancer malignancy. In order to migrate from the initial site, the cell undergoes a variety of morphological changes known as epithelial mesenchymal transition (EMT). This process is attributed to the conversion of epithelial cells into migratory cells of mesenchymal phenotypes [68]. In contrast to carcinoma epithelial cells that are tightly connected with intercellular connections, mesenchymal cells have junctions that permit the acquisition of a higher invasive and migratory ability [69]. In addition, mesenchymal cells have increased resistance to apoptosis and an increased expression of extracellular matrix (ECM) components [70]. It has been shown that the morphological changes of the migrating cell include polarization, elongation, and formation of new cytoplasmic protrusions at the leading edge [71]. These protrusions can be either broad lamellipodia, or spike-like filopodia [72]. The polarization of moving cells is promoted by a wide range of growth

factors inducing the stimulation of Rho family [73]. As a result, both the tumor cells and neighboring extracellular matrix (ECM) are adhered together [71]. However, the migration of the cancer cells is restricted by the extracellular matrix (ECM). ECM-degrading proteolytical enzymes known as metalloproteinases degrade extracellular matrix such as fibronectin and collagen components [74]. Thus, facilitating the movement of the cancer cell. Subsequently, neoplastic cells contract and detach from the extracellular matrix (ECM) [75]. In addition, published evidence shows that there is an established relationship between the increased level of metalloproteinase-1 and advanced colorectal tumor migration and invasion [76]. Thus, targeting metalloproteinase-1 emerged as a potential approach for the development of anti-cancer treatment [77].

c. Adhesion

Adhesion process is not only a hallmark of bowel cancer, but also one of the steps required for metastasis to occur. During this process, cells adhere to the extracellular matrix (ECM) of adjacent organs through proteins and make focal adhesion areas. These proteins are identified as cell adhesion molecules such as cadherins and integrins. Several lines of evidence indicated that there is an integral role between the altered expression of these specific molecules and the development of malignancy [78]. Once integrin is activated, it produces a signaling cascade involved in many biological processes including growth, proliferation and migration of malignant cells [79]. This action is mediated by the non-receptor tyrosine kinases; focal adhesion kinase (FAK) and steroid receptor coactivator (Src) [80]. Upon integrin clustering, focal adhesion kinase (FAK) undergoes

autophosphorylation at its tyrosine residue 397 (Y397) [80]. The phosphorylated (Y397) presents a binding site for steroid receptor coactivator (Src) which in turn phosphorylates more focal adhesion kinase sites [81, 82]. All of these signaling pathways result in an enhanced activity of focal adhesion kinase protein (FAK) [82]. A wide range of studies indicates that the overexpression of FAK is associated with intestinal cancer metastasis [83]. Consequently, the Fak-Src binary complex is formed and undergoes a cascade of intracellular phosphorylation processes [80]. Both paxillin and p130 Crk-associated substrate (CAS) are composed of a wide variety of motifs involved in protein-protein association and they are phosphorylated on their tyrosine residues by FAK-Src complex [84-86]. Interestingly, fibroblasts deprived from focal adhesion kinase (FAK) diminish the motility of tumor cells. Overall, the adhesion process should be inhibited in order to prevent colorectal cancer progression.

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d. Aggregation

One of the cancer hallmarks is the ability of malignant cells to lose cell to cell adhesion. This characteristic impairs the aggregation process in colorectal cancer cells. Physiologically, healthy cells stick to adjacent cells through adhesion cell molecules whereas malignant cells lack this adhesiveness in order to spread and reach distant organs [87]. It is well established that integrins and cadherins are implicated in cell aggregation. They are involved in the remodeling of actin cytoskeleton [88-90]. Integrins promote cell-extracellular matrix (ECM) adhesion whereas cadherins promote homotypic cell-cell adhesion [91]. Cadherins are calcium dependent proteins that promote calcium dependent

adhesion. Researchers suggest a tight relationship between the downregulation of E-cadherins and the progression of colorectal cancer [92]. In addition, soluble E-cadherin is increased in patients suffering from bowel cancer [93, 94]. Another adhesion cell molecule, vinculin, is also involved in the aggregation process. It is an actin-binding protein that mediates cellular adhesion. Different studies have revealed that the down expression of vinculin is correlated with worse outcomes. This downregulation is associated with cancer invasion and metastasis [95]. Interestingly, the normal level expression of vinculin plays a pivotal role in preventing bowel cancer progression [95].

Overall, the imbalance in the signaling pathways of several adhesion cell molecules is correlated with bowel cancer progression. The upregulation of these adhesion molecules might be an attractive chemopreventive strategy against bowel cancer.

e. Angiogenesis

Angiogenesis, also referred to as vascularization, is essential for tumor growth and metastasis., Targeting tumor angiogenesis has become a promising approach in the development of new anti-cancer drugs that limit cancer progression. Angiogenesis is a biological process that promotes the formation of new blood vessels. This process is regulated by an equilibrium between pro-angiogenic agents and anti-angiogenic agents [96]. Disruption of this equilibrium mediates colorectal neoplasm development and disrupts vascular homeostasis [96, 97]. In other words, when angiogenesis is dysregulated, it is responsible for intestinal cancer metastasis. The mechanism of angiogenesis is as follows: First of all, angiogenic factors bind to endothelial cells [98]. These cells are activated and

secrete metalloproteinase enzyme (MMP) [98]. Then, local damage occurs at the level of the basement membrane by the metalloproteinase enzyme leading to cell migration [98]. Migrating cells proliferate and induce neovascularization with the aid of integrins [99, 100]. Upon pathophysiological conditions tumor cells produce vascular endothelial growth factor (VEGF) and angiopoietin family which are considered as angiogenesis promoters [101]. However, thrombospondin-1 and angiostatin are known as anti-angiogenic factors acting as angiogenic inhibitors. Thus, in colorectal cancer, there is an overexpression of angiogenic activators by malignant cells and low expression of endogenous angiogenic inhibitors [102]. proteases antagonists, angiogenic growth factors inhibitors, and adhesion cell molecules inhibitors are important antagonistic agents used against angiogenesis in colorectal cancer [98]. As mentioned before, mAbs, VEGF antagonist, and bevacizumab are the first FDA-approved antiangiogenic drugs used against metastatic large bowel cancer [102].

CHAPTER II

RATIONALE OF STUDY AND AIMS

Despite major achievements in cancer research and treatment, colorectal cancer remains the third type of cancer that lead to death worldwide [103]. Proliferation, adhesion, migration, invasion, angiogenesis and apoptosis are the key hallmarks of cancer and play a pivotal role in metastasis. Nowadays, colorectal cancer metastasis is strongly associated with the increased risk of mortality and morbidity. Although the anti-metabolite drug, 5-fluorouracil, showed a high impact in colorectal cancer treatment, around eighty-five percent of patients treated with 5-fluorouracildid not demonstrate an increase in overall survival time [104] [105]. In addition, the resistance phenomenon of bowel cancer cells developed against 5-fluorouracil remains a major problem in cancer therapy. This mandates the need of novel approaches that fight against both colorectal cancer metastasis and resistance phenomenon.

The aim of this study was to investigate the effect of novel synthetic nucleobase analogues: Adenine, Thymine and Uracil analogues on human colorectal cancer cells. We hypothesize that nucleoside analogues modulate the metastatic phenotype of colorectal cancer cells by attenuating their viability, migratory capacity, and angiogenic potential. In addition, these analogues can increase the aggregation of colorectal cancer cell line.

CHAPTER III

MATERIALS AND METHODS

A. Cell lines and culture

HCT116 human colon cancer cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 Medium. The media was supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin. Cells were incubated at 37 °C in 5% CO_2. The culture medium was changed the next day. Once the cells reached 95% confluence, cells were split at a ratio 1 to 7.

B. MTT (3-(-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

HCT116 were grown in 96-well plates at a density of 6000 cells per well in 200 μM complete RPMI-1640 and placed at 37 °C in 5% CO_2 humidified incubator until 60% confluency. Twenty-four hours later, media was removed and HCT116 cells were treated with increasing concentration of nucleoside analogues (1-10-100-500- 1000 μM) for the different time points (24 hours, 48 hours,72 hours). However, cells incubated in culture medium alone served as a control for cell viability. Following treatment with nucleoside analogues, the culture media was removed and twenty microliters of MTT solution was added to each well. The cells were incubated in aluminum foil at 37 °C in 5% CO_2 for three hours. Then, the media in wells was removed, and 200 μL of dimethyl sulfoxide (DMSO) were added to each well. The plate was left under continuous shaking for 20 minutes in the

dark to dissolve the formazan crystals. Eventually, optical densities were measured on the Elisa Multiskan Reader (Thermo) at a wavelength of 595 nm. Absorbance is proportional to cell viability.

C. Monolayer Scratch Assay

HCT116 were seeded in 12-well plates at a density of 750000 cells per well in 1 ml complete RPMI-1640 and placed in 37°C in a 5% CO_2 humidified incubator until 95% confluency for 24 hours. By using a 10 μL sterile pipette tip, a uniform wound was scratched. The medium was removed, and cells were exposed to nucleoside analogues treatment with a concentration of 500 μM. However, cells incubated in culture medium alone with DMSO served as control. The wound healing was monitored at 0,2,4,6,8,12 and 24 hours. Several photomicrographs were taken using a Zeiss phase contrast microscope. ZEN imaging software (blue edition) from Zeiss was used to measure the width of the scratch.

D. Aggregation assay

HCT116 cells were seeded in a 35 mm plate at a density of 1,000,000 cells/plate. They were then incubated at 37°C/5% CO_2 for 24 hours. By using sterile 2 mM EDTA in Ca^{2+} /Mg^{2+} free PBS, cells were detached. After washing with EDTA, cells were centrifuged to get rid of EDTA. The pellet was resuspended in 1mL PBS with or without nucleoside analogues treatment. The

cells were left under continuous shaking (90 rpm) for (60,120,240 mins). Then, they were fixed with 1% formaldehyde. Several microscopic pictures were taken, and cells were counted.

E. Chorioallantoic membrane (CAM) assay

Fertilized eggs were cleaned with 70% ethanol and were incubated under conditions of 37°C with 50% humidity. The eggs were left under continuous rotation for 6 days. On day 6, a small window was opened above the CAM. Then, 30 μM of desired concentrations of control (vehicle) or nucleoside analogue was placed directly onto the CAM through the eggshell opening. The window was sealed with parafilm and the eggs were returned to the incubator at 37˚C and 50% humidity. After treatment, pictures were taken at 0 hours and after 24 hours and they were analyzed using AngioTool software in order to quantify the length of the vessels and number of junctions.

F. Western Blotting

HCT116 cells were seeded in a 12 wells-plate at a density of 500000 cells per well in 1 ml of RPMI 1640 and they were then incubated at 37°C in a 5% CO_2 for 24 hours until 70% confluency. After 24 hours, cells incubated in culture medium alone served as a control. However, treated cells with nucleoside analogue (500 μM) were incubated together for 10, 30 and 60 min. One hour later, HCT116 cells were washed with 200 μL of PBS and lysed using 100 μL of lysis buffer. Protein quantification was done using Bradford method

and 15-20 micrograms (equal protein quantities) were loaded in 10%-15% SDS-polyacrylamide gel electrophoresis. Proteins were transferred using PVDF (polyvinylidene difluoride) membrane. After blocking in 5% fat-free milk in TBS-T, 1 h at room temperature, the membrane was incubated overnight with the relevant primary antibody at 4 °C. The membrane was then washed three times with TBS-T for 10 min each and incubated with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature. The membrane was washed again (three times with TBS-T, 10 min each) and then developed using enhanced chemiluminescence (ECL clarity, Biorad) and quantified using Chemidoc MP Imaging system (Biorad).

