AMERICAN UNIVERSITY OF BEIRUT

SYNTHESIS OF NOVEL TRIAZINE-HYDRAZIDE BASED NUCLEOSIDE ANALOGUES AND THEIR ACTIVITY IN 5-FLUOROURACIL SENSITIVE OR RESISTANT COLORECTAL CANCER CELLS

by
ROLA HASSAN ABDALLAH

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AN ABSTRACT OF THE THESIS OF

Rola Hassan Abdallah for Master of Science
Major: Pharmacology and Therapeutics

Title: Synthesis of Novel Triazine-Hydrazide Based Nucleoside Analogues and their Activity in 5-Fluorouracil Sensitive or Resistant Colorectal Cancer Cells

Cancer continues to be the second leading cause of death worldwide, with colorectal cancer (CRC) being the second most common type. Despite significant advances in cancer therapies, current treatment of CRC remains inefficient. In addition, effectiveness of currently available chemotherapeutic drugs such as 5-Fluorouracil (5FU) is limited owing to developed resistance in CRC. Here, we provide novel schemes for synthesis of four novel nucleoside analogs, as well as describe their effects on proliferation, migration, aggregation, adhesion and de-adhesion of CRC cells, both 5-FU-sensitive and 5-FU-resistant HCT116. In either cell type, our synthesized novel analogs significantly inhibited cell viability in a concentration and time-dependent manner. Importantly, all the four analogues inhibited proliferation at a much lower concentration than that of 5FU, indicating higher potency of these analogs. In addition, these compounds inhibited cell migration in a time-dependent manner. They also inhibited adhesion and de-adhesion of both cell types to collagen, as well as promoted homotypic cell-cell interaction. Levels of ERK1/2, MMP-2 and MMP-9 were diminished by these analogs. Importantly, our analogs significantly inhibited angiogenesis, evident by decreased blood vessel density in Chick Chorioallantoic Membrane (CAM) assay. This was also concomitant with a decrease in the production of nitric oxide (NO) and vascular endothelial growth factor (VEGF). Taken together, by inhibiting these hallmarks of malignancy, our data highlight that the four analogs could act as potent chemotherapeutic drugs against CRC, including the 5-FU-resistant form.

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CHAPTER I

INTRODUCTION

A. Colorectal Cancer

1. Epidemiology

Cancer is the second cause of global death [1]. It accounted for 9.6 million of deaths in 2018 [1]. According to the World Cancer report, cancer rates are expected to increase 50% by 2020 [2].The basic phenomenon that defines cancer is aberrant proliferation of cells which become unresponsive to physiologic regulatory mechanisms. This is followed by metastasis to other parts of the body, which may eventually lead to death [3]. Despite significant advances in cancer research, colorectal cancer (CRC) continues to be the third common type of cancer worldwide, with 1.8 million reported cases [1]. It affects both genders, with higher a incidence in men than women [4]. Interestingly, CRC is more prevalent in industrialized nations with moderate and high Human Development Index (HDI) [5]. Moreover, the occurrence and mortality rates of CRC in Eastern Europe, Latin America and Asia have become higher than other nations [4].

2. *CRC screening*

Screening is a very important process when it comes to cancer prevention. It is considered an important element of the routine care for patients who are older than 50 years old or are at high risk for CRC [6]. Currently, two main categories for screening are available: invasive and non-invasive tests [7]. Invasive tests include colonoscopy and sigmoidoscopy [7]. Colonoscopy remains the first screening option for CRC in many countries, as it enables the detection and removal of the polyp at the same time [7]. Nonetheless, differences between left-sided and right-sided colon cancers have been recorded, where the latter is harder to detect and has higher incidence rates in women and senior populations [8, 9]. An enhanced version of colonoscopy, ADR (adenoma detection rate), has been employed. An increase of 1% in this indicator implies a 3% decrease in CRC risk [10, 11]. Non-invasive tests include blood and stool tests [7]. However, these tests are associated with a high rate of false positives. As such, non-invasive tests may not account for CRC specifically [7].

3. *CRC stages and symptoms*

Staging of CRC is key for treatment selection. Based on the stage of CRC, a special treatment is provided. There are many systems that stage CRC; however, the American Joint Committee on Cancer (AJCC) established an efficient way (TNM system) for this purpose. This system quantifies several factors including: size of the tumor (T), spread to the nearest lymph nodes (N), and metastasis to different parts and organs (M) [12]. CRC usually starts *in situ* where tumor cells have not grown beyond the mucosa, the inner layer of the colon; this is stage 0 [13]. When tumor cells pass to

the submucosa layer of the colon with no spread to lymph nodes or distant organs, it is referred to as stage I [13]. Stage II is divided into three sub-levels: tumor has reached the outer layer of the colon only (IIA); tumor passed through the wall of the colon without spreading to nearby tissues (IIB); or with spreading to nearby tissues (IIC) [13]. Stage (IV) is assigned to a tumor that has reached one distal part of the body such as the lungs or the liver (IVA), or more than one distal organ (IVB) [13].

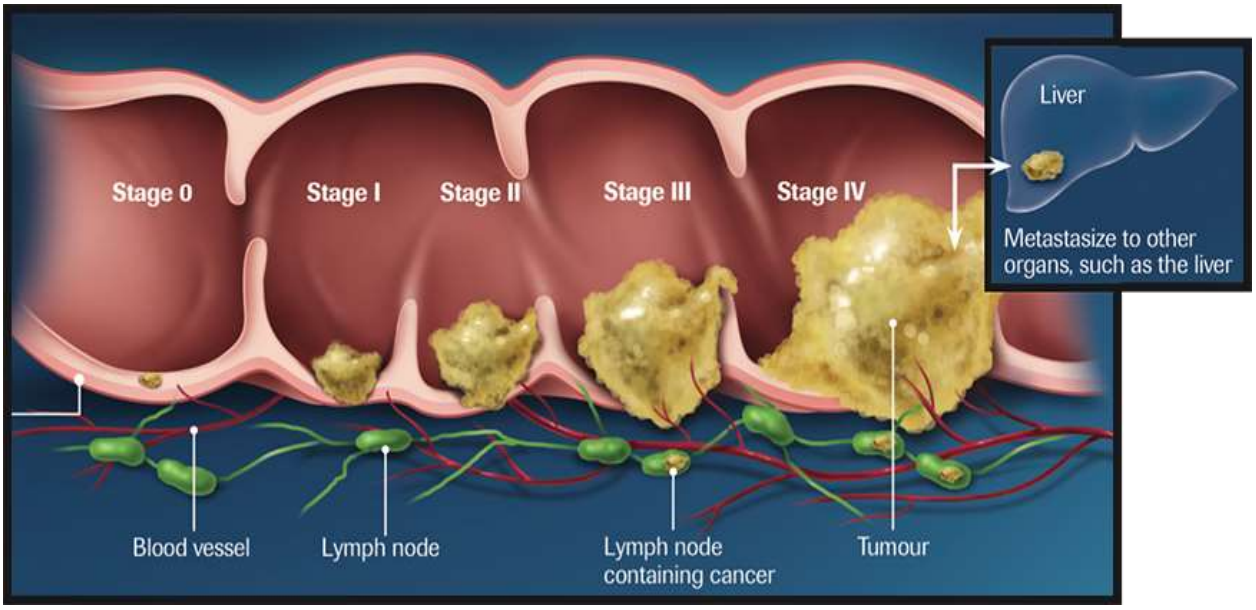


Figure 1: Stages of CRC. (Adopted from A Guide to CRC [14]).

Symptoms of CRC do not usually appear in its early stages [15]. Symptoms are usually reliant on the position of the main tumor. Irregular vasculature and pain from the fecal stream might lead to bleeding as the tumor develops in the intestinal lumen [6]. Usually, there is bleeding coming out with the stool but patients may not be aware

of it. Further, obstruction of the colonic lumen might lead to signs of abdominal enlargement, discomfort, diarrhea, and vomiting [16]. Otherwise, tumors of the anus, sigmoid colon and rectum might sometimes lead to hematochezia [17].

4. CRC risk factors

Initially a benign tumor, CRC proliferates in the form of a polyp over 10-20 years before becoming cancerous [18]. Nonetheless, this transformation can be prevented through screening. Most colorectal polyps are due to old age, and other non-modifiable predictors such as gender, geography, race, and ethnicity, with a small number of cases due to hereditary mutations. On the other hand, there are modifiable life-style factors which can encourage polyp development into cancer, including obesity, smoking, and lack of physical activity, as well as dietary choices like red and processed meat, alcohol, and certain medications. Modifying many of these behaviors may hold promise in reducing CRC risk and progression.

a. Non-Modifiable Risk Factors

i. Gender and Age

Colon cancer is more common in males than in females [4, 19]. However, studies report that CRC manifests in less aggressive forms in men compared to women [20], a fact that is confirmed by the results of sigmoidoscopy screenings [21]. Furthermore, in the US, rectal cancer is most common in those over 65 years old. However, over the last few decades, its incidence has decreased in people older than 50

years old and increased in people younger than 50 years old [22], so, in order to be identified earlier, the screening age has been reduced to 45 years old [23].

ii. Hereditary Mutations

Only 7-10% of CRC cases are hereditary. Having a first-degree relative with CRC confers a 2-3 times higher risk [24]. Some syndromes predispose the patient to higher risks. For instance, Lynch syndrome (HNPCC), present in 2–4% of all cases, is the most prevalent hereditary syndrome [25, 26]. Other common predisposing syndromes are familial adenomatous polyposis (FAP), along with MUTYH-associated polyposis (MAP), which account for less than 1% of all cases [27]. Despite being rare, conditions of hamartomatous polyps such as Peutz-Jeghers syndrome (PJS), juvenile polyposis syndrome (JPS), and PTEN hamartoma tumor syndrome (PHTS) are also associated with hereditary CRC [28].

iii. Inflammatory Bowel Disease (IBD)

IBD, which is manifested as increased inflammation of the colon, is also associated with increased CRC incidence. Ulcerative Colitis and Crohn's disease are the main subtypes of IBD [29]. Studies show that people with ulcerative colitis are at an increased risk of developing bowel cancer when compared to those with Crohn's disease [30].

b. Modifiable Life-Style Factors

i. Obesity and Physical Inactivity

The most notable factor to promote the rise of CRC is physical inactivity, which leads to obesity [31]. Analysis shows that obese individuals and those physically inactive are separately at an elevated risk for colon cyst [32]. Not to mention that these two disease contributors also reduce the rate of survival [33].

ii. Lifestyle

Most cancer deaths are well-known to be due to smoking tobacco, and colon adenoma is not exceptional to this fact [34, 35]. Moreover, prevalence of CRC is revealed to go hand in hand with patients' daily food intake choices (irrespective of weight) [36] In fact, processed and red meats have proven to enhance exposure to colorectal neoplasm [37]. On the other hand, researchers believe that folate and fibers present in fruits and vegetables, as well as calcium and vitamin D have demonstrated a protective effect against CRC [38]. In addition, researchers have detected that alcohol consumption plays an adverse role in proliferation of CRC, especially in men who tend to under-report the quantity they consume [39].