G. General procedure for the synthesis of the nucleobase-hydrazide Triazine derivatives

A solution of nucleobase-hydrazide (2 equiv, 1.35 mmol), 2-amino-4,6-dichloro-1,3,5-triazine (1equiv, 0.67 mmol) and $NaHCO_3$ (2 equiv, 1.35 mmol) was stirred in a sealed tube in DMF (6 ml) for 2 - 4 h at 110°C. The reaction mixture was cooled to Room Temperature, washed with acetone (3x30 ml) and centrifuged. The formed precipitate was washed again with Acetone/Methanol 8:2 (6-10 x10 ml), centrifuged and dried under reduced pressure to give the pure product as a solid. The four products were identified on the basis of their NMR spectral data. The ^{1}H and ^{13}C NMR of all the synthesized compounds are provided in the experimental part.

H. Chemicals and instruments

Reagents used for the synthesis were purchased from the Aldrich Chemical Company, ACROS Chemicals, Fisher Scientific Company and were used as received. Thin layer chromatography (TLC) was performed on pre-coated TLC-sheets ALUGRAM SIL G/UV254 silica gel sheets (used directly as received). Flash chromatography employed Aldrich silica gel (60 Å, 230–400 mesh). Melting points were measured on a DigiMelt apparatus and were uncorrected. NMR spectra were obtained from deuterated solvents with tetramethylsilane (TMS) as an internal standard on a Bruker Avance III HD 500 NMR spectrometer. Chemical shifts (δ) are quoted in ppm downfield relative to TMS. Coupling constants (*J*) are stated in Hz and the employed resonance multiplicity abbreviations are s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet.

I. Synthesis of the dinucleobase-hydrazide triazine derivatives

1. *General procedure for the synthesis of the nucleobase ester derivatives 2a, 2b, and 2c with the general formula, Nu-$(CH_2)_2COOCH_2CH_3$, via Michael addition, where NuH is uracil, thymine, or adenine nucleobase*

To a suspension of the corresponding nucleobase (for compound **1a** 10 g, 89.2 mmol; for compound **1b** 10 g, 79.3 mmol; for compound **1c** 10 g, 74 mmol) in Ethanol (100 ml), sodium ethanoate NaOEt (for compound **1a** 4.2 mL, 53.5 mmol; for compound **1b** 3.7 mL, 47.6 mmol; for compound **1c** 3.5 mL, 44.4 mmoL) was added at room temperature, followed by addition of ethyl acrylate (for compound **1a** 11 mL, 102.6 mmol;

for compound **1b** 9.8 mL, 91.2 mmol; for compound **1c** 9.2 mL, 85.1 mmol). The resultant mixture was refluxed overnight after which the reaction was allowed to cool to r.t. The solution was reduced to minimal volume to afford a quantitative white precipitate. The obtained white solid was collected via suction filtration, washed thoroughly with cold ethanol and dried under reduced pressure to yield the desired product.

Synthesis of the Guanine ester derivative 2d

A round bottom flask was charged with guanine (10 g, 66.22 mmol) in *N*, *N*-dimethylacetamide (DMA, 30 mL) and acetic anhydride (16.5 mL, 168 mmol). The reaction mixture was stirring overnight at 165°C after which it was cooled to room temperature. The precipitate was collected and triturated with water/Ethanol 1:1 (30 mL) at 80°C for 2-3 hrs. The precipitate was collected and dried under reduced pressure at 70°C to yield a white solid. The solid was transferred to a 500 mL round bottom flask with DMF (50 mL). An ethanolic solution of sodium ethoxide NaOEt (2 mL, 25.9 mmol) was added and the mixture was stirred for 15 min. Ethyl acrylate (3.8 mL, 34.96 mmol) was added and the mixture was refluxed at 165 °C for 1h. The solvent was evaporated, and the residue was dissolved in methylene chloride and washed with distilled water. The organic layer was separated, dried with anhydrous $MgSO_4$, filtered and the solvent evaporated under reduced pressure. The crude was then triturated with toluene (250 mL) with stirring overnight at room temperature. The precipitate was collected, dried under reduced pressure and purified by column chromatography on silica gel CH_2Cl_2/MeOH (98:2) to yield 2d as a white solid (7.03 g).

Ethyl 3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl) propanoate (2a). Color: white. yield: 76 %. mp: 102.9–103.6 °C (lit.[106] mp 101.3–101.6 °C). ^{1}H NMR (500 MHz, DMSO-d_6, δ): 11.27 (s, 1H), 7.52 (s, 1H), 4.06 (q, *J* = 7.1 Hz, 2H), 3.86 (t, *J* = 6.8 Hz, 2H), 2.67 (t, *J* = 6.8 Hz, 2H), 1.74 (s, 3H), 1.17 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6, δ): 171.2, 164.8, 151.3, 142.3, 108.6, 60.7, 44.3, 33.3, 14.5, 12.4. HPLC/ESI-MS (*m/z*): calcd for $C_{10}H_{14}N_2O_4$ 226.10, found 249.0 $(M + Na)^+$, 475.1 $(2M + Na)^+$. FT-IR (KBr): 760, 797, 879, 928, 1013, 1065, 1137, 1201, 1288, 1323, 1350, 1380, 1416, 1463, 1693, 1707, 2088, 3043, 3168 cm^{-1}. The spectroscopic data agrees with the one reported.[107]

Ethyl 3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl) propanoate (2b). Color: white. yield: 65 %. mp 154.4–155.6 °C (lit.[106] mp 154.6–155.1 °C). ^{1}H NMR (500 MHz, DMSO-d_6, δ): 11.27 (s, 1H), 7.52 (s, 1H), 4.06 (q, *J* = 7.1 Hz, 2H), 3.86 (t, *J* = 6.8 Hz, 2H), 2.67 (t, *J* = 6.8 Hz, 2H), 1.74 (s, 3H), 1.17 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6, δ): 171.2, 164.8, 151.3, 142.3, 108.6, 60.7, 44.3, 33.3, 14.5, 12.4. HPLC/ESI-MS (*m/z*): calcd for $C_{10}H_{14}N_2O_4$ 226.10, found 249.0 $(M + Na)^+$, 475.1 $(2M + Na)^+$. FT-IR (KBr): 760, 797, 879, 928, 1013, 1065, 1137, 1201, 1288, 1323, 1350, 1380, 1416, 1463, 1693, 1707, 2088, 3043, 3168 cm^{-1}. The spectroscopic data agrees with the one reported. [108]

Ethyl 3-(6-amino-9H-purin-9-yl) propanoate (2c). Color: White. Yield: 90%. mp: 167-168 °C. ^{1}H NMR (300 MHz, CDCl3, δ, ppm): 1.20-1.25 (t, 3H, CH_3, CH_3-CH_2-O), 2.91-2.93 (t, 2H, CH_2, N-CH_2-CH_2-CO), 4.09-4.17 (q, 2H, CH_2, CH_3-CH_2-O), 4.48-4.50 (t, 2H, CH_2, N-CH_2-CH_2-CO), 5.69 (s, 2H, NH_2, C-NH_2), 7.91 (s, 1H, Ar-CH), 8.36 (s, 1H, Ar-

CH). ^{13}C NMR (75 MHz, CDCl3, δ, ppm): 14.1 (1C, CH_3, CH_3-CH_2-O), 34.16, 39.44, 61.55 (3C, 3CH_2, N-CH_2-CH_2-COO-CH_2-CH_3), 141.28, 153.00, 155.37 (3C, purine ring), 170.92 (1C, C=O, -CH_2-COO-CH_2-CH_3). FT-IR (KBr, ν, cm^{-1}): 1733 ν(C=O) (ester), 1204 ν(C-O) (ester), 1609, 3315 ν(NH) (amine), 1481 ν(C=N) (Ar-imine). The spectroscopic data are in agreement with the literature.[109, 110]

Ethyl 3-(2-acetamido-6-oxo-1,6-dihydro-9H-purin-9-yl) propanoate (2d). Color: White. Yield: 38%. mp: 215-217 °C. FT-IR (KBr, ν, cm-1): ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 12 (s, 1H), 11.73 (s, 1H), 7.95 (s, 1H), 4.28 (t, *J* = 7.0 Hz, 2H), 4.04 (q, *J* = 7.1 Hz, 2H), 2.91 (t, *J* = 7.0 Hz, 2H), 2.16 (s, 3H), 1.12 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6, δ, ppm): 173.5, 170.4, 154.8, 148.6, 147.7, 139.8, 120.0, 60.4, 38.9, 33.6, 23.7, 13.9.

2. *General procedure for the synthesis of the nucleobase-hydrazide derivatives 3a, 3b, 3c and 3d*

The nucleobase hydrazides were prepared by refluxing the corresponding ester (for compound **2a**, 14 g, 66 mmol; for compound **2b** 9 g, 40 mmol; for compound **2c** 10 g, 42.5 mmol; for compound **2d** 7 g, 23.9 mmol) with hydrazine monohydrate (for compound **2a** 11.1 mL, 231.1 mmol; for compound **2b** 6.7 mL, 140 mmol,; for compound **2c**, 7.1 mL, 149 mmol; for compound **2d**, 4.1 mL, 83.6 mmol) in ethanol (20 mL for 1 g ester) for 24 hrs. An off-white to white precipitate formed upon cooling, was filtered under reduced pressure, washed with cold ethanol and dried under vacuum to give the desired product.

3-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl) propanehydrazide (3a). Color: off-White. Yield: 77%. mp 184.3–184.9 ˚C (lit.[111] >300 ˚C). ^{1}H NMR (500 MHz, DMSO-d_6, δ): 10.99 (s, 1H), 9.11 (s, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 5.50 (d, *J* = 7.8 Hz, 1H), 4.29 (s, 2H), 3.85 (t, *J* = 6.7 Hz, 2H), 2.40 (t, *J* = 6.7 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6, δ): 169.3, 164.3, 151.2, 146.6, 100.9, 45.1, 32.8. HPLC/ESI-MS (*m/z*): calcd for $C_7H_{10}N_4O_3$ 198.08, found 221.0 $(M + Na)^+$, 419.0 $(2M + Na)^+$, 197.5 $(M – H)^–$, 417.0 $(2M + Na – 2H)^–$. FT-IR (KBr): 458, 510, 552, 584, 678, 726, 765, 793, 831, 903, 952, 993, 1010, 1034, 1057, 1114, 1157, 1190, 1202, 1255, 1285, 1333, 1362, 1387, 1398, 1430, 1446, 1468, 1550, 1614, 1689, 2783, 2880, 2974, 3066, 3181, 3312 cm^{-1}. The spectroscopic data agrees with the one reported.[112]

3-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl) propanehydrazide (3b). Color: White. Yield: 92 %). mp 193.5–195.8 ˚C (lit.[111] 189–190 ˚C). ^{1}H NMR (500 MHz, DMSO-d_6, δ):11.24 (s, 1H), 9.08 (s, 1H), 7.42 (s, 1H), 4.21 (s, 2H), 3.82 (t, *J* = 6.8 Hz, 2H), 2.39 (t, *J* = 6.8 Hz, 2H), 1.73 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6, δ): 169.4, 164.8, 151.2, 142.4, 108.5, 44.9, 32.9, 12.4. HPLC/ESI-MS (*m/z*): calcd for $C_8H_{12}N_4O_3$ 212.09, found 235.0 $(M + Na)^+$, 447.1 $(2M + Na)^+$. FT-IR (KBr): 451, 493, 540, 562, 635, 654, 693, 880, 915, 950, 996, 1011, 1050, 1113, 1224, 1248, 1272, 1346, 1367, 1422, 1486, 1522, 1636, 1667, 1695, 2824, 2925, 3019, 3103, 3155, 3347 cm^{-1}. The spectroscopic data agrees with the one reported.[113]

3-(6-Amino-9H-purin-9-yl) propanehydrazide (3c). Color: White. Yield: 98 %. mp 269-271 °C. ^{1}H NMR (300 MHz, D_2O-D_2SO_4, δ, ppm) 3.02-3.03 (t, 2H, CH_2, N-CH_2-CH_2-CO), 4.63-4.66 (t, 2H, CH_2, N-CH_2-CH_2-CO), 8.39 (s, 1H, Ar-CH), 8.48 (s, 1H, Ar-CH).

^{13}C NMR (75 MHz, D_2O-D_2SO_4, δ, ppm): 35.3, 42.5 (2CH_2, N-CH_2-CH_2-CO), 120.5, 147.0, 147.5, 151.1, 152.3 (5C, purine ring), 172.9 (1C, C=O). FT-IR (KBr, ν, cm-1): 1679 ν(C=O) (amide), 1647, 3325 ν(N-H) (amine), 1422 ν(C-N) (amide), 1480 ν(C=N) (Ar-imine). The spectroscopic data are in agreement with the literature[114]

3-(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl) propanehydrazide (3d). Color: White. Yield: 98 %. mp >260 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 10.49 (s, 1H), 9.04 (s, 1H), 7.54 (s, 1H), 6.49 (s, 2H), 4.22 – 4.19 (s, 2H), 4.14 (t, J = 6.9 Hz, 2H), 3.36 (t, 2H). ^{13}C NMR (126 MHz, DMSO-d_6, δ, ppm): 168.7, 156.8, 153.5, 151.0, 137.4, 116.5, 39.5, 33.5. HRMS (m/z): Calcd. for $C_8H_{11}N_7O_2$ 237.09; found 238.105 $[M+H]^+$.

3. *Synthesis of the dinucleobase-hydrazide Triazine derivatives 5a, 5b, 5c and 5d*

N',N'''-(6-amino-1,3,5-triazine-2,4-diyl)bis(3-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl) propanehydrazide) (5a).

A 50 mL sealed tube was charged with the nucleobase hydrazide 3a (400 mg, 2 mmol) and 2-amino-4,6-dichloro-1,3,5-triazine 4 (156 mg, 0.95 mmol) in the presence of sodium bicarbonate $NaHCO_3$ (160 mg, 1.9 mmol) in DMF (8 mL). The reaction mixture was stirring at 110°C for 1h. The resulting solution was then allowed to cool to r.t., washed with acetone (3x30 ml) and centrifuged in a 50 mL conic tube. The precipitate was washed again in a 15 mL conic tube with a mixture of acetone/MeOH 9:1 (6x10 ml), centrifuged, and dried under reduced pressure to yield the desired product 5a as white solid (283 mg; 61%). mp >260 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ): 11.2 (s, 1H x2), 9.6 (s, 1H x2), 8.2

(s, 1H x2), 7.5 (d, *J* = 7.8 Hz, 1H x2), 6.2 (s, 2H), 5.4 (d, *J* = 7.8 Hz, 1H x2), 3.8 (t, *J* = 6.8 Hz, 2H x2), 2.4 (t, 2H x2). ^{13}C NMR (126 MHz, DMSO-d_6, δ): 174.4, 169.3 (x2), 167.8 (x2), 163.8 (x2), 150.8 (x2), 146.5 (x2), 100.4 (x2), 44.7 (x2), 32.3 (x2).

N',N'''-(6-amino-1,3,5-triazine-2,4-diyl)bis(3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)propanehydrazide) (5b). A 50 mL sealed tube was charged with the nucleobase hydrazide **3b** (400 mg, 1.9 mmol) and 2-amino-4,6-dichloro-1,3,5-triazine **4** (148 mg, 0.9 mmol) in the presence of sodium bicarbonate $NaHCO_3$ (151 mg, 1.8 mmol) in DMF (8 mL). The reaction mixture was stirring at 110°C for 1.5h. The resulting solution was then allowed to cool to r.t., washed with acetone (3x30 ml) and centrifuged in a 50 mL conic tube. The precipitate was washed again in a 15 mL conic tube with a mixture of acetone/MeOH 8:2 (5x10 ml), centrifuged, and dried under reduced pressure to yield the desired product **5b** as yellowish pale solid (310 mg; 67%). mp >260 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ): 11.2 (s, 1H x2), 9.6 (s, 1H x2), 8.2 (s, 1H x2), 7.4 (s, 1H x2), 6.2 (s, 5H), 3.8 (t, *J* = 6.8 Hz, 2H x2), 2.4 (m, 2H x2), 1.7 (s, 3H x2); ^{13}C NMR (126 MHz, DMSO-d_6, δ): 174.4, 169.3 (x2), 167.8 (x2), 164.4 (x2), 150.8 (x2), 142.4 (x2), 107.8 (x2), 44.6 (x2), 32.4 (x2), 11.9 (x2).

N',N'''-(6-amino-1,3,5-triazine-2,4-diyl)bis(3-(6-amino-9H-purin-9-yl)propanehydrazide) (5c). A 50 mL sealed tube was charged with the nucleobase hydrazide **3c** (300 mg, 1.35 mmol) and 2-amino-4,6-dichloro-1,3,5-triazine **4** (105 mg, 0.64 mmol) in the presence of sodium bicarbonate $NaHCO_3$ (108 mg, 1.28 mmol) in DMF (6 mL). The reaction mixture was stirring at 110°C for 3h. The resulting solution was then

allowed to cool to r.t., washed with acetone (3x45 ml) and centrifuged in a 50 mL conic tube. The precipitate was washed again under reflux with a mixture of acetone/MeOH 90:10 (3x50 ml), hot filtrated, and dried under reduced pressure to give the desired product **5c** as white solid (191 mg; 56%). mp >260 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ): 9.0 (s, 1H x2), 8.1 (s, 1H x2), 7.9 (s, 1H x2), 7.1 (s, 2H x2), 6.2 (s, 2H), 4.3 (t, J = 6.6 Hz, 2H x2), 2.6 (t, J = 6.6 Hz, 2H x2); HRMS (m/z).^{13}C NMR (126 MHz, DMSO-d_6, δ): 168.7 (x2), 155.9 (x2), 152.3 (x2), 149.3 (x2), 140.9 (x2), 118.6 (x2), 39.2 (x2), 33.2 (x2).

N',N'''-(6-amino-1,3,5-triazine-2,4-diyl)bis(3-(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl)propanehydrazide) (5d). A 50 mL round bottom flask was charged with the nucleobase hydrazide **3d** (100 mg, 0.42 mmol) and 2-amino-4,6-dichloro-1,3,5-triazine **4** (33 mg, 0.2 mmol) in the presence of sodium bicarbonate $NaHCO_3$ (35 mg, 0.42 mmol) in distilled water (20 mL). The reaction mixture was stirring at 100 °C for 2h. The resulting solution was allowed to cool to r.t., washed under stirring with 40 mL of acetone and dried under reduced pressure. The precipitate was washed again in a 15 mL conic tube with a mixture of acetone/MeOH 8:2 (4x10 ml), centrifuged and dried under reduced pressure to yield the desired product **5d** as white solid (80 mg; 72%). mp >260 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ): 7.8 (s, 1H x2), 7.2 (s, 2H), 6.6 (s, 2H x2), 4.3 (t, J = 6.6 Hz, 2H x2), 2.3 (t, J = 6.6 Hz, 2H x2); ^{13}C NMR (126 MHz, DMSO-d_6, δ): 174.5, 169.1(x2), 167.5(x2), 160.5(x2), 154.3(x2), 152.1(x2), 143.8(x2), 108.3(x2), 44.7(x2), 39.3(x2).

D. Statistical Analysis

Statistical analysis was performed by a student's t-test for either paired or unpaired observations using GraphPad Prism. The average of the triplicate from each experiment (individual mean) was calculated, and these means were then averaged. Data were presented as mean ± standard error of the mean (SEM) When more than two means are used for comparison, ANOVA was used: either one-way ANOVA (with Dunnett's post hoc test) or two-way ANOVA (with Tukey-Kramer's post hoc test). A *p*-value of less than 0.05 was considered as significant

CHAPTER IV

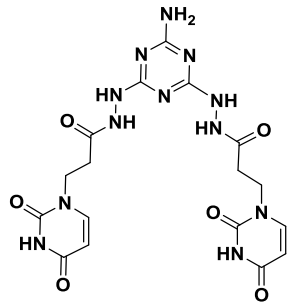
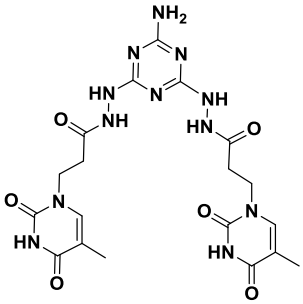
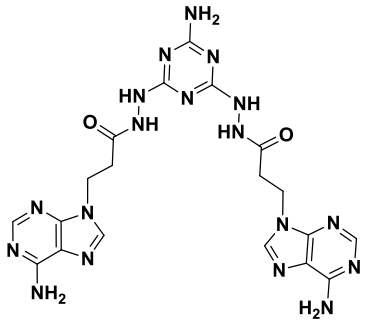
RESULTS

A. Synthesis

The reaction of 2-amino-4,6-dichloro-1,3,5-triazine **4** with nucleobase-hydrazides 3a-d proceeds by a nucleophilic substitution reaction. The formation of the desired dinucleobase-hydrazide triazine derivatives **5a-d** was achieved by diamination of 2-amino-4,6-dichloro-1,3,5-triazine 4 with the nucleobase-hydrazides. The starting nucleobase-hydrazides 3a-d were prepared in excellent yields from the corresponding esters **2a-d** by reaction with excess of hydrazine monohydrate in refluxing ethanol according to an established literature procedure[108]. The esters **2a-d** were afforded in very good yields from the corresponding nucleobases following a modified literature procedure[113, 115]. The method employed a base catalyzed Michael-type addition reaction between the nucleobase and ethyl acrylate in refluxing ethanol.

Reaction of the resultant hydrazides **3a-d** with 2-amino-4,6-dichloro-1,3,5-triazine in DMF at 110°C from 2-4 hours led to substitution of the two chloro-substituents with the nucleophilic hydrazide. At first, our attempts to react 2-amino-4,6-dichloro-1,3,5-triazine with the various nucleobase-hydrazides in several refluxing solvents, including ethanol, resulted in failure due to the poor solubility of these hydrazides in the common solvents. We found it was essential to perform the coupling reaction in the dissolving solvent DMF at 110 °C in the presence of sodium bicarbonate $NaHCO_3$. In fact, the products dihydrazide

triazines are very polar as seen on TLC and column chromatography, bind tightly to silica which made purification by chromatography impractical. Ultimately, the dihydrazide triazine derivatives **5a-d** were obtained as solids in moderate yields after washing with a mixture of acetone and methanol, and centrifugation (Table 1). The synthesized compounds were well characterized by ^{1}H NMR and ^{13}C NMR techniques.