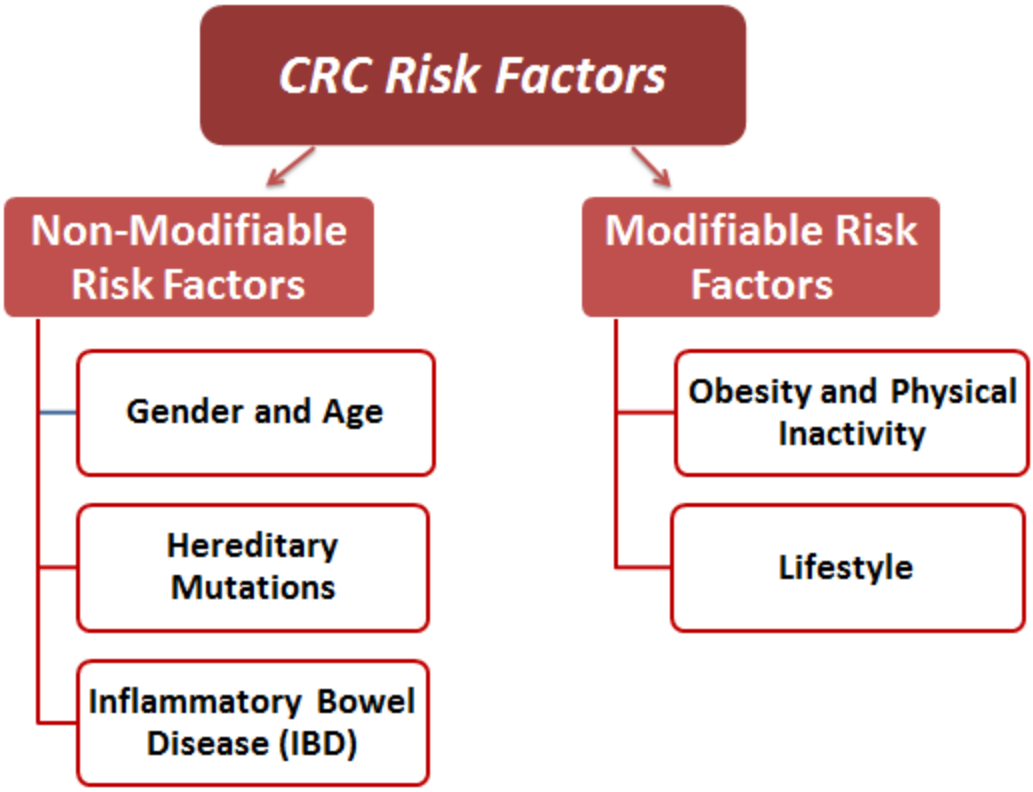


Figure 2: Risk factors of CRC.

5. CRC treatments

If not prevented, CRC can be treated, but that depends on early detection and the stage at which it was diagnosed.

a. Surgery

If colorectal malignancy is detected during early stages I or II, it can be treated by surgical removal of the carcinoma, as well as that of local lymph nodes where the cancer has metastasized [40]. There are several approaches that can be utilized including endoscopic resection, endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD) or segmental colonic resection [41, 42].

b. Chemotherapy

Chemotherapy for the treatment of CRC is administered for stages II or higher. It is worth mentioning that although chemotherapy is not favored in stage II CRC, it is approved in case of high risk features such as poorly differentiated cancers, lympho-vascular invasion, perineural invasion, bowel obstruction, localized perforation and positive margins [43].

Chemotherapy becomes vital for stages III and IV, stages at which bowel cancer is metastatic and survival is low [44]. In contrast, while attempting to lengthen life longevity, the main chemical agents in therapy are 5-FU (5-Fluorouracil) and LV (Biomodulator Leucovorin)[45]. They were proved to be successful, granting CRC patients a range of 12 extra months of life [46]. Beyond that, more agents were added, Oxaliplatin and Irinotecan, which resulted in development of 2 combinations: FOLFOX (5-FU/LV infused with oxaliplatin) and FOLFIRI (5-FU/LV infused with irinotecan), prolonging patients' life span to a range of 20 months [47-49].

Alternatively, newer agents for treatment of advanced CRC have been tested in order to develop new regimens with less toxicity than that of 5-FU/LV, oxaliplatin, and irinotecan combined [50]. They consist of anti-VEGF agents, anti-epidermal growth factor (EGF) agents, new cytotoxic agents and radiation. Bevacizumab is the sole anti-VEGF agent that is commercially available. Upon infusing bevacizumab to IFL (irinotecan with 5-FU/LV), a median prolonged life span of 20 months is achieved, same as previous regimens, whereas toxicity level has not subsided [51].

6. CRC Complications

In reference to the aforementioned risk factors, CRC is one of the most prevailing malignancies that will proliferate with time until it achieves metastasis, thus dwindling survival rates as is the case in 50% of patients. Notwithstanding, advanced colon cancer treatment have improved markedly over the last years, and survival rates have increased. However, these anti-tumor regimens come at the risk of cardiovascular complications, re-occurrence of CRC by means of metastasis, or even drug resistance.

Patients are exposed to cardiovascular complications which might rise amid or even following treatment of metastatic CRC, whether it was surgery or chemotherapy[52]. Notably, while assurance against these complications deteriorates with age [53], the application of any regimen of anti-tumor agents has its own adverse risk. For example, cardiovascular problems following 5-FU utilization fluctuate between 1.2% to 18% [54], despite enhancing patients recovery from mCRC when incorporated with other cytotoxic agents [55].

In addition, bleeding, obstruction, and perforation have been reported as mCRC surgical complications [56].

B. Hallmarks of CRC

1. Proliferation

In healthy individuals, there's equilibrium between intestinal cell proliferation and cell death. After the cells are proliferated, they will differentiate into different kinds of cells including enteroendocrine cells and enterocytes. Then, they may undergo cell death or apoptosis. On the other hand, sustained cellular proliferation and escaping cell death are the main hallmarks of cancer including CRC. Studies have demonstrated various pathways that induce cellular proliferation in CRC. One of the major pathways is the mitogen-activated protein kinase (MAPK) pathway. It is a series of three kinases, where the activation of the first kinase (MAPKKK) by phosphorylation, activates directly the other kinase (MAPKK), which in return activates the last kinase (MAPK – ERK1/2) [57]. Once activated, different physiological mechanisms are promoted, including cellular proliferation, differentiation and migration [58, 59]. Indeed, it is well noted that this pathway is aberrantly activated in CRC progression [59]. Thus, this pathway should be target for CRC treatment.

Other pathways that contribute to increased cellular proliferation in CRC include the Wnt pathway, PI3K/Akt pathway, and the Notch pathway. These pathways have been reported to be over activated in CRC especially the advanced ones [59].

In addition, it is worth mentioning that during drug resistance, several pathways are constituently activated including the ERK signaling and other pathways that induce proliferation [57]. Furthermore, in CRC chemoresistance, ERK upregulate the expression of the main efflux transporter for chemotherapeutic drugs, P-glycoprotein,

by stabilizing the hypoxia-inducible factor-1α (HIF-1α) [60]. They also maintain anti-apoptosis by increasing the levels of some of Bcl-2 family that are anti-apoptotic [61].

2. Migration

Another hallmark of advanced cancer is cell migration. This is especially observed in metastatic colon malignancy [62]. In advanced CRC, cancer cells migrate from the initial cyst to a further collateral organ [63, 64], and then to other distant organs especially the liver [65].

For the cell to migrate, it has to interact with its surrounding. This should be preceded by some modification in its structure and stiffness. The migrating cell first becomes elongated and polarized. This could be done by the help of the Rho family where they show different localized activity in the front and the back of the cell [66]. Then, cell actin-protrusions are formed at the leading edge; such as lamellipoda, or filopoda [66, 67]. These would help the migrating cell to propagate.

In addition, the extracellular membrane (ECM) provides a barrier for cell migration. However, matrix metalloproteinases (MMPs) could solve this issue. They are zinc dependent enzymes that degrade the main components of the ECM such as collagen and fibronectin [67, 68]. Thus, cell migration and invasion will be favored. In fact, it is well documented that MMPs level especially MMP-2 and MMP-9 are upregulated in CRC [69].

Overall, cell migration is a prerequisite step for metastasis, which in turn is dependent on the expression of MMPs.

3. Adhesion

Cell adhesion is also a hallmark in metastatic cancer. It occurs when corrupted cells attach onto the extracellular matrix (ECM) of collateral organs in order to create a link between neighboring cells and ECM, in addition to assembly of focal adhesions [70]. Beyond that, cytoplasmic proteins are drafted, illustrated by focal adhesion kinase (FAK) and c-Src, which forge a complex that prevents focal adhesion dismantlement by regulating the phosphorylation of paxillin, an integrin-assembly protein, on tyrosine sites Tyr31 and Tyr118 [71]. After that, migration is allowed through phosphorylation of PI3K upon merging of FAK with the p85 subunit of P13k [72, 73]. Last but not least, cell protrusions are established and they guide migration through actin-related protein (Arp2/3) polymerization [74, 75]. In light of this, cancerous cell adhesion should be avoided to cease the progression of CRC.

In addition, collagen, which is the main structural protein of the ECM, may help in CRC metastasis [76]. Studies revealed that collagen presence upholds and expedites CRC growth and spread through triggering the integrin α2β1/PI3K/AKT/snail signal [77]. So, to cease tumor metastasis, blocking integrin α2β1 would reverse the effect of type I collagen.

However, although integrin, a major adhesive protein, plays a role in regulation of attachment of colorectal malignant cells to ECM, yet its expression alone is not enough [78]. Further investigation revealed that the cytoskeletal elements are essential, where microfilaments and intermediate filaments stabilize the cell-ECM connection and

signal transduction [78]. Therefore, integrin-regulated virulent colon cancer cells are also dependent on cytoskeletal interactions.

4. Aggregation

Additional hallmarks of cancer include decrease cell aggregation or the loss of cell-to-cell adhesion. In healthy individuals, homeostasis is upheld through adherence of cells to each other and to the ECM. This adherence involves physical attachment and exchanged signal transduction maintained through proteins known as adhesion molecules, cadherins and integrins in particular. As mentioned before, integrins are responsible for cell-ECM adhesions where as cadherins regulate homotypic cell-cell adhesions [79-81]. Cadherins are calcium dependent proteins that play important roles in tissue remodeling and barriers.

Also, vinculin has a role in aggregation. It is an actin-binding protein that is present in integrin-mediated cell-ECM junctions and cadherin-mediated cell-cell junctions [82]. Vinculin bolsters homotypic cell-cell adhesions [83], and its absence in tumorigenesis hints that it may have anti-metastatic impacts on cancer. Studies showed that CRC progression and migration was hindered due to vinculin overexpression, through which the membrane bound β-catenin was upregulated [83]. Hence, because it maintains adhesion between cells, vinculin could be utilized in advancement of tumor treatment through β-catenin-dependent pathway.

Last is the E-cadherin's influence on cancer susceptibility to treatment. Studies revealed that upon E-cad activation, due to its affiliation with intercellular adhesion, it

down-regulates β-catenin [84]. Once β-catenin decreases, phosphorylation of p38/ERK1/2 is downregulated, resulting in increase of metastatic CRC chemosensitivity [84].

5. Angiogenesis

Under normal condition, angiogenesis, or the formation of new blood vessels, is maintained by equilibrium between pro-angiogenic and anti-angiogenic factors. However, any signal that favors uncontrolled angiogenesis is a hallmark of cancer. Signals may include tumor needs of nutrients and oxygen for cancer progression. In fact, without angiogenesis, cancer cells will extend to only 1-2 mm^3 and then they may undergo apoptosis [85, 86].

Angiogenesis is a four step mechanism [86]. First, the pro-angiogenic factors activate the endothelial cells which in return secret MMPs for ECM degradation. Then, the activated endothelial cells start to migrate to the surroundings. Third, they are stabilized with the help of Angiotensin-1, -2 and start to multiply [86, 87]. Finally, they will form mature blood vessels with the aid of adhesion molecules such as the integrins [88]. During this, the pro-angiogenic factors keep inducing angiogenesis and the anti-angiogenic factors are inhibited [86].

The major pro-angiogenic element is vascular endothelial growth factor (VEGF). It enhances neo-vascular permeabilization and interactions in CRC [89]. In reverse to this fact, Bevacizumab antibody has been authorized for cancer treatment

because of its antiangiogenic properties that operate by binding to VEGF-A (a ligand for VEGFR1 and VEGFR2) [90].