Product	Structural Formula	Reaction Time	Yield %	m.p. (°C)	Molecular Formula
5a		1 h	61%	>260	$C_{17}H_{20}N_{12}O_6$
5b		1.5 h	67%	>260	$C_{19}H_{24}N_{12}O_6$
5c		3 h	56%	>260	$C_{19}H_{22}N_{18}O_2$

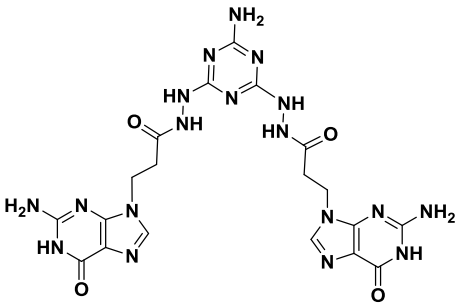
5d		2 h	72%	>260	$C_{19}H_{22}N_{18}O_4$

Table 1:Synthesis of dinucleobase-hydrazide triazine derivatives

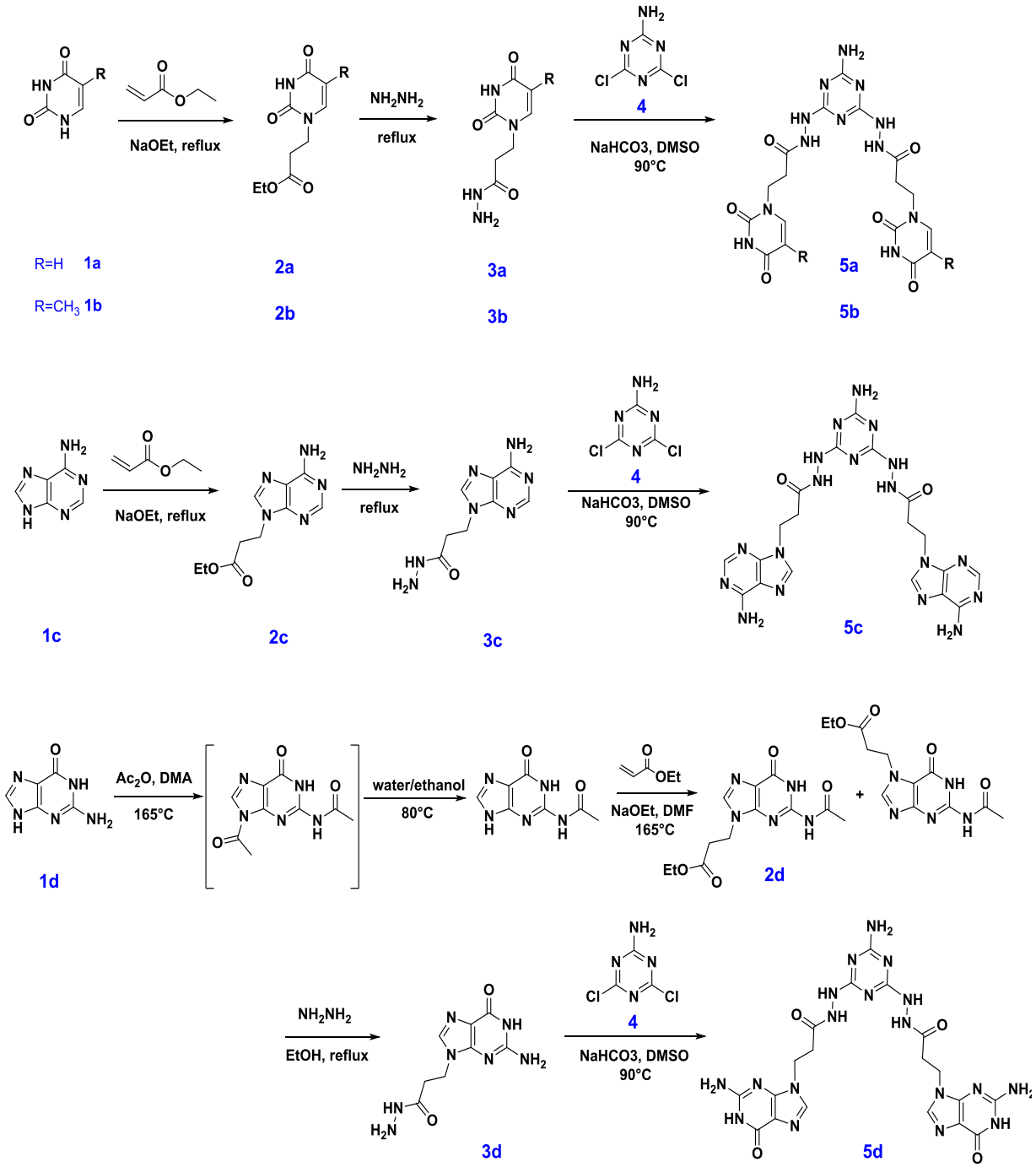


Figure 3: General scheme for dinucleobase-hydrazide triazine derivatives synthesis

The synthesized dinucleobase-hydrazide triazines **5a-d** were white in color with 61%, 67%, 56% and 72% yields respectively.

The ^{1}H NMR spectra of triazines **5a–d** exhibit following types of signals; a) two sharp singlets around 9.6 ppm and 8.2 ppm corresponding to the secondary amine groups protons for the hydrazide; b) a broad singlet between 6.2-6.4 ppm corresponding to the primary amine protons for the triazine; (c) two characteristic triplets around 2.6 ppm and 4 ppm, corresponding to the two methylene group protons. ^{13}C NMR results are consistent with literature; the signals corresponding to the hydrazide function appear at 169.3, 44.7 and 32.3. The amino triazine carbons appear at 174.4 and 167.8. The guanine carbons appear at 157.4, 154.1, 151.4, 138.5 and 116.7. The Adenine carbons appear at 156.3, 152.8, 149.8, 141.9, 119. The uracil carbons appear at 164.5, 151.4, 147.1, 100.9. The thymine carbons appear at 165, 151.3, 142.9, 108.3, 12.4.

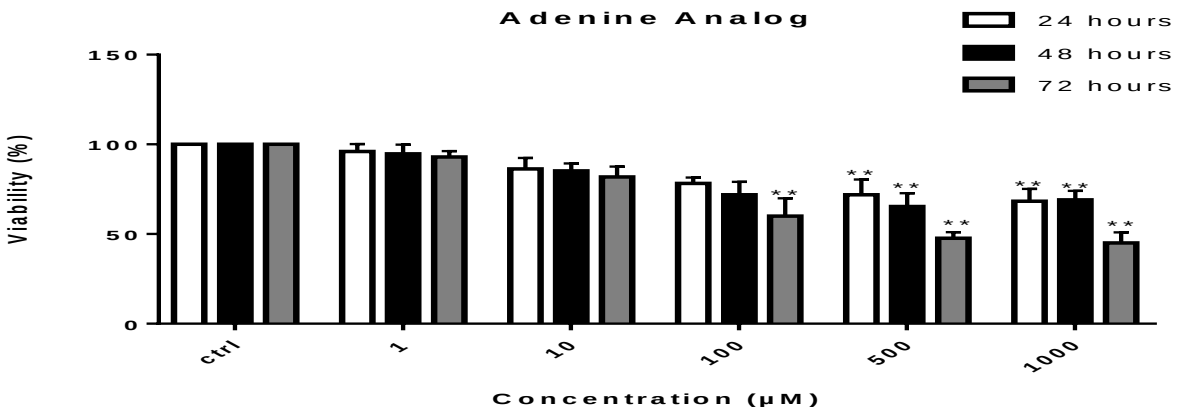
B. Biological Activity

1. Dinucleobase-hydrazide triazine decreases the viability of HCT116 cells

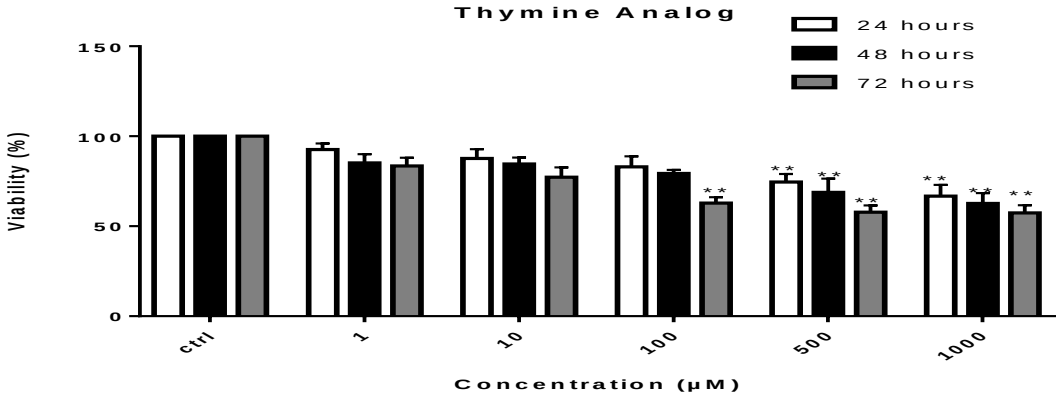
The effect of nucleoside analogues on HCT116 proliferation was studied using MTT assay. Human colon cancer cell lines HCT116 were cultured with increasing concentrations of Adenine, thymine and uracil analogues (0.1,1, 10,100,500 and 1000 μM) for three time points (24,48 and 72 hours). Figure 4 shows that these three nucleoside analogues decreased the viability of HCT116 cell lines in a concentration- and time- dependent

manner. It is noteworthy to mention that there was a significant decrease in the viability of human colorectal cells starting at a concentration of 500 μM.

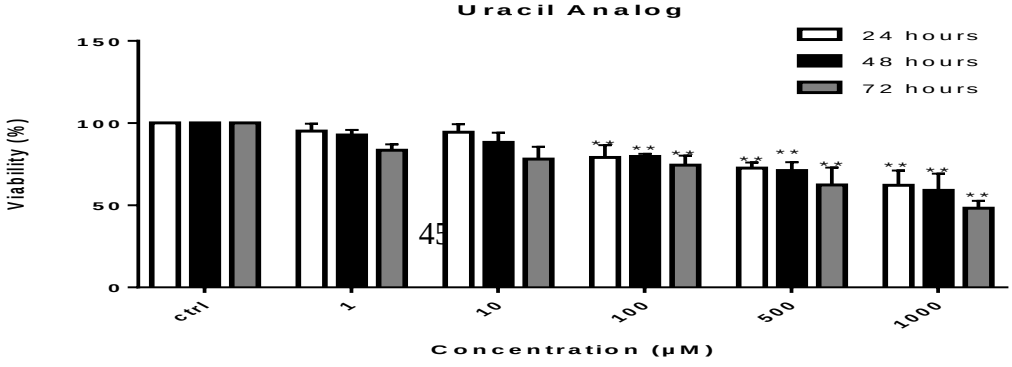
A.



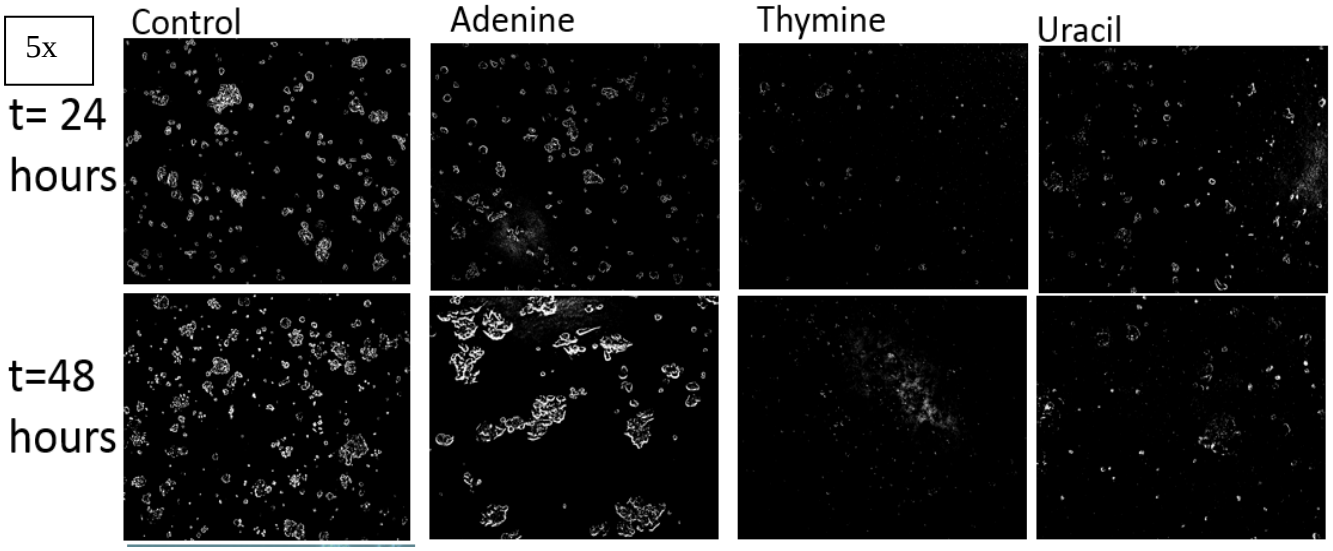
B.



C.



D.


5x
Control
Adenine
Thymine
Uracil
t= 24 hours
t=48 hours

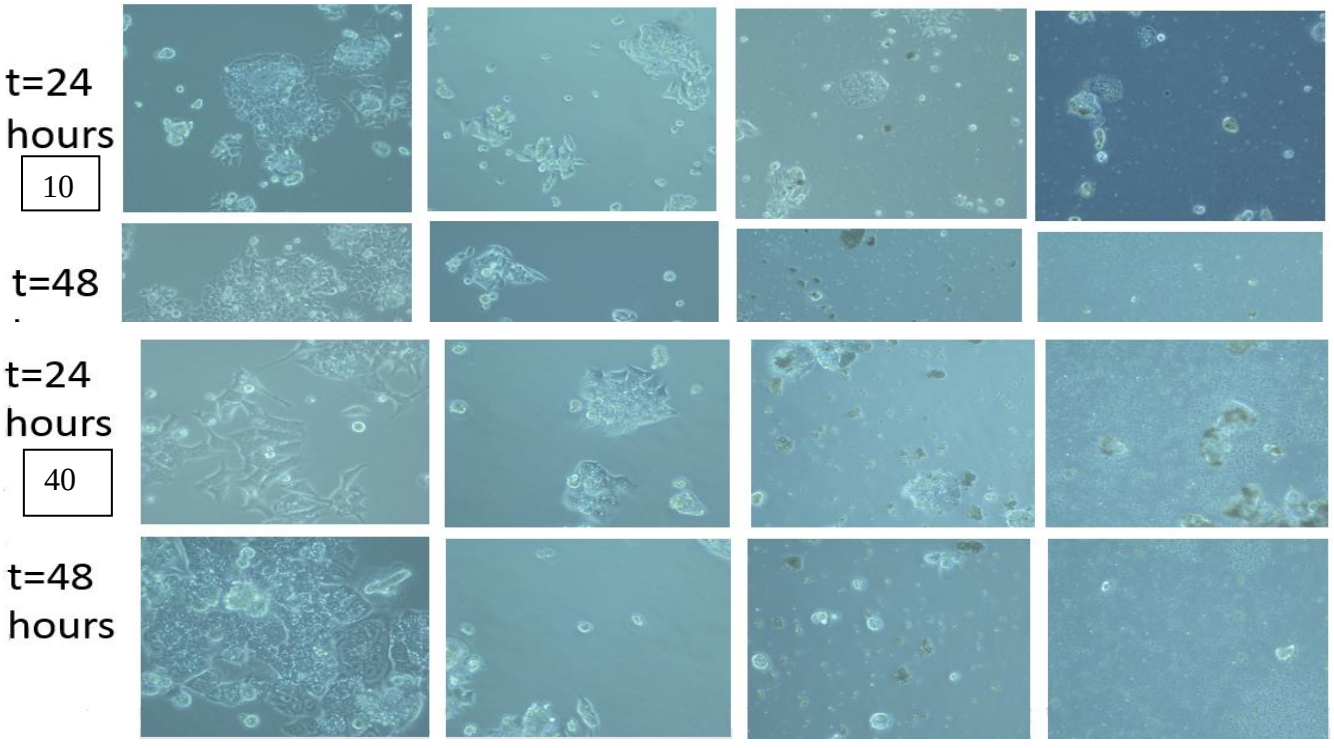

t=24 hours
10
t=48
t=24 hours
40
t=48 hours

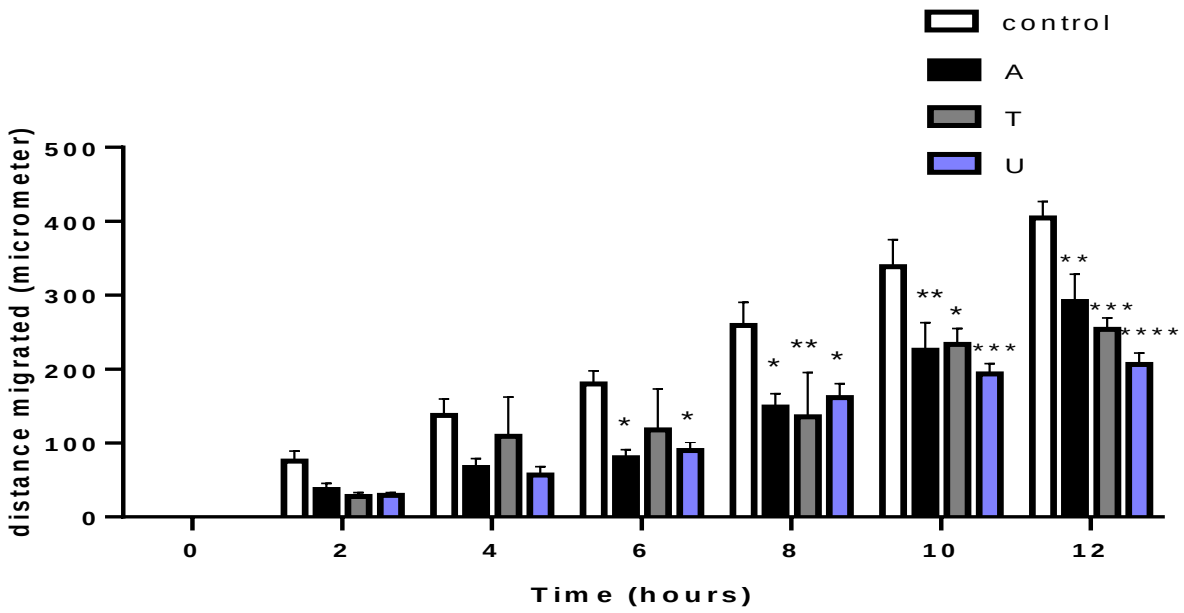
Figure 4: Effect of Dinucleobase-hydrazide triazine on cell proliferation in HCT116 colorectal cancer cells.

Figure 4 (A, B and C): MTT assay was performed. (A) HCT 116, after 24, 48 and 72 hours of Adenine analogue treatment). (B) HCT 116, after 24, 48 and 72 hours of Thymine analogue treatment). (C) HCT 116, after 24, 48 and 72 hours of Uracil analogue treatment). Increasing concentrations of dinucleobase-hydrazide triazine (0.1,1,10,100,500 and 1000 µM) significantly reduced the viability of colorectal cancer cells at 24,48 and 72 hours respectively in concentration and time dependent manner. Viability values are calculated as % of the corresponding control value and represented as mean ± SEM of triplicates (n=3). Figure 4 (D): HCT116 cells were incubated without or with 500 µM dinucleobase-hydrazide triazine at 24 and 48 hours. These cells were photographed under phase-contrast microscope (x5, x10 and x40 magnification). The concentration of 500 µM of three dinucleobase-hydrazide triazine significantly reduced the viability of colorectal cancer cells at 24and 48 hours.

2. Dinucleobase-hydrazide triazine decreases the migration of HCT116 cells

Cancer cells are characterized by their increased migratory ability. Migratory ability is considered as a crucial step for cancer cell metastasis. To examine the effect of nucleoside analogues on HCT116 migration, wound healing scratch assay was performed. Dinucleobase-hydrazide triazine at a concentration of 500 µM attenuated cells migration in a time-dependent manner (Figure 5). 24 hours post-treatment, Adenine, Thymine and Uracil analogues significantly inhibited the closure of the scratch. However; there is a complete scratch closure in non-treated HCT116 cell line.

A.


control
A
T
U
distance migrated (micrometer)
500
400
300
200
100
0
0
2
4
6
8
10
12
Time (hours)

B.

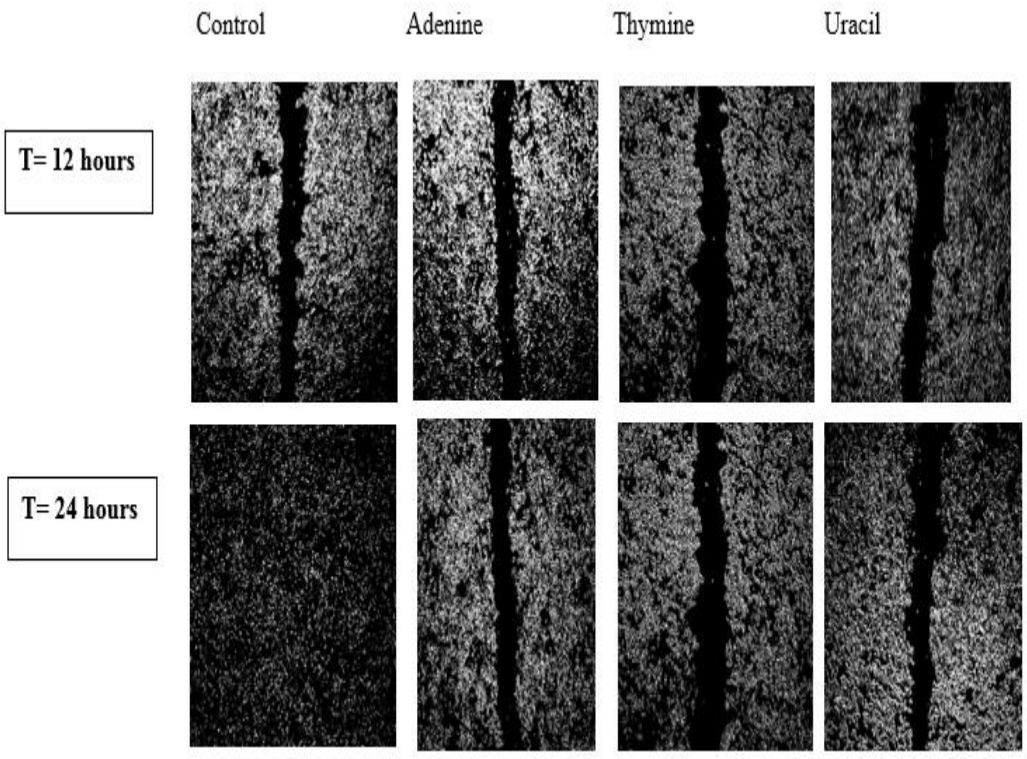

Control
Adenine
Thymine
Uracil
T= 12 hours
T= 24 hours

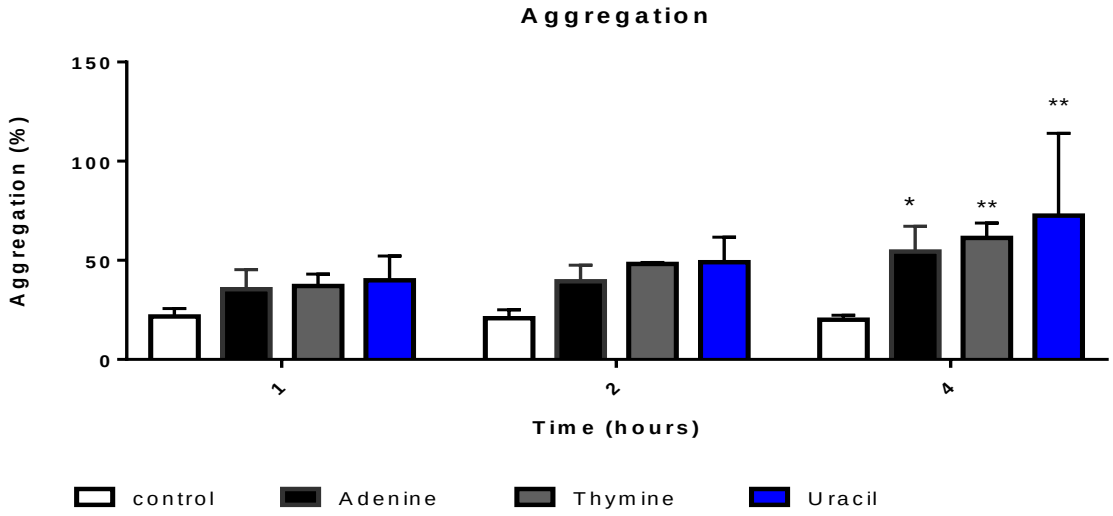
Figure 5: Effect of dinucleobase-hydrazide triazine on cell migration in HCT116 colorectal cancer cells.