In addition, VEGF will also stimulate the release of nitric oxide (NO) [91]. The later molecule, in addition to its role in inducing many physiological mechanisms such as cellular proliferation and migration, it induces vasodilation [91]. This in return will support tumor metastasis [92].

C. Nucleoside Analogs

Nucleoside analogues is a family of important agents that have anticancer, antiviral, and immunosuppressant properties [93]. The anticancer drugs include different purine and pyrimidine analogs. These agents are close to the original biological molecules from a functional and structural point of view. They are chemically modified by substitution, addition or deletion of groups so they can have better reactivity.

All these analogs share a similar mechanism of action [94]. After diffusion into the cell, they are phosphorylated into their active metabolites [94]. These metabolites can then interfere with DNA/RNA synthesis, causing DNA/RNA damage and apoptosis [95]. Despite these similarities, there is an apparent diversity in the clinical activity of these drugs [96]. For example, while purine analogs, including ,thiopurines, fludarabine, cladribine and pentostatin, and the pyrimidine analog, cytarabine, are used in treating different types of hematological malignant diseases, the other pyrimidine

analogs, such as Fluorouracil, Capecitabine and gemcitabine show cytotoxicity in solid tumors especially colorectal and breast cancer [97].

1. Fluoropyrimidines

Fluoropyrimidines, including 5-fluorouracil, are considered antineoplastic (antitumor) drugs that are commonly used in treating different types of cancer [98, 99]. 5-FU increased the survival rates in patients with breast cancer, head and neck cancer and CRC [100]. Increased knowledge of 5-FU mechanism of action, led to developing drugs with increased antitumor activity [101]. These prodrugs are Capecitabine and Tegafur [101].

Figure 3: Chemical structure of 5-FU.

a. Mechanism of action

5-FU enters the cell using transporters as uracil [100]. Inside the cell, it is converted into three active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) [100]. FdUMP covalently inhibits thymidylate synthase (TS) [100]. Normally, TS catalyzes

the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), and this later product is further converted to deoxythymidine triphosphate (dTTP) [97]. dTTP is important for DNA synthesis [97]. Thus, inhibiting TS leads to inhibition of DNA synthesis [97]. Moreover, dUMP accumulation will lead to increased levels of dUTP. Both dUTP and FdUTP can incorporate into DNA leading to DNA damage [100]. Furthermore, FUTP can incorporate into the RNA strand causing RNA damage [100]. This incorporation can inhibit the maturation of rRNA, the post-transcriptional modification of tRNA and the splicing of pre-mRNA [100].

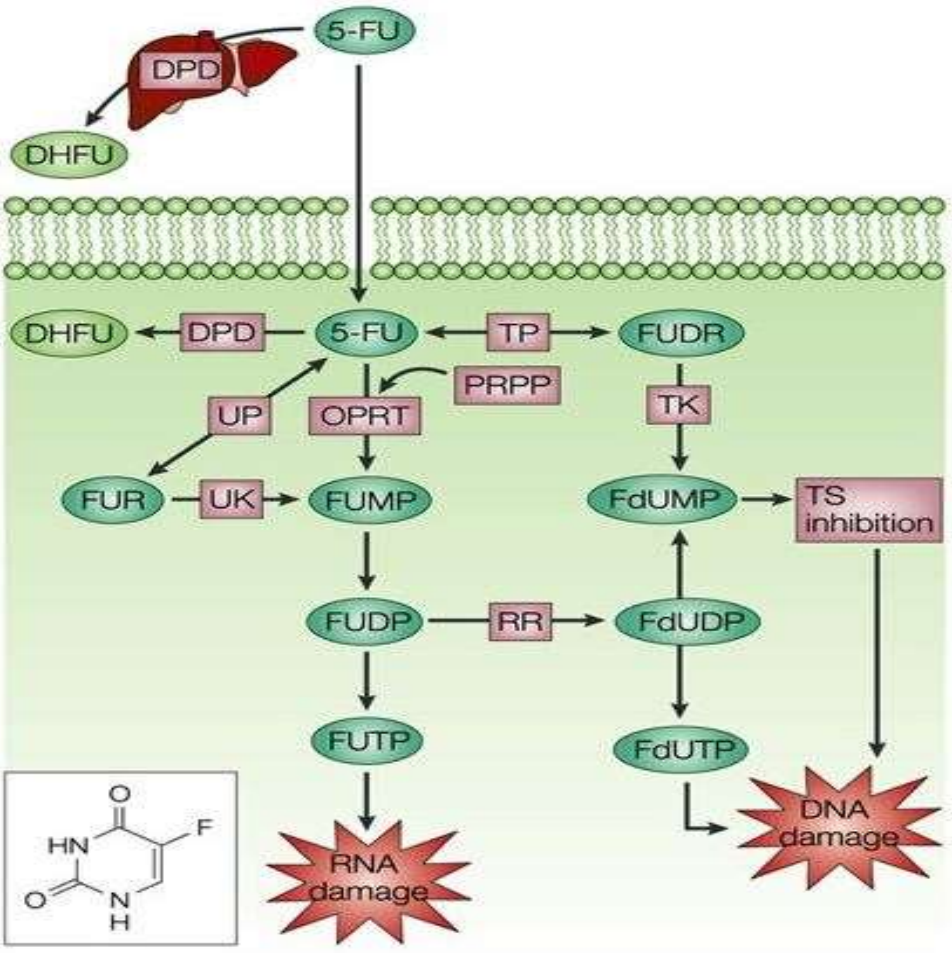


Figure 4: Mechanism of action of 5-FU. (Adopted from Longley et tal [100]).

b. 5-FU prodrugs

5-FU is rapidly degraded by the action of dihydropyrimidine dehydrogenase (DPD) [100]. Thus, it has a poor bioavailability. Also, it can cause gastrointestinal tract toxicity [102]. In order to overcome these issues, 5-FU prodrugs were designed.

Capecitabine is a 5-FU prodrug that minimizes the GI tract toxicity [102]. Capecitabine undergoes several conversions before exerting its cytotoxic effect. What is special about capecitabine is that the final conversion is done by the enzyme thymidine phosphorylase [97]. This enzyme is more expressed by tumor tissues than normal tissues, thus increasing tumor selectivity and susceptibility to capecitabine [97].

Tegafur is another 5-FU prodrug that is metabolized in the liver and converted to 5-FU [101]. Usually, it is given with uracil, a competitive inhibitor of DPD, in order to increase 5-FU bioavailability [101].

2. 5-FU chemoresistance

Although chemotherapy serves as the typical treatment for metastatic CRC, and despite its breakthrough with 5-Fluorouracil, Oxaliplatin and Irinotecan, yet it still does not achieve a high success rate. This is speculated to be due to the colon cancer stem cells (CSCs) that have demonstrated the ability to escape the toxic effect of the therapeutic chemical agents: 5-FU and Oxaliplatin. In addition, increased 5-FU metabolite efflux, enhanced 5-FU inactivation and Epithelial-mesenchymal transition (EMT) phenotype are also associated with 5-FU resistance in CRC [103].

Apart from this, the tumor mass response is generally negative to treatment, which discloses that the CSCs can influence tumor cells via the release of soluble factors that cultivate growth and endurance into the micro setting of the neoplasm [104]. Further investigations have revealed that these soluble factors may include a secreted glycoprotein for growth called Progranulin that is linked to the development of CSC drug resistance [104]. However, treatments targeting CRCs and their intracellular signaling pathways solely were proved to be insufficient.

3. 5-FU resistance mechanism of action

5-FU resistance is affiliated with several determinants [105]:

a. TS

There are three prospects by which the TS is involved in 5-FU resistance. To begin with, inhibition of TS, which activates thymidine kinase activity for thymidylate retrieval from thymidine, illustrates the first method of 5-FU resistance [106, 107]. Next is the intrinsic resistance of 5-FU as an aftereffect of over-expression of TS in the absence of CH2THF. This leads to weak inhibition due to the establishment of a precarious binary complex using FdUMP [108, 109]. Last is the acquired resistance of 5-FU via mutations in the TS gene and its amplification [110, 111].

b. DPD

According to Danenberg et al., the combative impression of 5-FU is affiliated with the mRNA expression of DPD in advanced colon cancer upon its analysis [112],

backed by the fact that 5-FU could jeopardize low-DPD swellings [113]. As a consequence, 5-FU resistance is achieved by means of heightened DPD activity and the reciprocal catabolism of 5-FU.

c. DNA and RNA Misincorporation

Another technique for cancer resistance is the eradication of 5-FU from DNA through alienating the MMR hMutS alpha complex from 5-FU-modified DNA [114]. This could cause the MSI to be more resistant to 5-FU in the tumor cells.

d. Anti-apoptosis

5-FU resistance through anti-apoptosis goes back to the over-expression of the anti-apoptotic proteins BCL-2 and BCL-XL, which upon the increase of BMI-1, are upregulated by hRFI during treatment [115]. Consequently, this is supported by the activation of NF-jB (nuclear factor-jB), which if arrested, has the ability to efficiently reverse the resistance to apoptosis as well as the upregulation of Bcl-2 and Bcl-XL in the hRFI transfectant [116]. In addition, increased levels of IAP surviving, a major apoptosis inhibitor, is associated with 5-FU resistance [117].

e. Cell cycle

Disruption of the cell cycle is linked with 5-FU resistance. It affords tumor cells with ample time to correct the incorporation of 5-FU metabolites into DNA and

develop resistance against 5-FU [118]. In fact, resistant cells are known to have increased doubling time and delays in G1- G1/S cell cycle [118].

f. NO

NO production process is hindered by 5-FU through NF-κB suppression, which in turn weakens iNOS protein production [119]. This suggests that an excessive quantity of NO prompts immunity to anti-cancer treatment [120].

g. Mitochondria

Upon analysis of 5-FU antagonistic cells, results reveal that ATP is under-expressed. This means that ATP synthase action is diminished and intracellular ATP composition is minimized [121]. Thus, ATP synthase is associated with anti-tumor treatment resistance.

h. Oxidative stress

5-FU-induced suppression of tumor cell progression is retaliated by cells that attain the power to adjust to oxidative stress. This cellular adaptive response can be provoked by Romo1 siRNA treatment which intercepts ROS production stimulated by fluorouracil [122].

i. Wnt Pathway

Upon culturing 5-FU-resistant CRCs (HCT-8 cells), He et al. found abundant quantities of TCF4 and β-catenin [123]. This implies that the Wnt pathway is enlarged, and thus applied microarray analysis implies that CHK1 (checkpoint kinase 1) pathway is intercepted [123]. Consequently, He et al. conclude that communication between Wnt pathway and CHK1 pathway affords a unique mechanism for 5-FU resistance [123].

j. TGF-β pathway

TGF-β pathway's role in carcinogenesis and metastasis in CRC is studied. Upon 5-FU incorporation in tumor treatment, TGF-β pathway is triggered resulting in overexpression of SMAD3 and transcription of distinct genes [124]. However, when TGF-β pathway is blocked through TGF-βRI inhibitor, 5FU-genes transcription was halted, which resulted in recovering the susceptibility of 5-FU-resistant cells to chemotherapy [124]. This means that TGF-β pathway activation builds up 5-FU resistance and cuts down chemosensitivity of advanced CRC cells [124].