Figure 5 (A): Migration assay was performed. The wound healing assay showing that the concentration of 500 μM of three dinucleobase-hydrazide triazine reduced the migration ability of colorectal cancer cells in time dependent manner. Distance migrated values are represented as mean ± SEM of triplicates (n=3). Figure 5 (B): Cultured HCT116 cells were scratching using the tip of P20 pipette and incubated without or with 500 μM nucleoside analogues for 0,6,12 and 24 hours. These cells were photographed under phase-contrast microscope (x5 magnification) and the wound was measured. Confirming that the three nucleoside analogues with a concentration of 500 μM significantly reduced the migratory ability of colorectal cancer cells at 6, 12 and 24 hours.

3. Dinucleobase-hydrazide triazine increases the aggregation of HCT116 cells

Since nucleoside analogues showed anti-growth and anti-migratory effect on colorectal cancer cells, we next wanted to determine whether our synthesized drugs affect the aggregation behavior of HCT116 colorectal cancer cells. For this purpose, aggregation assay was performed. Aggregation assay showed that Adenine, Thymine and Uracil analogues enhanced aggregate formation in a time-dependent manner. As shown in figure (6), at 1 hour, our nucleoside analogues at a concentration of 500 μM initiated the formation of homotypic cell-cell aggregates. Interestingly, the percentage of aggregates in nucleoside analogues-treated group significantly increased 4 hours post-treatment. These results indicate that these analogs may potentially inhibit tumor progression and metastasis.

A.



B.

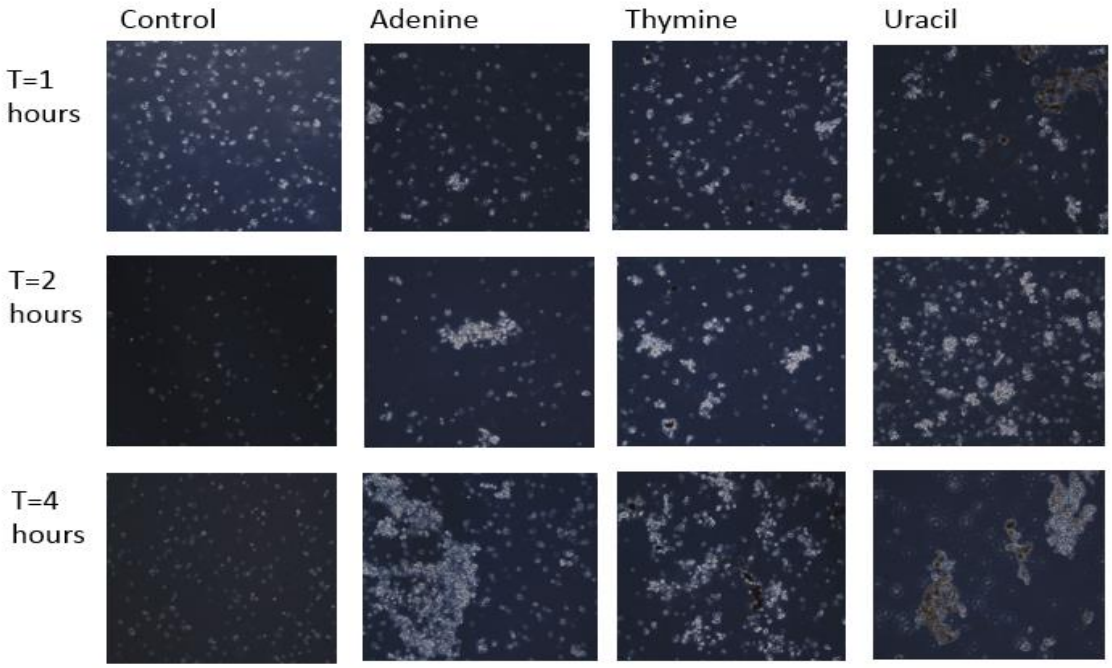


Figure 6: Effect of dinucleobase-hydrazide triazine on cell-cell aggregation in HCT116 colorectal cancer cells.

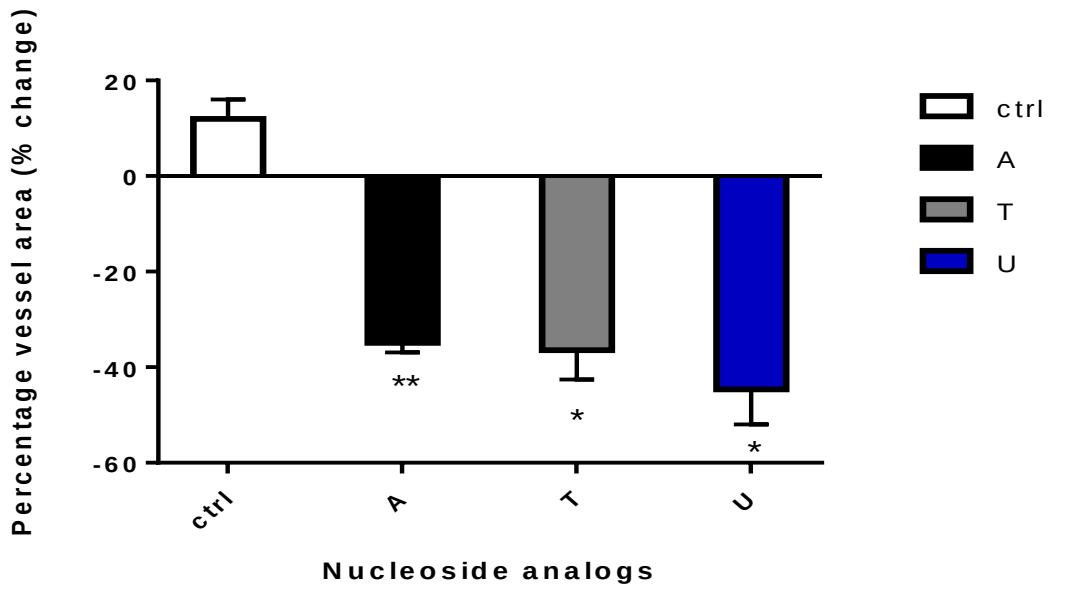
Figure 6 (A): Aggregation assay was performed. The concentration of 500 μM of three dinucleobase-hydrazide triazine promoted the aggregation ability of colorectal cancer cells in time dependent manner. Cell

aggregation (%) values are represented as mean ± SEM of triplicates (n=3). Figure 6 (B): HCT116 cells were incubated without or with 500 μM nucleoside analogues for 1,2 and 4 hours. Photomicrographs were taken under phase-contrast microscope (x5 magnification). The three nucleoside analogues with a concentration of 500 μM significantly increased the aggregation ability of colorectal cancer cells at 1, 2 and 4 hours.

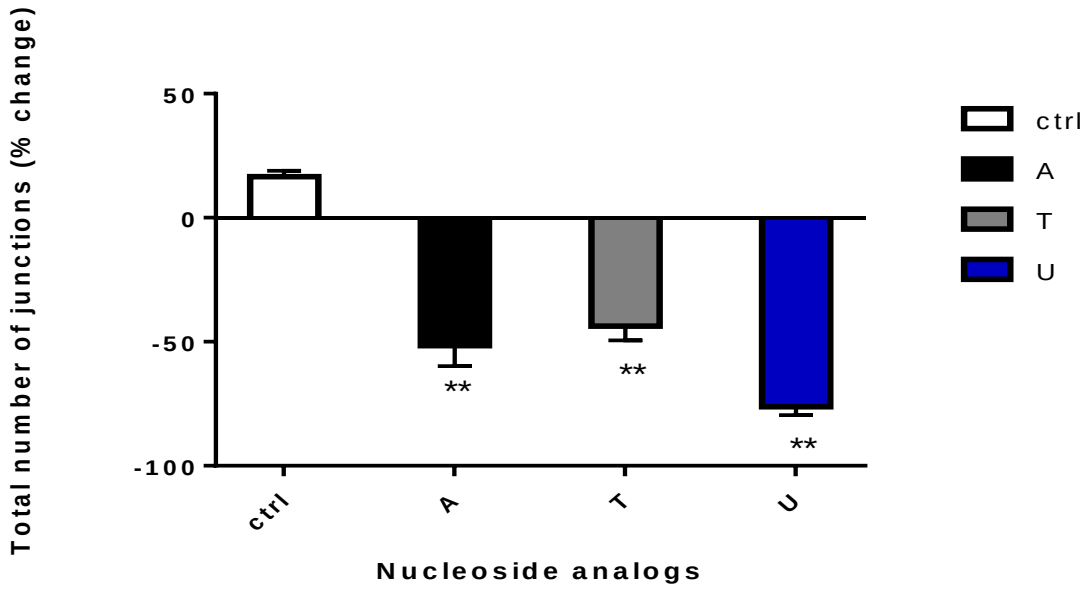
4. Dinucleobase-hydrazide triazine decreases the angiogenesis of HCT116 cells

Overwhelming evidence that angiogenesis plays a central role in tumor growth and metastasis. To assess whether nucleoside analogues affect angiogenesis, in ovo chicken chorio-allantoic membrane (CAM) assay was performed. Adenine, Thymine or Uracil analogs (500 μM) was applied on the chorio-allontoic membrane of eggs developing embryo. 24 hours post-treatment, these analogs significantly reduced total number of blood vessels and total number of junctions. However, non-treated eggs showed an increase of 10% in the number of blood vessels and 30% in the number junctions.

A.


Percentage vessel area (% change)
20
0
-20
-40
-60
**
*
*
ctrl
A
T
U
ctrl
A
T
U
Nucleoside analogs

B.


Total number of junctions (% change)
50
0
-50
-100
**
**
**
ctrl
A
T
U
ctrl
A
T
U
Nucleoside analogs

C.