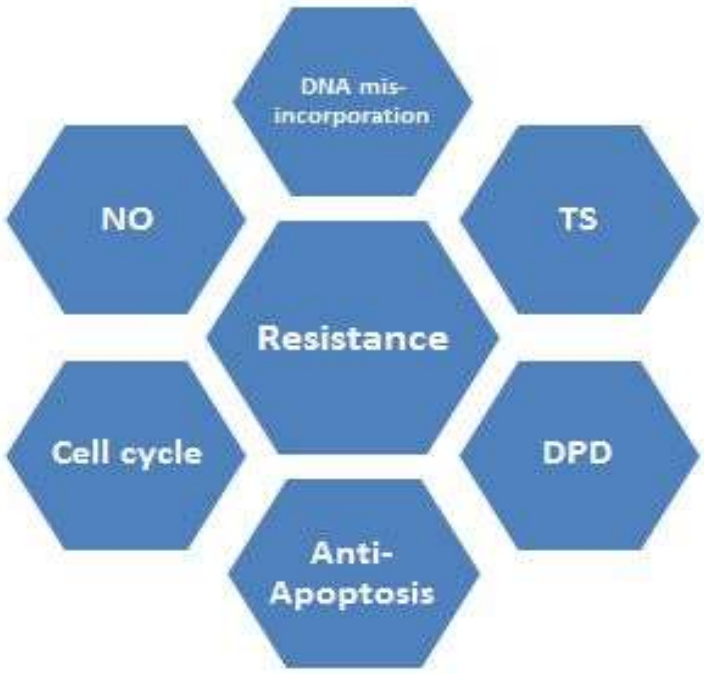


Figure 5: Mechanism of 5-FU resistance.

CHAPTER II

RATIONALE OF STUDY AND AIMS

Despite major advances in cancer treatment, CRC remains one of the leading causes of mortality worldwide [1]. 5-FU is the drug of choice for the last five decades for CRC treatment, however; its effectiveness is decreasing due to drug resistance. Therefore, there is an increasing interest to move away from the common drugs and synthesize more potent ones that could treat CRC and overcome drug resistance.

Nucleoside analogs have been heavily investigated and employed in treating different types of cancer. Their increased efficacy and reactivity seem to emanate from various chemical modifications that include substitution, addition or deletion of groups. In fact, the presence of their nucleic bases that constitute the cores of DNA and RNA structures is the main cause of their activity. Despite the achievements of nucleoside analogs in treating CRC, more potent therapeutic drugs are needed to be developed especially that resistance in CRC is increasing.

The aim of this study was to synthesize four nucleoside analogs (two purines and two pyrimidines) and investigate their biological effect on two types of CRC cells: HCT116 5-FU sensitive and HCT116 5-FU resistant types. We hypothesized that these synthesized nucleoside analogs are more potent than the common chemotherapeutic drug, 5FU, in treating CRC. Indeed, in comparison to 5-FU, these NAs, with low

concentrations, attenuated the hallmarks of CRC, including proliferation, migration, adhesion to ECM, aggregation, and angiogenesis.

CHAPTER III

MATERIALS AND METHODS

A. Cell line and Culture:

The parental HCT116 human CRC cell line was purchased from the American Tissue Culture Collection (ATCC). The 5-FU- resistant HCT1116 was kindly provided by Dr. Nadine Darwiche and were generated by continuous exposure to increasing doses of 5FU up to eight months to achieve resistance at clinically relevant doses (Rana El-Samad, American Journal of Cancer Research 2018). Cells were maintained in RPMI-1640 supplemented with 10% Fetal bovine serum (FBS), 1% Penicillin/Streptomycin and 1% Sodium Pyruvate. Cells were kept in the incubator at 37°C and 5% CO_2. Until 95% confluency, cells were split in a ratio of 1 to 10 for the HCT116 and 1 to 4 for the 5FU-Resistant HCT116 cells.

B. MTT assay:

Cells were seeded in a 96-well plate at a density of 7000/well and incubated at 37°C/5% CO_2 for 24 hours. After reaching 40 % confluency, media was removed and treatments of increasing concentrations (0.001 till 100 μM) of NAs or 5-FU (0.1 till 200 μM) were added for 3 time points (24, 48, and 72 hours). Then, MTT solution (5mg/ml PBS) was added and the plates were incubated in dark in the

incubator for 1 hour. After that, media was removed and the obtained formazan crystals were dissolved in 200 mL DMSO and kept in dark on the shaker for 20 mins. Then, optical densities were measured on Elisa Multiskan Reader (Thermo) at 595nm wavelength. The control was assumed to be 100% and the results were determined as a percentage of the control.

C. Monolayer Scratch Assay

Cells were seeded in a 12-well plate at a density of 750,000 cells/well and incubated at 37°C/5% CO_2 for 24 hours where a monolayer is formed. By using 10 μL pipette tip, a uniform wound scratch was made to each well. Then media was removed, and culture media containing the treatments of concentration 0.1 μM for the NAs, and 10 μM for 5-FU were added. Control had media containing the vehicle (DMSO). Cells were kept in the incubator for 24 hours, with microscopic pictures taken in different time points (0, 2, 4, 6, 8, 12, and 24) at specific marked places. Pictures were analyzed using ZEN software in order to quantify the wound healing.

D. Adhesion Assay

Cells were seeded in a 24-well plate pre-coated with Collagen at a density of 50,000 cells/well. Cells were incubated at 37°C/5% CO_2 with the treatments (NAs or 5-FU) for different time points (10, 20 and 130 mins). Then, media was removed and cells were washed with PBS to remove any floating cells. Attached cells were

stained with crystal violet staining and microscopic pictures were taken and cells were counted.

E. De-adhesion Assay

In a Collagen pre-coated 24-well plate, cells were seeded at a density of 50000 cells/well and incubated at 37°C/5% CO_2 for 24 hours. Then media was removed, and culture media containing NAs or 5-FU was added. After 24 hours, media was removed, cells were washed with PBS, and warmed typsin was added. Microscopic pictures were taken every 10 seconds and until the cells are rounded or detached.

F. Aggregation Assay

Cells were seeded in a 35 mm plate at a density of 1,000,000 cells/plate and incubated at 37°C/5% CO_2 for 24 hours. Then, cells were detached using sterile 2mM EDTA in Ca^{2+} /Mg^{2+} free PBS. Then cells were centrifuged to remove the EDTA and the pellet was re-suspended in 1mL PBS with or without treatment. After that, cells were allowed to shake (90 rpm) for (60,120,240 mins) at 37°C. Then, they were fixed with 1% formaldehyde, microscopic pictures were taken and cells were counted.

G. Scanning electronic microscopy (SEM)

Cells were seeded on coverslips in 12-well plate and incubated at 37°C/5% CO_2. When reaching 80% confluency, media was removed and culture media containing NAs or 5-FU was added. After 24 hours, cells were washed twice with

PBS, 5 mins each, and fixed with 4% paraformaldehyde (PFA) for one hour at 4°C. Then, cells were washed three times again with PBS, two mins each, and post-fixed with 1% osmium tetroxide (OsO4). Then, after washing the cells with PBS, they were dehydrated using a graded series of ethanol with increasing concentration (25, 50, 75, 95, and 100 μM) for 5 mins each. Then, the coverslips were mounted on SEM stubs and covered with gold. Images were taken using Tescan SEM (MIRA3 software).

H. Chorioallantoic membrane (CAM) assay

Fertilized eggs were cleaned with 70% ethanol and incubated at 37°C with 50% humidity and they were rotated. On day 6, a small window is cut above the CAM. Then, 30 μL of desired concentrations of Control (vehicle), NAs, or 5-FU were added above the CAM and the window was sealed with parafilm and returned back to the incubator at 37°C/50% humidity with no rotation. 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.

I. MMP-2 and MMP-9 measurement

Cells were seeded and allowed to reach sub-confluent level. Then media was removed and new media containing the desired concentrations of Control (vehicle), NAs, or 5-FU were added. Then, medium was collected and levels of MMP-2 and MMP-9 were detected using ELISA kits (R&D Systems).

J. Quantification of NO production

The amount of NO production was determined with a colorimetric ELISA kit (Cayman Chemical, Ann Arbor, Michigan, USA), which is based on the Griess reaction, according to the manufacturer's instructions. The value of NO presented is the total value measured in the presence of cells minus the value determined from the media alone in the absence of any growing cells. Assays were performed in triplicates and three independent experiments were performed. Data are presented as mean values ± SEM.

K. Quantitative Immunoassay for Human Vascular Endothelial Growth Factor (VEGF)

Cells (1.5×10^5/well) were seeded in 24-well plates with culture media overnight. Then, cells were treated with vehicle (DMSO) or indicated concentrations of NAs and 5-FU and the conditioned media was collected at 24 h. The level of VEGF therein was measured using a VEGF enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The optical density was at 570 nm.

L. In-Cell ELISA

Cells were seeded in a 96-well plate and incubated at 37°C/5% CO_2. Cells were treated with the desired concentration of NAs or 5FU. Then, cells were fixed with PFA for 15 min, washed with 1X TBS, permeabilized for 20 mins, and then the blocking solution was added for 2 hours. Then after washing, the primary anti-body was added overnight at 4°C. Then, they were washed again and incubated with Diluted HRP Conjugate for 1 hour at room temperature. Then, after washing three times with PBS, TMB substrate was added in dark. Then the reaction was stopped and measured at 450 nm absorbance.

M. Statistical analysis

Statistical analyses were performed by student's *t*-test for unpaired observations. For multiple comparisons, ANOVA was used—either one-way ANOVA (with Dunnett's post hoc test) or two-way ANOVA (with Tukey-Kramer's post hoc test) using GraphPad Prism software. 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).

CHAPTER IV

RESULTS

A. Synthesis

The reaction of 2-chloro-4,6-diamino-1,3,5-triazine (4) with nucleobase-hydrazides (3a-d) proceeds by a nucleophilic substitution reaction. The formation of the desired nucleobase-hydrazide triazines derivatives (5a-d) was achieved by amination of 2-chloro-4,6-diamino-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 [125]. The esters (2a-d) were afforded in very good yields from the corresponding nucleobase following a modified literature procedure [126, 127]. The method employed a base catalyzed Michael-type addition reaction between the nucleobase and ethyl acrylate in refluxing ethanol. The synthesized compounds were well characterized by ^{1}H NMR, ^{13}C NMR and HRMS techniques.

The ^{1}H- NMR spectra of (5a–d) exhibit following types of signals; a) two sharp singlets around 9.7 ppm and 8.3 ppm corresponding to the secondary amine groups protons for the hydrazide; b) a broad singlet between 6.3-6.5 ppm corresponding to the primary amine groups 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 174.9, 45.1 and 32.9. 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, and 119. The uracil carbons appear at 164.5, 151.4, 147.1, and 100.9. The thymine carbons appear at 165, 151.3, 142.9, 108.3, and 12.4.

The thermal properties of the nucleobase-hydrazide triazines (5a-d) were evaluated using thermogravimetric analysis (TGA) at a heating rate of 10°C min up to 1100°C under a nitrogen atmosphere. The nucleobase hydrazide triazines 5a-d exhibits good thermal stability with high decomposition temperature. These derivatives exhibit 5% decomposition temperature below 300°C, which shows that these compounds are robust.

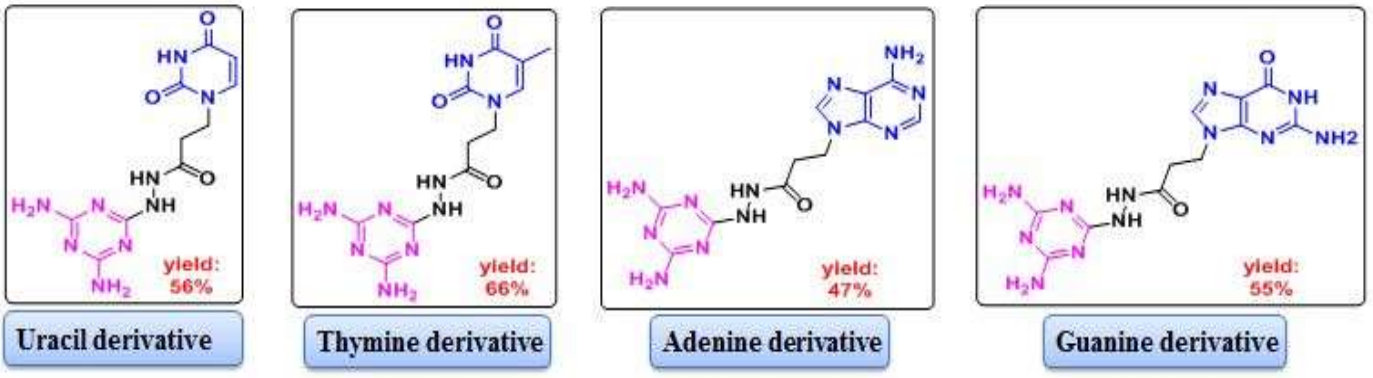


Figure 6: Chemical structure of the synthesized nucleoside analogs.

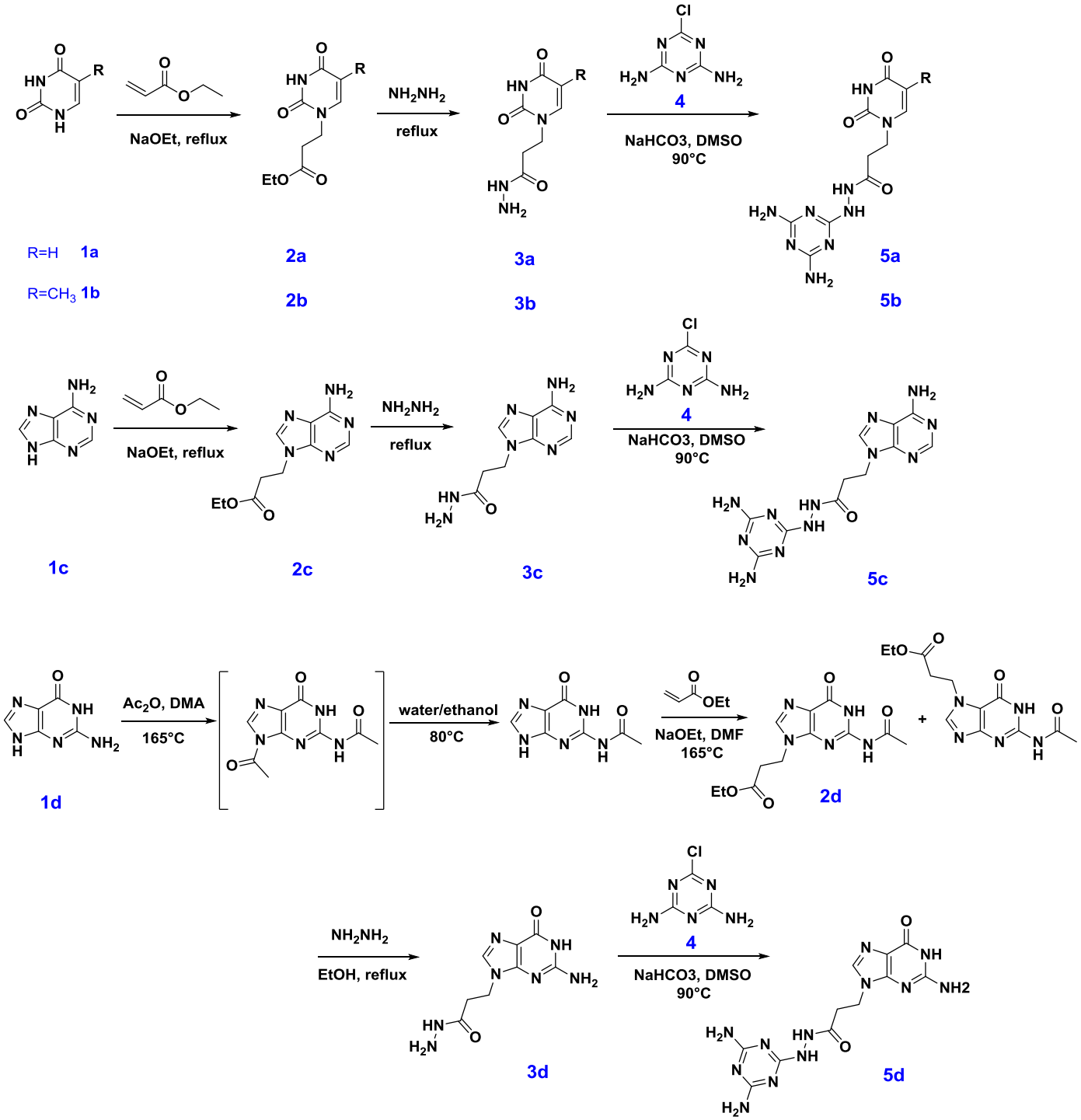
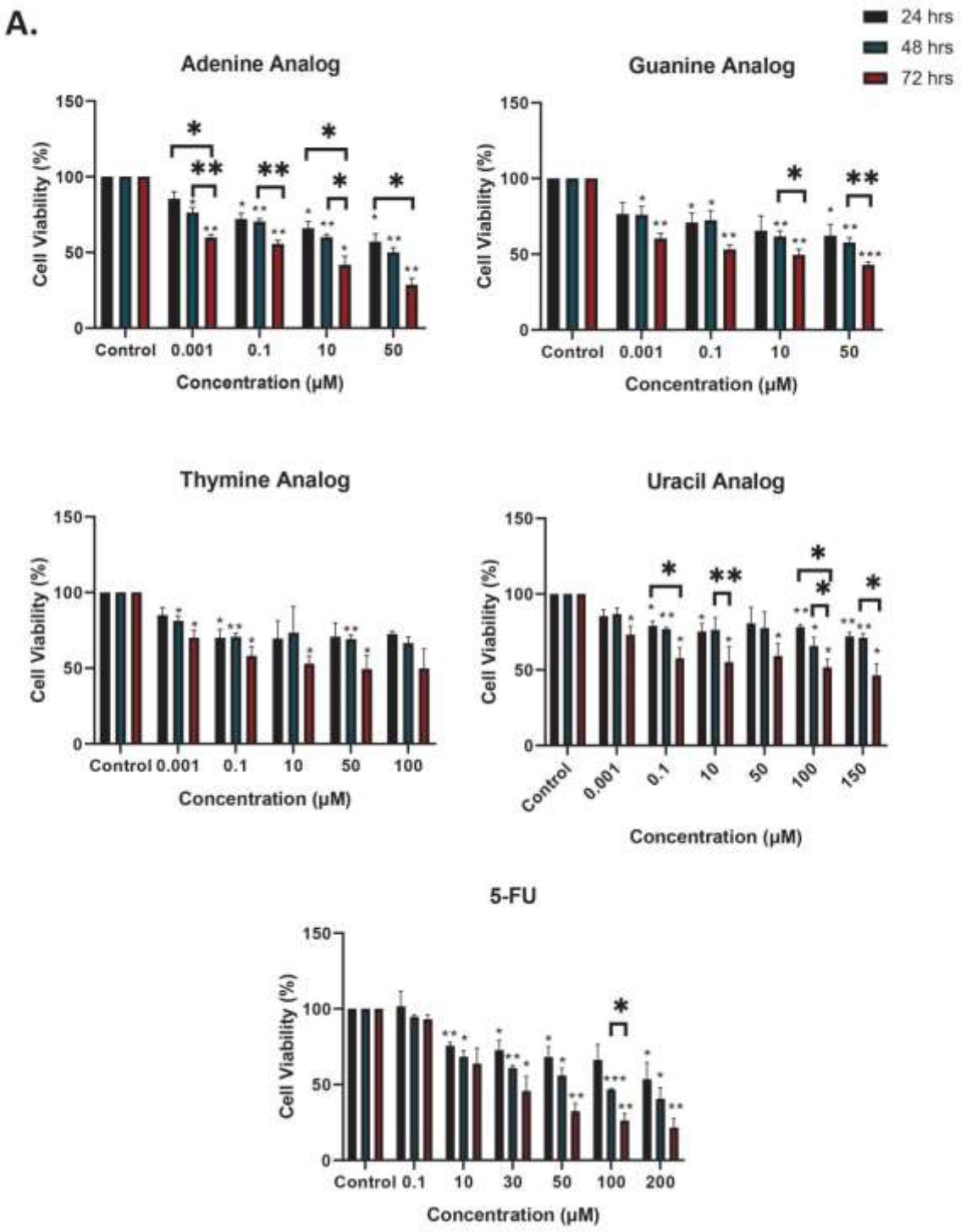


Figure 7: General scheme for NAS synthesis.

B. Biological Activity

1. NAs inhibit CRC cells proliferation in both cell types

In order to evaluate the effect of our synthesized nucleoside analogs on CRC, MTT assay was performed. HCT116 cells of both types, 5-FU –sensitive and resistant, were treated with increasing concentrations of NAs (0.001, 0.1, 10, and 50 μM) and 5-FU (10, 30, 50, 100, and 200 μM) respectively, for 24, 48 and 72 hours. In both cell lines, all NAs exhibited significant inhibition of cell proliferation in a time and concentration dependent manners. However, Adenine (A) and Guanine (G) analogs were found to be the most potent ones. As shown in figure (8), a 25% inhibition of the viability of the HCT116-5-FU-sensitive cells was caused by the lowest concentration (1 nM) of A after 48 hours. This was likely achieved by a 5-FU concentration of 10 μM, as depicted in figure (8). In addition, while 1 nM concentration of A caused a 30% reduction in the cell viability of the HCT116-5-FU-sensitive after 48 hours, it also caused a 22% reduction in the cell viability of the 5-FU resistant type. This indicates that the anti-proliferative effect of NAs is not affected by the 5-FU resistance status.


A.
24 hrs
48 hrs
72 hrs
Adenine Analog
Guanine Analog
Thymine Analog
Uracil Analog
5-FU
Cell Viability (%)
Concentration (μM)
Control
0.001
0.1
10
50
100
150
200
30

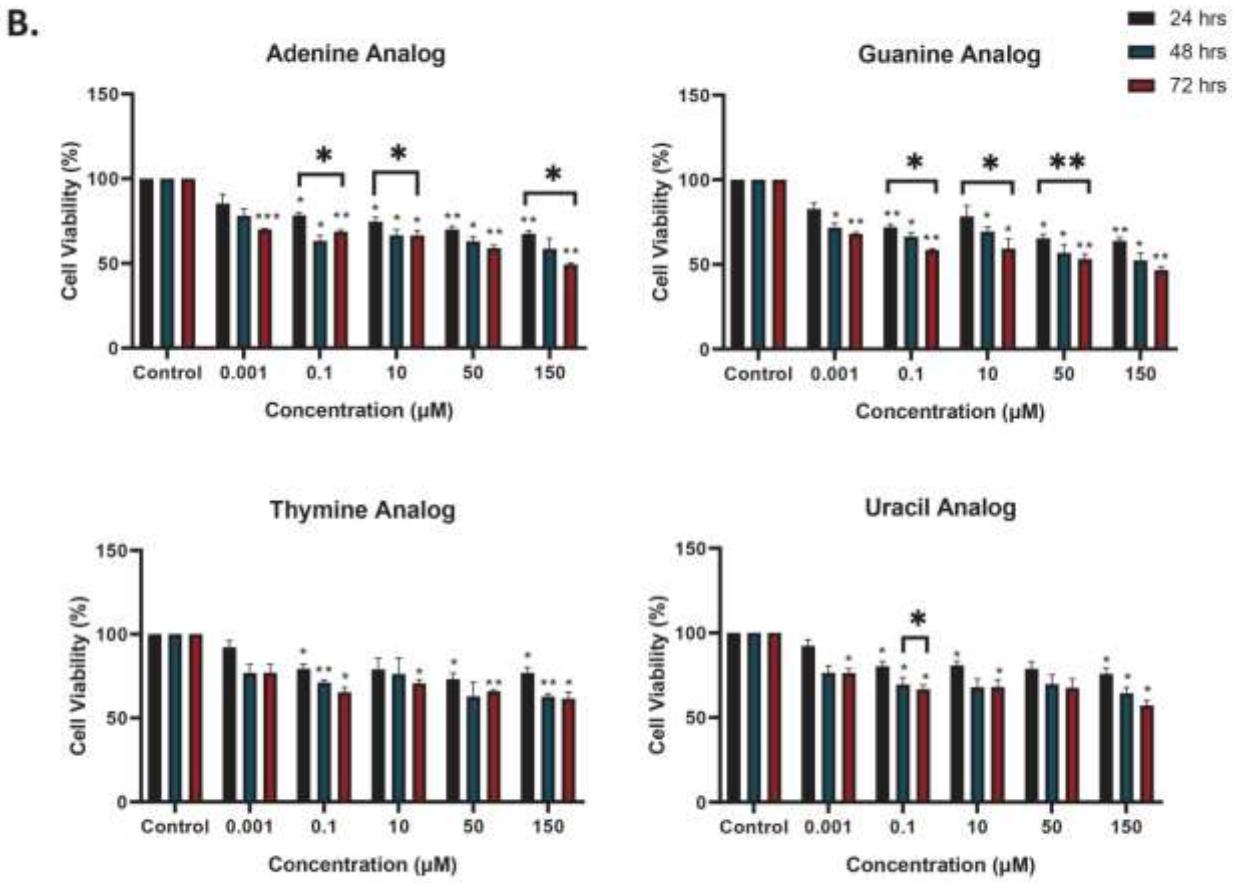


Figure 8: Nucleoside analogs (NAs) decrease the cellular viability of both types of HCT116 cells, in time and concentration dependent manners.