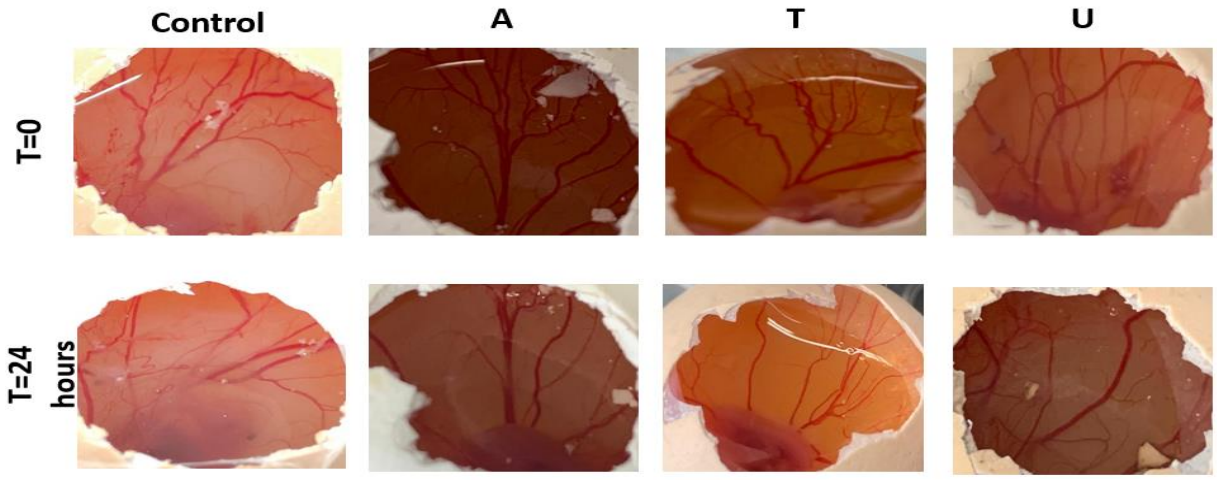


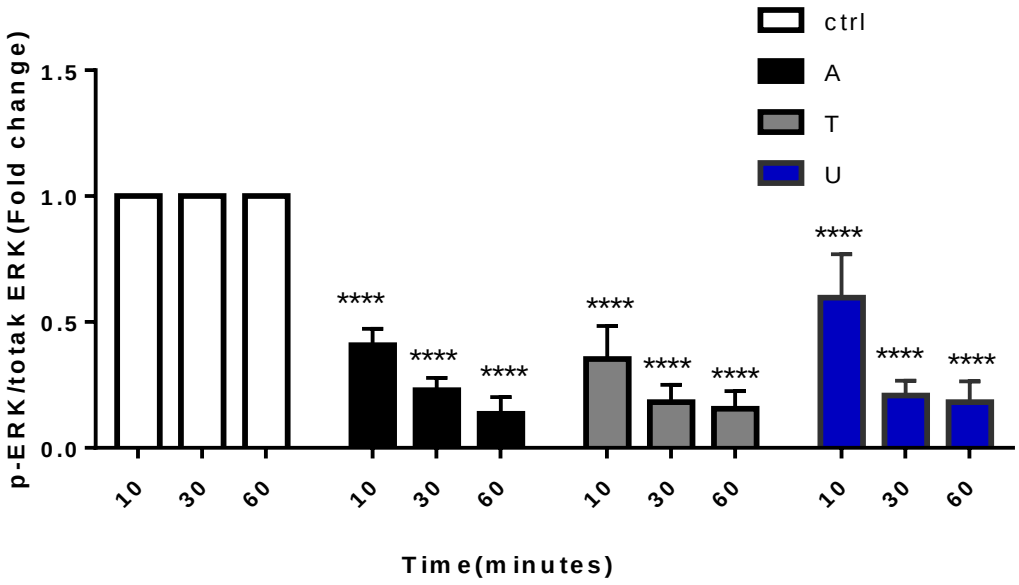
Figure 7:Effect of dinucleobase-hydrazide triazine on angiogenesis on the chorio-allontoic membrane of eggs developing embryo.

Figure 7 (A): CAM assay was performed. The three nucleoside analogues with a concentration of 500 µM inhibited the formation ability of new blood vessels 24 hours post-treatment. Figure 7 (B): In addition, results showed that there is a decrease in total number of junctions in treated eggs compared to control. Percentage vessel area and the total number of junctions (%) values are represented as mean ± SEM of triplicates(n=3). Figure 7 (C): Chorio-allontoic membrane of eggs developing embryo were incubated without or with 500 µM nucleoside analogues for 24 hours. Photomicrographs were taken with the mobile phone. The three nucleoside analogues with a concentration of 500 µM significantly inhibited angiogenesis process.

5. Dinucleobase-hydrazide triazine decreases ERK 1/2 activity in HCT116 cells

The mitogenic effect of ERK 1/2 has been established in series of cell lines. Having established a decrease in proliferation ability, we sought to determine whether the nucleoside analogues inhibit ERK 1/2 phosphorylation. Indeed, as we can see in figure (8), there was a significant reduction in ERK 1/2 phosphorylation. This downregulation was time dependent. The maximum decrease (90%) was achieved after 60 minutes.

A.



B.

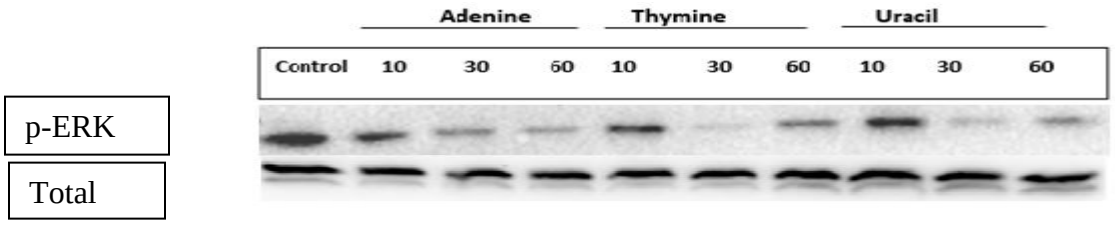


Figure 8: Effect of dinucleobase-hydrazide triazine on ERK ½ activity in HCT116 colorectal cancer cells.

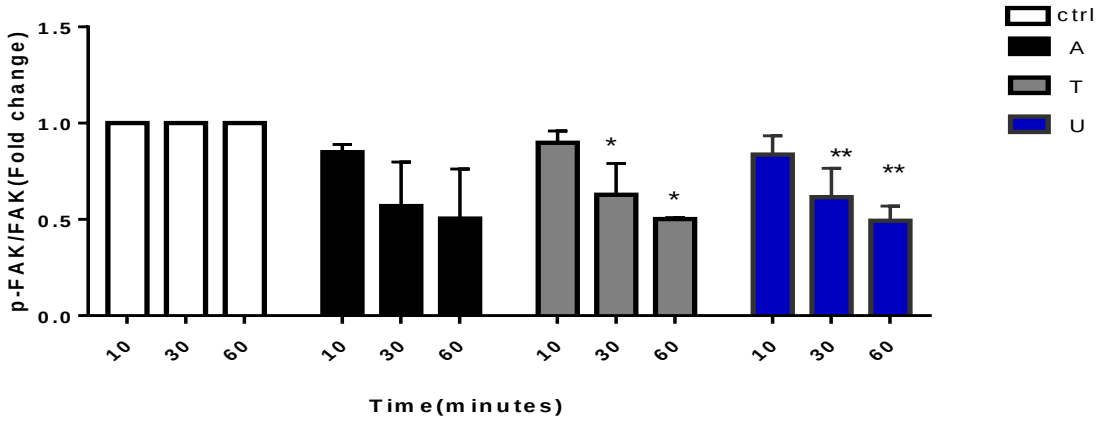
Figure 8 (A): Western blotting was performed. Histogram showing quantification of phosphorylated ERK1/2 / ERK1/2. Figure 8 (B): The concentration of 500 µM of three nucleoside analogues reduced the expression of phosphorylated ERK1/2 of colorectal cancer cells in time dependent manner. Fold change values are represented as mean ± SEM of triplicates (n=3)

6. Dinucleobase-hydrazide triazine decreases FAK activity in HCT116 cells

Since FAk is a critical kinase that is implicated in migration of several cell lines, we sought to determine FAk phosphorylation level using western blotting. HCT116 cells were

treated with Adenine, Thymine and Uracil analogues for 10, 30 and 60 minutes. Our three synthesized drugs at concentration of 500 μM significantly reduced phosphorylated FAK. The maximum decrease was achieved after 60 minutes where there was a reduction of 60% for adenine, thymine and uracil treated cells.

A.



B.

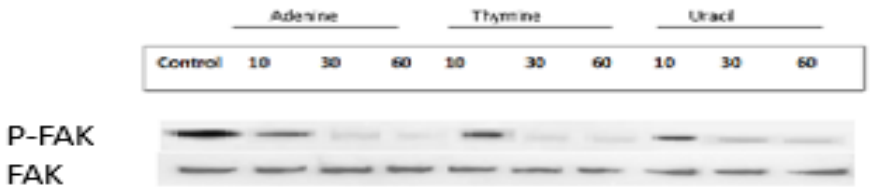
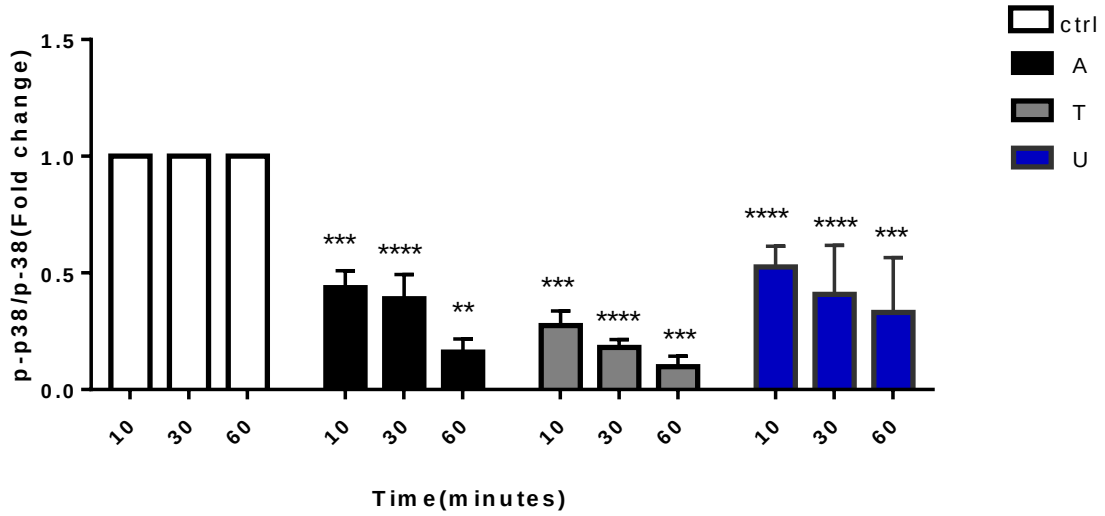


Figure 9: Effect of dinucleobase-hydrazide triazine on FAK activity in HCT116 colorectal cancer cells. Figure 9 (A): Western blotting was performed. Histogram showing quantification of phosphorylated FAK/ FAK. Figure 9 (B): The concentration of 500 μM of three nucleoside analogues reduced the expression of phosphorylated FAK of colorectal cancer cells in time dependent manner. Fold change values are represented as mean ± SEM of triplicates (n=3).

7. Dinucleobase-hydrazide triazine decreases p-38 activity in HCT116 cells

In addition to FAK, it has been demonstrated that p-38 also plays an essential role in cancer cell migration in a variety of cell types. Thus, we sought to determine whether the nucleoside analogues also inhibit the migration ability through p-38 pathway. Indeed, as we can see in figure (10), there was a significant reduction in p-38 phosphorylation in a time-dependent manner. The maximum decrease was achieved after 60 minutes where there was a reduction of 90% for adenine and thymine-treated cells and 60% for uracil treated cells.

A.



B.

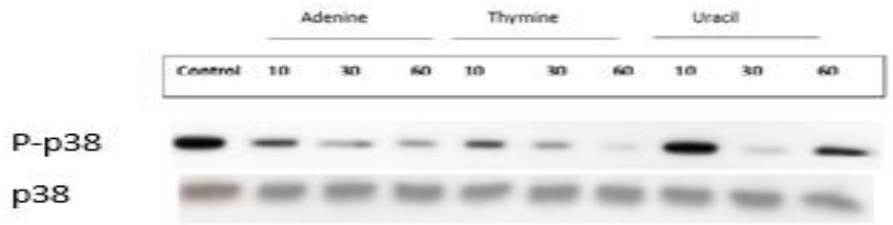


Figure 10: Effect of dinucleobase-hydrazide triazine on p-38 activity in HCT116 colorectal cancer cells.