(A): 5-FU-sensitive HCT116 cells were treated with increasing concentrations of NAs (0.001, 0.1, 10, 50 μM) or 5-FU (0.1, 10, 30, 50, 100, 200 μM) for 24, 48, 72 hours, and cellular viability was determined by MTT. *p < 0.05, **p < 0.01 and ***p < 0.005. (B): 5-fu resistant HCT116 cells were treated with increasing concentrations of NAs (0.001, 0.1, 10, 50, 150 μM) for 24, 48, 72 hours, and cellular viability was determined by MTT. *p < 0.05, **p < 0.01 and ***p < 0.005.

2. NAs decrease the ratio of phospho- to total ERK1/2 in both cell types

ERK1/2 plays a key role in cell activity including proliferation, migration and angiogenesis. Thus, we treated, both cell types, with 0.1 μM of NAs or 10 μM of 5-FU and assessed their effect on the ratio of phospho- to total ERK1/2 by in-cell ELISA. As shown in fig (9), NAs significantly decreased the ratio levels of ERK1/2

not only in the HCT116-5-FU-sensitive cells, but also in the 5-FU resistant cells. While 0.1 μM of NAs decreased about 50% of ERK1/2 levels as compared to control, 10 μM of 5-FU decreased only 40 % (figure 9-A). In addition, with the same concentration, 0.1 μM, these NAs were able to decrease also 50% of ERK1/2 ratio level in the 5-FU resistant cells (figure 9-B).

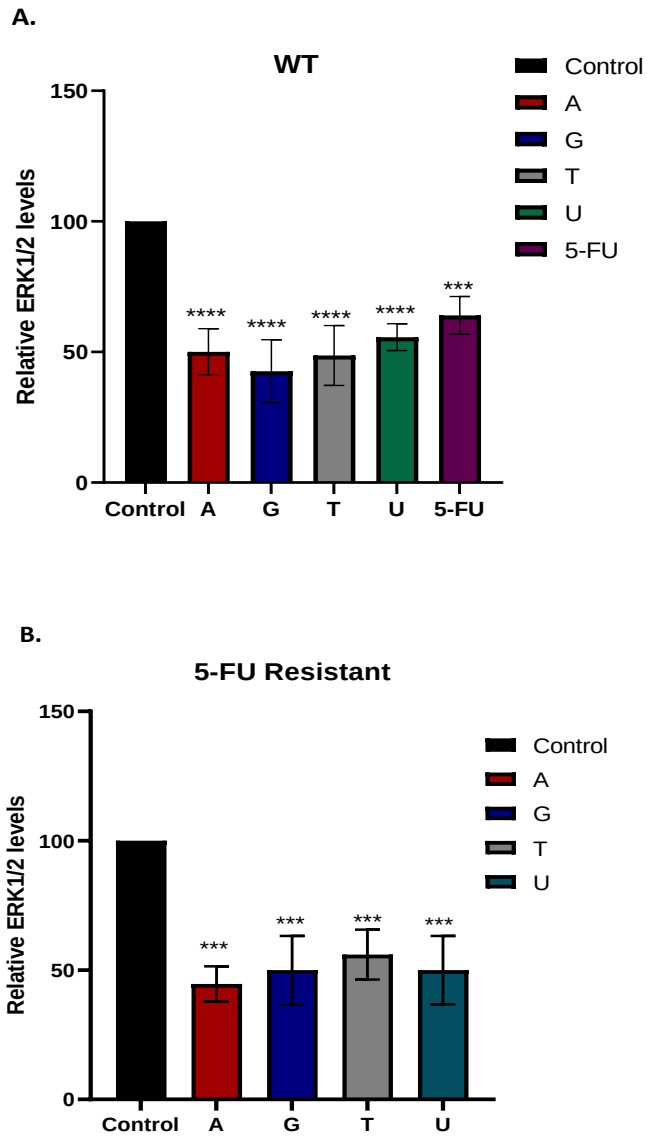


Figure 9: Nucleoside analogs decrease the ratio of phospho- to total ERK1/2 in both cell types.

HCT116 of both cell types were treated with 0.1 μM of NAs or 10 μM 5-FU and ERK1/2 level was determined by IN-CELL-ELISA. (A): ratio levels of ERK1/2 in HCT116-5-FU-sensitive treated with NAs/5-FU as compared to control (vehicle). ***$p < 0.005$ and ****$p < 0.0001$. (B): ratio levels of ERK1/2 in 5-FU resistant HCT116 treated with NAs as compared to control (vehicle). ***$p < 0.005$.

3. NAs induce morphological changes of cells

It is well-known that changes in cell morphology affect cellular functions. So, we wanted to check the effect of our NAs on cellular morphology. Both cell types were treated with NAs (0.1 μM), 5-FU (10 μM), or control (vehicle) for 24 hours, then microscopic pictures were taken using SEM. As shown in figure (10), unlike control cells, treated cells had cytoplasmic protrusions with a flattened shape, thus indicating less migratory cells. Further, it was well noticed the difference in cellular morphology between the HCT116 cells of 5-FU-sensitive and 5-FU resistant types. Fewer and less pronounced cytoplasmic protrusions were developed by the treated 5-FU resistant cells when compared to the treated HCT116-5-FU-sensitive cells. Additionally, the treated 5-FU- resistant cells were more elongated than the 5-FU resistant control cells (figure 10-B).

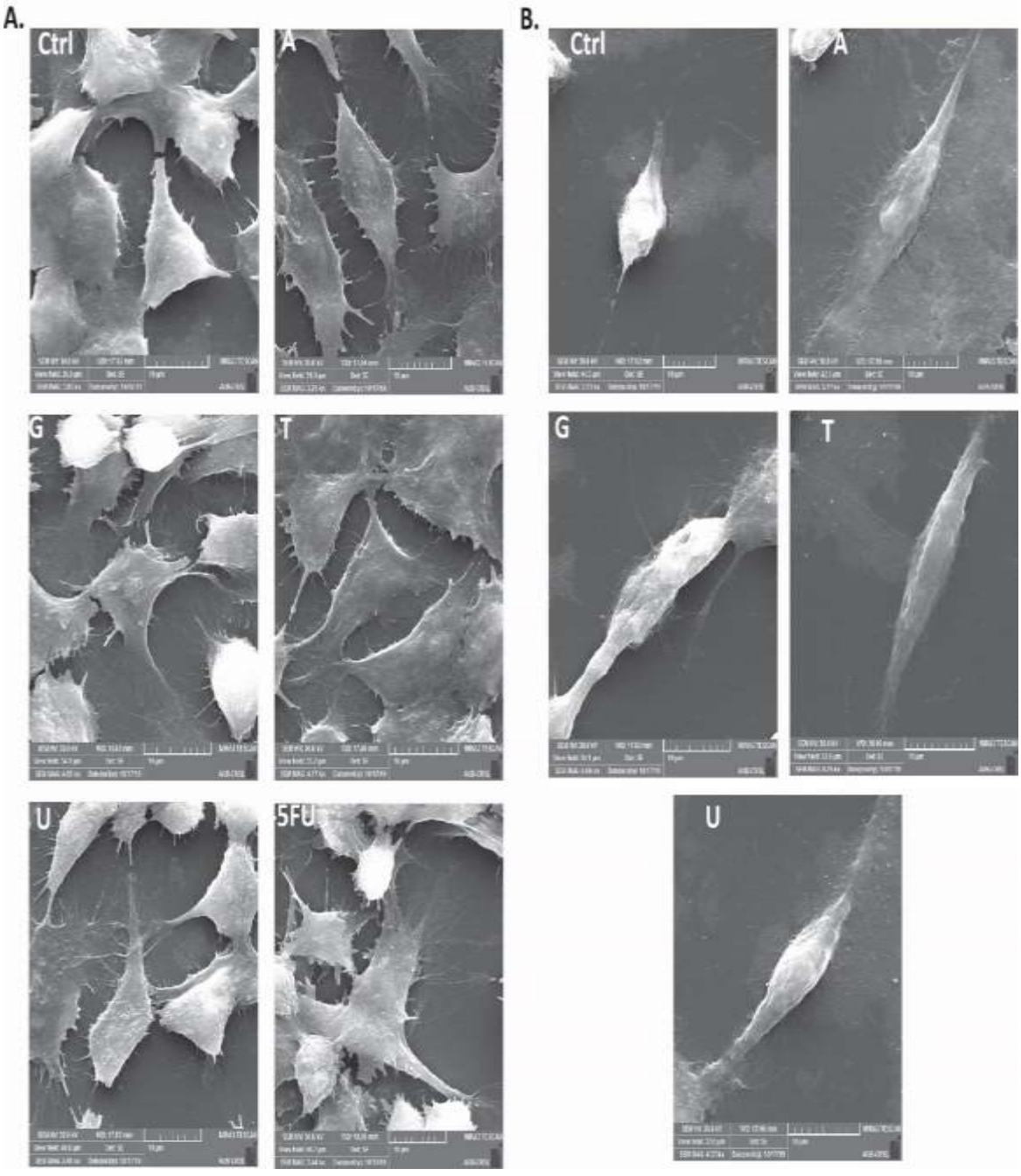


Figure 10: NAs induce morphological changes in both cell types of HCT116.

Cells were treated with vehicle (control), 0.1 μM NAs, or 10 μM 5-FU for 24 hours. (A): representative SEM pictures of HCT116-5-FU-sensitive cells with scale bar 10 μm. (B): representative SEM pictures of HCT116- 5-FU resistant type with scale bar 10 μm.

4. NAs inhibit cell migration in both cell types

Cell migration is a pre-requisite step for tumor metastasis. Thus, inhibiting migration could inhibit cell metastasis. The effect of our NAs on cells migration was determined using the wound healing scratch assay. With concentrations of 0.1 μM of NAs or 10 μM of 5-FU, cell migration was inhibited in a time dependent manner. Starting with t=6 hours, A, G, U and 5-FU significantly inhibited cell migration in both cell types with no significant decrease in cell viability, as seen in figure (11). This shows that our NAs have an anti-migratory effect in addition to their abovementioned anti-proliferative effect. On the other hand, T showed no significant inhibition of the cell migration of the HCT116-5-FU-sensitive cells; however, it significantly inhibited migration of the 5-FU resistant type starting at 6 hours (figure 11-A/B).