Figure 10 (A): Western blotting was performed. Histogram showing quantification of phosphorylated p-38/ p-38. Figure 10 (B): The concentration of 500 μM of three nucleoside analogues reduced the expression of phosphorylated p-38 of colorectal cancer cells in time dependent manner. Fold change values are represented as mean ± SEM of triplicates (n=3).

CHAPTER V

DISCUSSION

Despite the major advances in the diagnosis and treatment of colorectal cancer, colorectal cancer remains a major contributor to cancer-related deaths worldwide. Recently, enormous efforts have been made to develop new anti-cancer therapeutic agents in order to treat cancer at early stages [116]. An example of these agents is 5-fluorouracil, which proved to be potent and efficient in CRC treatment. However, due to cellular drug resistance, clinical prescription of 5-fluorouracil has decreased[116]. This is a clear indication for the urgent need of developing new anti-cancer drugs that overcome resistance. In our laboratory, we synthesized novel nucleosides analogues and tested their anti-cancer effect on colorectal cancer cells. Our analogs attenuated the hallmarks of cancer such as proliferation, migration, adhesion and angiogenesis. Our findings demonstrate that treatment of HCT116 cancer lines with our nucleoside analogues play a central role in inhibiting the malignant phenotype of colorectal carcinoma. First, they exert an anti-proliferative effect on HCT116 cells. Second, they decrease the migratory capacity of tumor cells. Third, they increase the homotypic cell to cell aggregation ability. Fourth, they eventually inhibit angiogenesis. Finally, they decrease the phosphorylated levels of ERK1/2, FAK and p-38.

The resistance of neoplastic cells to chemotherapeutics drugs remains a chief factor that limits cancer therapy [117]. As a result, this phenomenon establishes a major cause of cancer relapses. The resistance problem is either due to an intrinsic factor, existing prior to

drug administration and known as innate resistance, or acquired factor, produced after anti-cancer drug exposition[117]. Thus, a growing body of evidence suggests several strategies that fight against drug resistance and improve health outcomes. As cancer tumors are heterogeneous in gene expression and form a poly-clonal complexity, the tumor constitutes of numerous reasons that lead to the ineffectiveness of pharmaceutical therapy. So, the first strategy is the alternative therapy that replaces the treatment with only one drug with more than two drugs aiming at targeting both sensitive and resistant cancer cells [117]. Studies showed that treatment with according to the intermittent theory, from high to low dose, might also induce longer life expectancy and retard resistance phenomenon [117]. Moreover, another study indicated that Zn (II) coordination compound and multifunctional gold nanoparticles inhibited the proliferation of resistant colorectal cancer cells and induced their apoptosis [118].

In our present study, we synthesized novel compounds and assessed their efficacy against colorectal carcinoma cells. These compounds showed cytotoxic activity against HCT116 cancer cell line.

Mitogen- activated protein kinases (MAP Ks) are involved in regulating many cellular physiological processes such as proliferation and migration [119]. ERK1/2 represents the key downstream effector of MEK1/2 and one of the last serine/threonine kinases (MAPKs) members [121]. The seminal work of Longping Huang and Si Chen led to the development of agent known as PD98059 which was indicated to cause the inhibition of extracellular-signal- regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling[122]. It is therefore anticipated that PD98059 induce the

detraction of both proliferation and migration mechanisms in HCT116 human colorectal carcinoma cells with basic leucine zipper and W2 domains 2 (BZW2) overexpression [122]. Earlier literature showed that the deregulation of MAP kinases induces further types of cancers rather than colorectal carcinoma. For instance, yohimbine, an alpha 2 adrenoceptor antagonist, suppresses pancreatic tumor cell lines proliferation by deregulating signal transduction pathways, MAPK, and lead to their self-death [123]. This suggests that alpha 2 adrenoceptor antagonist might be used as an attractive therapeutic approach for pancreatic cells apoptosis [123]. Other study have demonstrated that the use of DNA methyl transferase (DNMT1) antagonist, laccaic acid (LA), in combination with 5-fluorouracil (5-FU) exerted anti- intestinal cancer activity including cancer cell proliferation inhibition[124]. This simultaneous combination might also overcome the resistance phenomenon [124]. This is concomitant with our findings, since nucleoside analogues significantly reduced HCT116 colorectal cell viability starting from a concentration of 500 micromolar. This could be mediated through the attenuation of ERK1/2 signaling pathway. Ultimately, our results suggest that nucleoside analogues cause carcinoma cell proliferation arrest and they can also be used as anti-cancer therapeutic drugs. However, the uncontrollable proliferation of cancer cells allows them to acquire a high number of mutations that improve their migration speed [125]. Thus, additional studies are needed to better determine the relationship between proliferation and migration processes.

Under pathophysiological conditions, the cell migration process is a crucial step in metastasis. The upregulation of matrix metalloproteinases (MMPs) is a key biomarker of

colorectal tumor progression especially invasion [77]. In addition, MMPs trigger the degradation of extracellular matrix in order to promote migration. Ample evidence has indicated that there is an established favorable link between matrix metalloproteinases-1(MMP-1) and matrix metalloproteinases-2 (MMP-2) levels and worse colorectal cancer prognosis [77]. For example, patients with lymph node metastasis showed higher expression level of MMP-9 [77]. Remarkably, once endogenous tissue inhibitors of metalloproteinases (TIMP) bind to matrix metalloproteinase, the proteolytic activity of MMP is impeded and the degradation of extracellular matrix is reduced [126]. This suggests that TIMP downregulate the expression level of metalloproteinases. Thus, the migratory capacity of colorectal carcinoma cells is attenuated which indicates that the synthesis of TIMP analogues is needed. Additionally, previous studies revealed that the use of AB0041 and AB0046 monoclonal antibodies also impede colorectal cancer cell migration through decreasing the expression level of MMP-9 [127]. Interestingly, further pioneering work by Fujun Dai and Yihua Chen reported the anti-metastatic activity of YH-306, a novel synthetic compound [128]. A strong negative association has been reported between YH-306 agent and almost all key processes of colorectal malignancy including proliferation, adhesion, migration and apoptosis [128]. First, YH-306 showed a greater decrease in colorectal carcinoma cells viability. Thus cancer cell proliferation is inhibited through the decrease in MMP2- and MMP-9 levels [128]. Second, this novel drug decreased the amount of adhesions between colorectal cancer cells and extracellular matrix (ECM)[128]. Neither protrusions nor polarity are founded between CRC-cancer cell lines, HCT 116 and HC-29 cells, and ECM results in adhesion suppression [128]. Third, YH-306 reduced the overexpression of FAK and thus inhibited colorectal carcinoma cells migration

[128]. It is important to mention than FAK is a critical kinase implicated in the migration of several cell lines. Therefore, all these effects may lead to metastasis suppression.

As mentioned earlier, liver is considered as the first prevalent site of metastatic spread. Remarkably, YH-306 exerts anti-metastatic effect and prevents the colorectal cancer cells from occupying the liver or the lungs [128].

Other studies showed the role of p-38 in promoting cancer cell migration. This is a clear indication for the urgent need for a drug that exerts anti-migratory capacity through p-38 pathway. In our results, HCT116 cell migration was attenuated upon exposure to nucleoside analogues was observed. This was accompanied with the downregulation of MMP-2 and MMP-9 as well as decrease in FAK and p-38 activity. Thus, nucleosides analogues are effective therapeutic agents that can be considered as inhibitors for cancer cell migration.

Cell angiogenesis is considered an important hallmark of cancer due to their pivotal role in a wide variety of physiological and pathophysiological biological processes. Recently, a study demonstrated that there is high expression of vascular endothelial growth factor (VEGF) in fifty percent of patients suffering from bowel cancer compared to low or no expression in healthy colonic mucosa [129]. Moreover, the expression level of the proangiogenic factor, (VEGF), appears to be higher in the stage III and IV of colorectal cancer compared to stages I and II [129]. As a result, VEGF is recognized as a biomarker of colorectal cancer [129]. It is evident in literature that a strong correlation has been found between the signaling pathway ERK/MAPK and angiogenesis process [130, 131]. Interestingly, the inhibition of this signaling pathway lead to suppress the formation of new

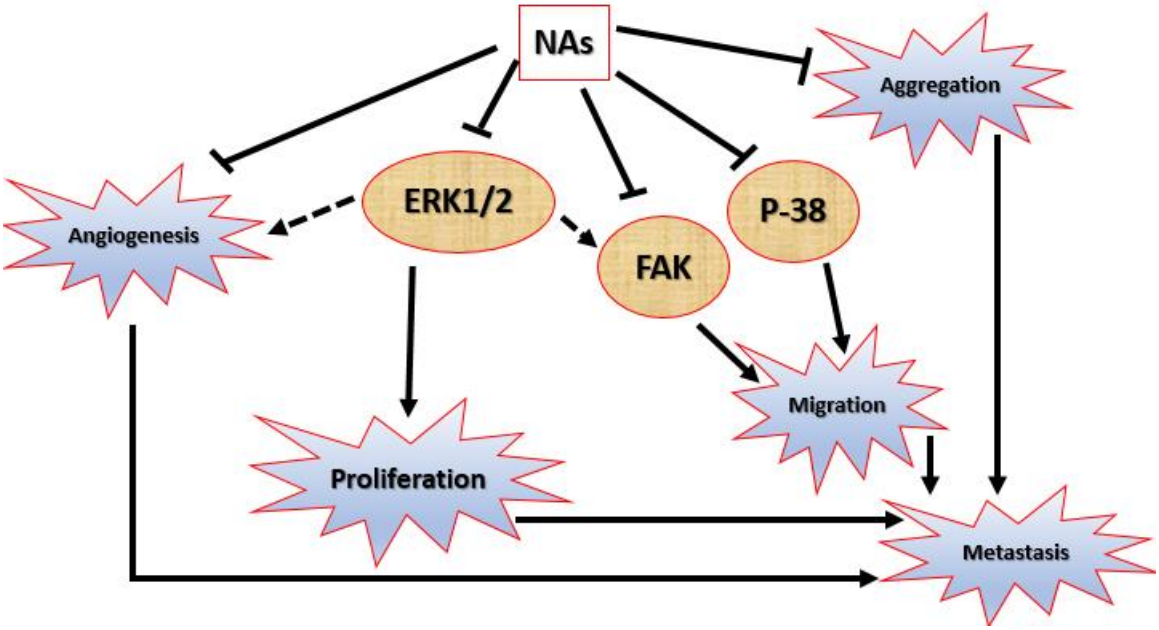
blood vessels [132]. Reports showed that nucleoside analogues decrease the expression level of VEGF and suppress tumor neovasculature through the downregulation of ERK/MAP kinase signaling pathway.

As the poorly differentiated cancer cell lines HCT116 represent a good model for in vitro studies relating to colorectal cancer, the in-ovo chick embryo chorioallantoic membrane (CAM) assay was used to assess the effect of the nucleoside analogues on angiogenesis. Our study showed that nucleoside analogues significantly decreased the total density of vessels in treated eggs whereas there was a normal microvasculature formation in the non-treated eggs. During metastasis, novel blood vessels undergo splitting from pre-existing vessels in the tumor[135]. However, the CAM treated with nucleoside analogues, showed a great decrease in the total number of junctions and the branching points of blood vessels. Consequently, the treatment with nucleoside analogues inhibited angiogenesis and exhibited a direct anti-metastatic effect.

CHAPTER VI

CONCLUSION

In conclusion, this study reveals the importance of nucleoside analogues in suppressing the malignant phenotype of colorectal cancer. Our findings show that nucleoside analogues, Adenine, Thymine, and Uracil, attenuate colorectal carcinoma hallmarks including proliferation, migration, invasion, aggregation, and angiogenesis. Therefore, our novel nucleoside analogues represent an attractive approach for the development of new types of anti-cancer treatments.



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