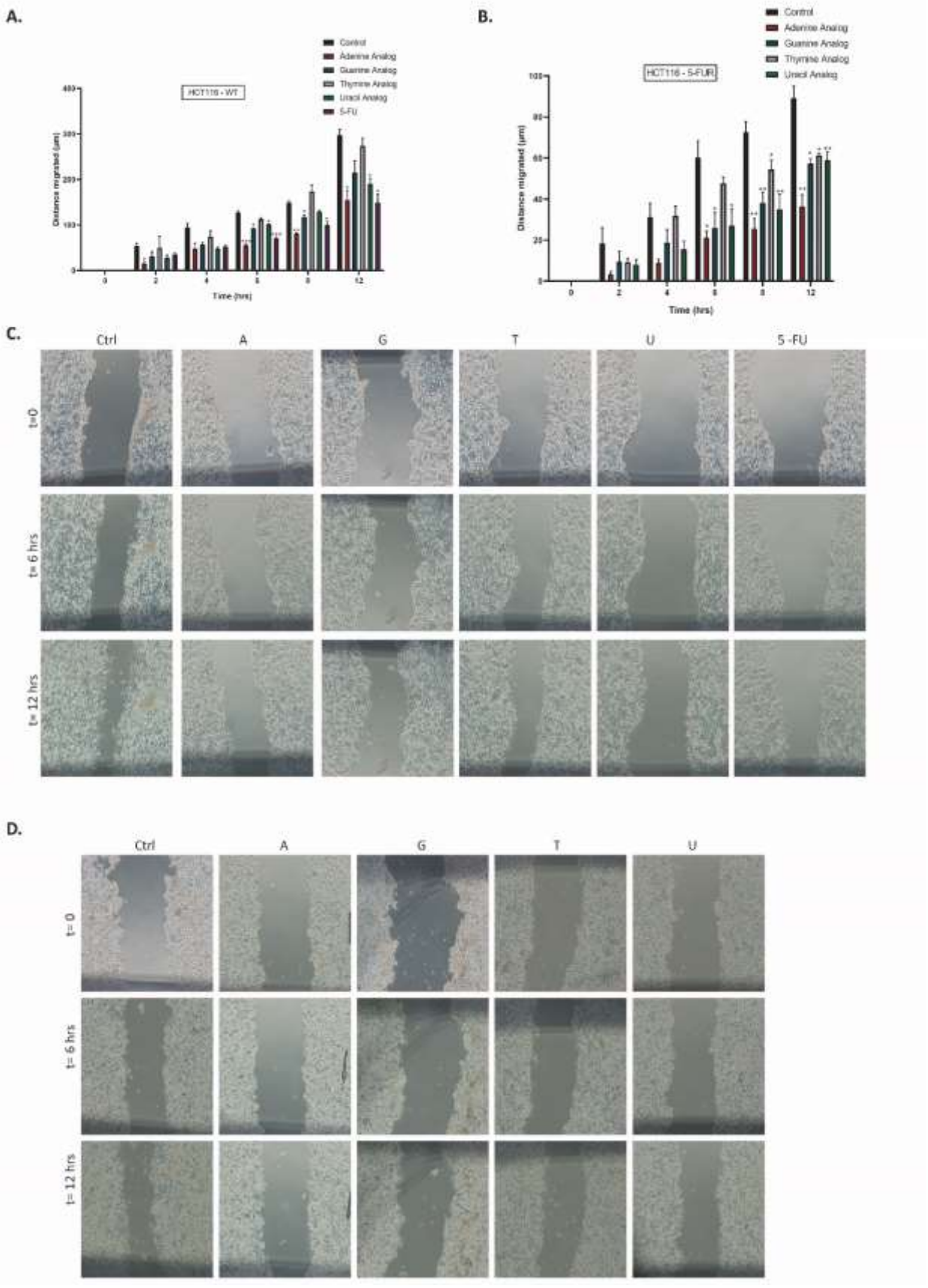
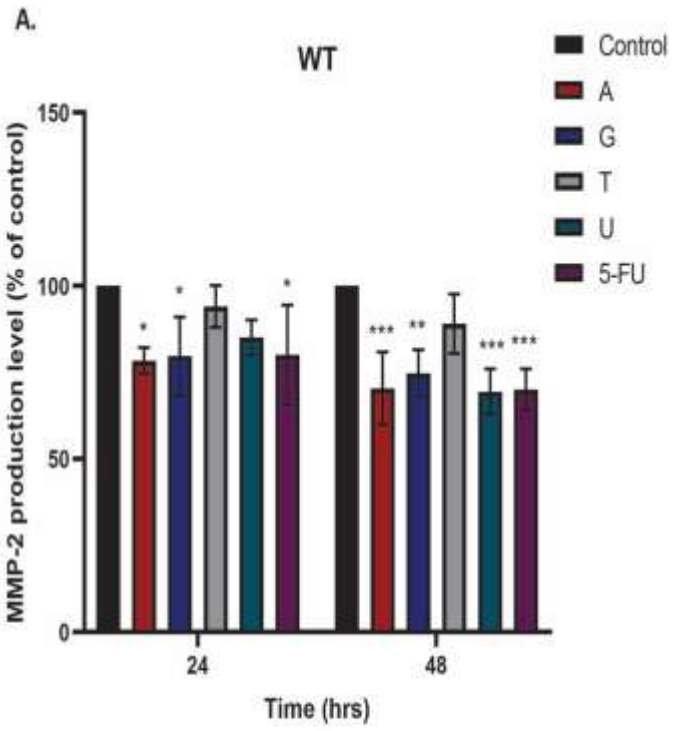

A.
HCT116 - WT
Control
Adenine Analog
Guanine Analog
Thymine Analog
Uracil Analog
5-FU
Distance migrated (µm)
Time (hrs)
B.
HCT116 - 5-FUR
Control
Adenine Analog
Guanine Analog
Thymine Analog
Uracil Analog
Distance migrated (µm)
Time (hrs)
C.
Ctrl
A
G
T
U
5 -FU
t=0
t= 6 hrs
t= 12 hrs
D.
Ctrl
A
G
T
U
t= 0
t= 6 hrs
t= 12 hrs

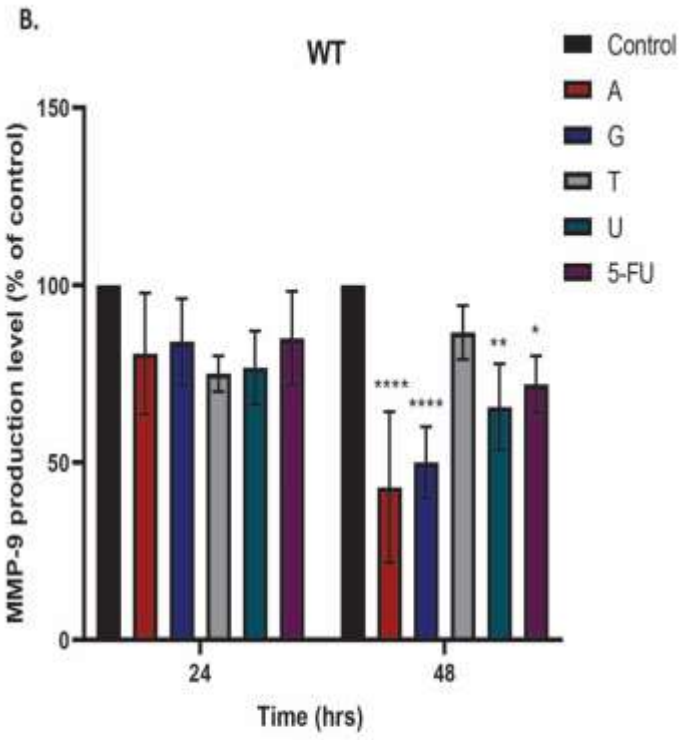
Figure 11: NAs inhibit cellular migration of both types of HCT116 cells in time dependent manner.

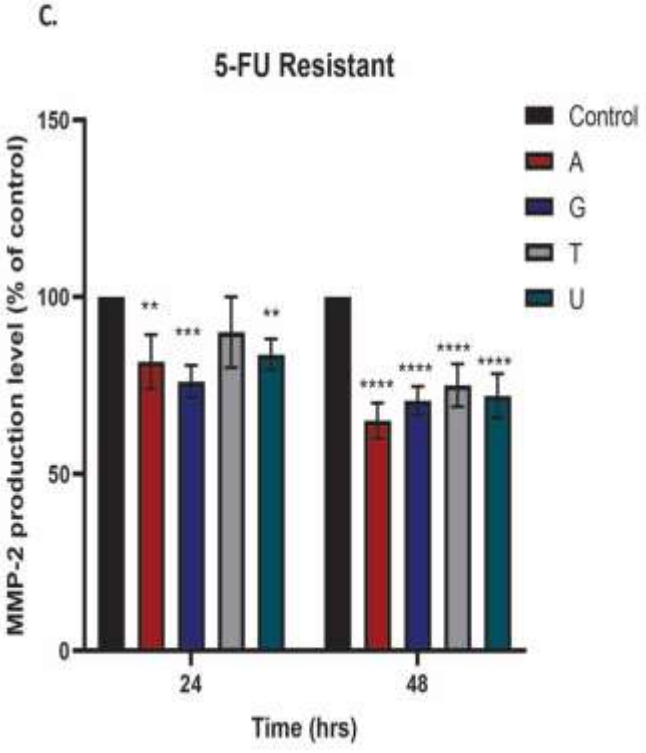
Cells were treated with NAs (0.1 μM) or 5-FU (10 μM) and cell migration was assessed using wound healing assay. (A): distance migrated of HCT116 5-FU-sensitive cells were represented as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.005. (B): distance migrated of HCT116 5-FU resistant cells were represented as mean ± SEM. *p < 0.05 and **p < 0.01 (C): representative figures of HCT116- 5-FU-sensitive cells for scratch assay. (D): representative figures of HCT116-5-FUR cells for scratch assay.

5. NAs decreased the levels of MMP-2 and MMP-9 in both cell lines

Matrix metalloproteinases proteases (MMPs) constitute a family of enzymes involved in ECM degradation, and also in tumor cells migration and invasion. Thus, the inhibition of these MMPs, especially MMP-2 and MMP-9, would decrease cell migration. We treated, both types of cells, with 0.1 μM of NAs, or 10 μM of 5-FU and checked the levels of MMP-2 and MMP-9. As shown in the figure (12), all NAs significantly decreased the levels of MMP-2 and MMP-9 not only in the 5-FU-sensitive HCT116 cells, but also in the 5-FU resistant ones. For instance, while 0.1 μM of A decreased the expression of MMP-2 and MMP-9 by 35% and 55 % respectively, it decreased the levels of MMP-2 and MMP-9 by 30% and 40% respectively in the 5-FU resistant cells after 48 hours.


A.
WT
Control
A
G
T
U
5-FU
MMP-2 production level (% of control)
150
100
50
0
24
48
Time (hrs)


B.
WT
Control
A
G
T
U
5-FU
MMP-9 production level (% of control)
150
100
50
0
24
48
Time (hrs)


C.
5-FU Resistant
Control
A
G
T
U
MMP-2 production level (% of control)
150
100
50
0
24
48
Time (hrs)

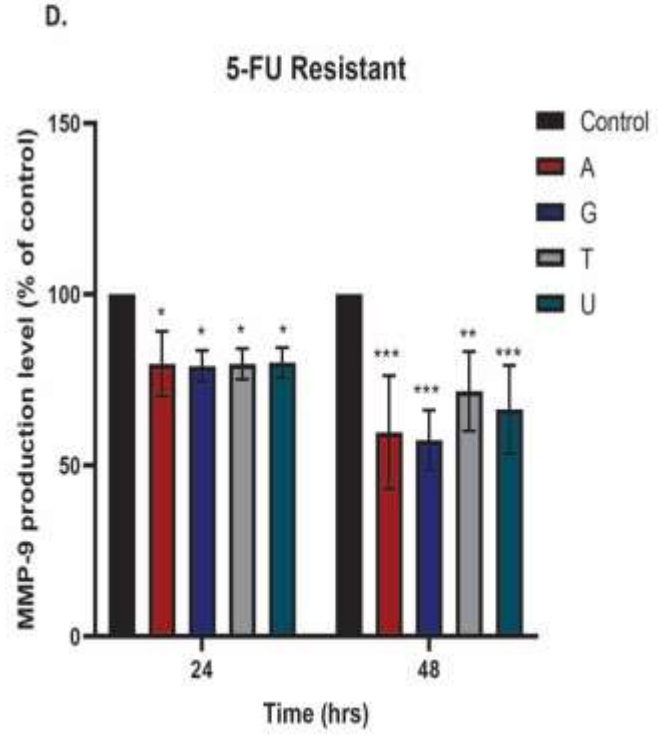

D.
5-FU Resistant
Control
A
G
T
U
MMP-9 production level (% of control)
150
100
50
0
24
48
Time (hrs)

Figure 12: NAs decreased the levels of MMP-2 and MMP-9 in both cell lines.

Cells were treated with NAs (0.1 μM) or 5-FU (10 μM) for 24 and 48 hours and levels of MMP-2 and MMP-9 were determined. (A): levels of MMP-2 as a % of control of HCT116-5-FU-sensitive was represented as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.005. (B): levels of MMP-9 as a % of control of HCT116-5-FU-sensitive was represented as mean ± SEM. *p < 0.05, **p < 0.01 and ****p < 0.0001. (C): levels of MMP-2 as a % of control of HCT116-5-FUR was represented as mean ± SEM. **p < 0.01, ***p < 0.005 and ****p < 0.0001. (D): levels of MMP-9 as a % of control of HCT116-5-FUR was represented as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.005.

6. NAs decrease the adhesion of HCT116 cells on Collagen in both cell lines

Cancer cells are believed to have an enhanced ability to adhere to the extracellular matrix. This facilitates their migration to new sites. Thus, we desired to determine the effect of our NAs on cell adhesion to collagen; one of the ECM components. In both cell types, 5-FU-sensitive and 5-FU resistant HCT116 cells, NAs with the same concentration (0.1 μM) significantly inhibited cell adhesion in a time dependent manner. After 30 mins, there was a 60% decrease in cell adhesion for cells treated with A not only in the HCT116-5-FU-sensitive cells, but also in the 5-FU resistant ones. In addition, while 0.1 μM of T caused a 30% decrease in the adhesion of the HCT116-5-FU-sensitive cells after 30 mins, it caused a more potent effect in the 5-FU resistant type (50 % inhibition) as seen in figure (13 A-B).

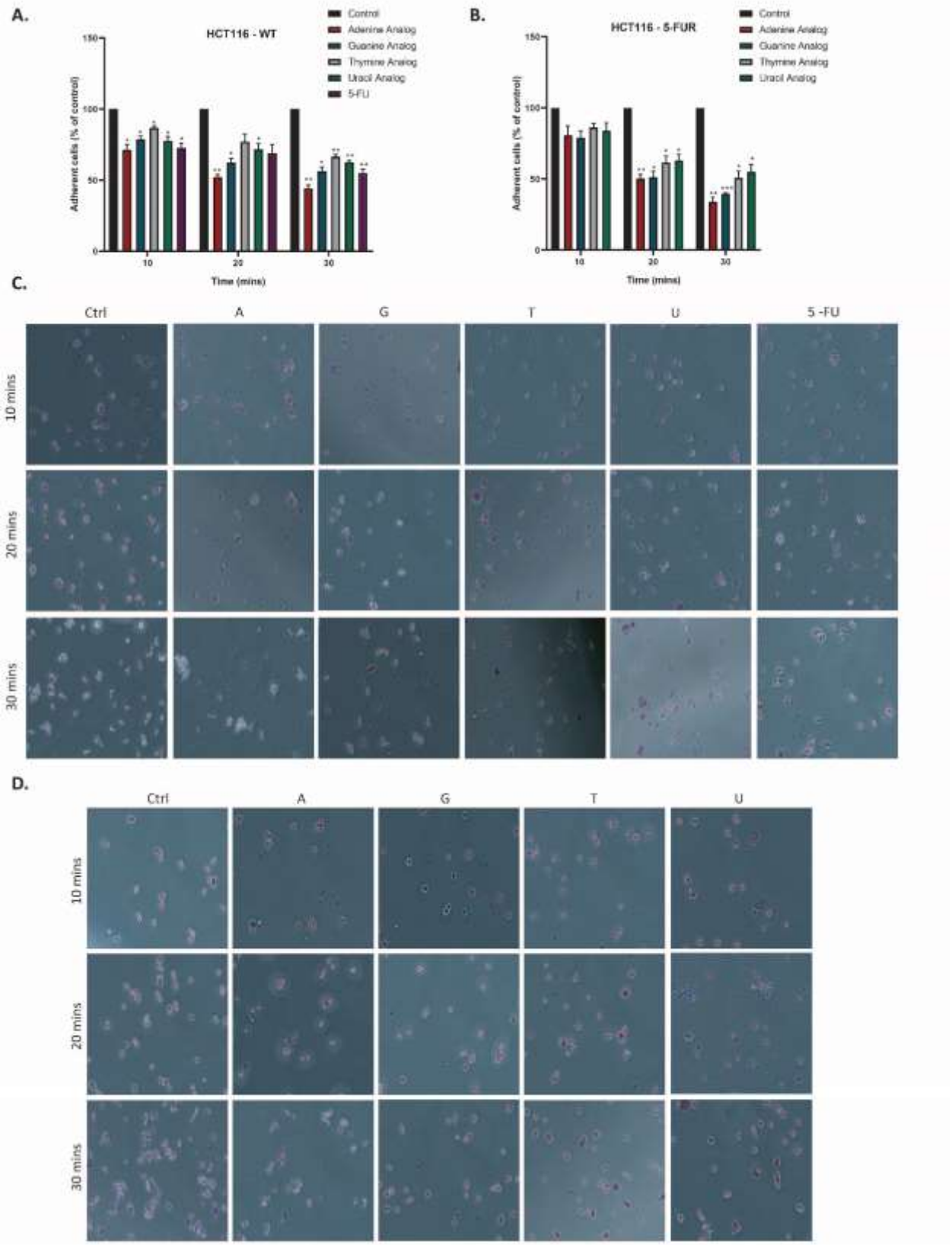
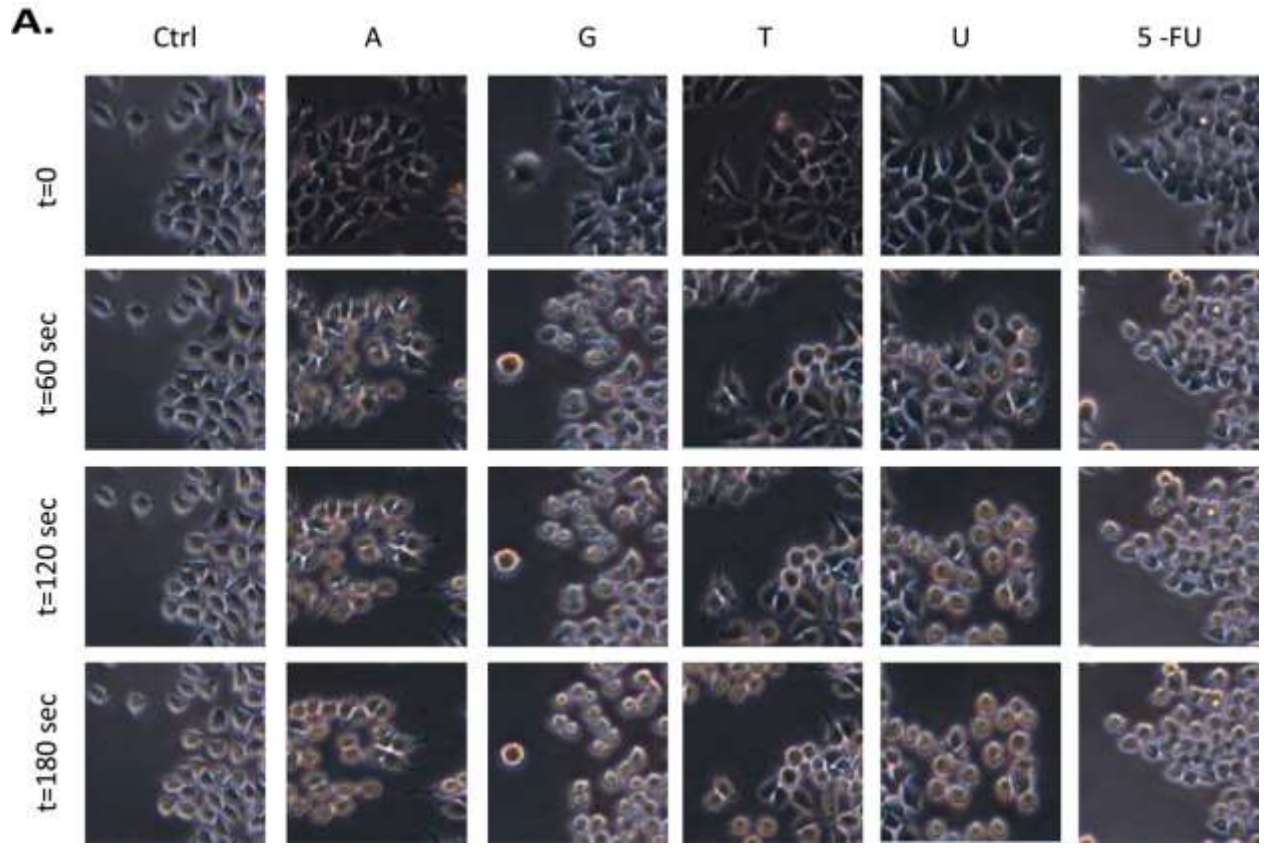

A.
HCT116 - WT
Control
Adenine Analog
Guanine Analog
Thymine Analog
Uracil Analog
5-FU
Adherent cells (% of control)
Time (mins)
B.
HCT116 - 5-FUR
C.
Ctrl
A
G
T
U
5 -FU
10 mins
20 mins
30 mins
D.

Figure 13: NAs inhibit the adhesion of both types of HCT116 cells to collagen in a time dependent manner.

Cells were treated with NAs (0.1 μM) or 5-FU (10 μM) for 10, 20, and 30 mins and % adhesion was determined. (A): % of adhesion of HCT116-5-FU-sensitive was represented as mean ± SEM. *$p < 0.05$ and **$p < 0.01$. (B): % of adhesion of HCT116-5-FUR was represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$. (C): representative figures of HCT116-5-FU-sensitive cells for adhesion assay. (D): representative figures of HCT116-5-FUR cells for adhesion assay.

7. NAs increase the de-adhesion rate of HCT116 cell on Collagen in both cell lines

Next, we wanted to check the effect of our NAs on the de-adhesion rate of the CRC cells of both types. As shown in figure (14 A-B), the de-adhesion rate was higher in the treated cells as compared to the control. While control cells took 120 secs to detach, treated cell detached more quickly (within 60 secs), see figure (14). In addition, with the same concentration of NAs (0.1 μM), NAs were able to increase the detachment rate not only of the HCT116-5-FU-sensitive cells, but also the 5-FU resistant ones.


A.
Ctrl
A
G
T
U
5 -FU
t=0
t=60 sec
t=120 sec
t=180 sec

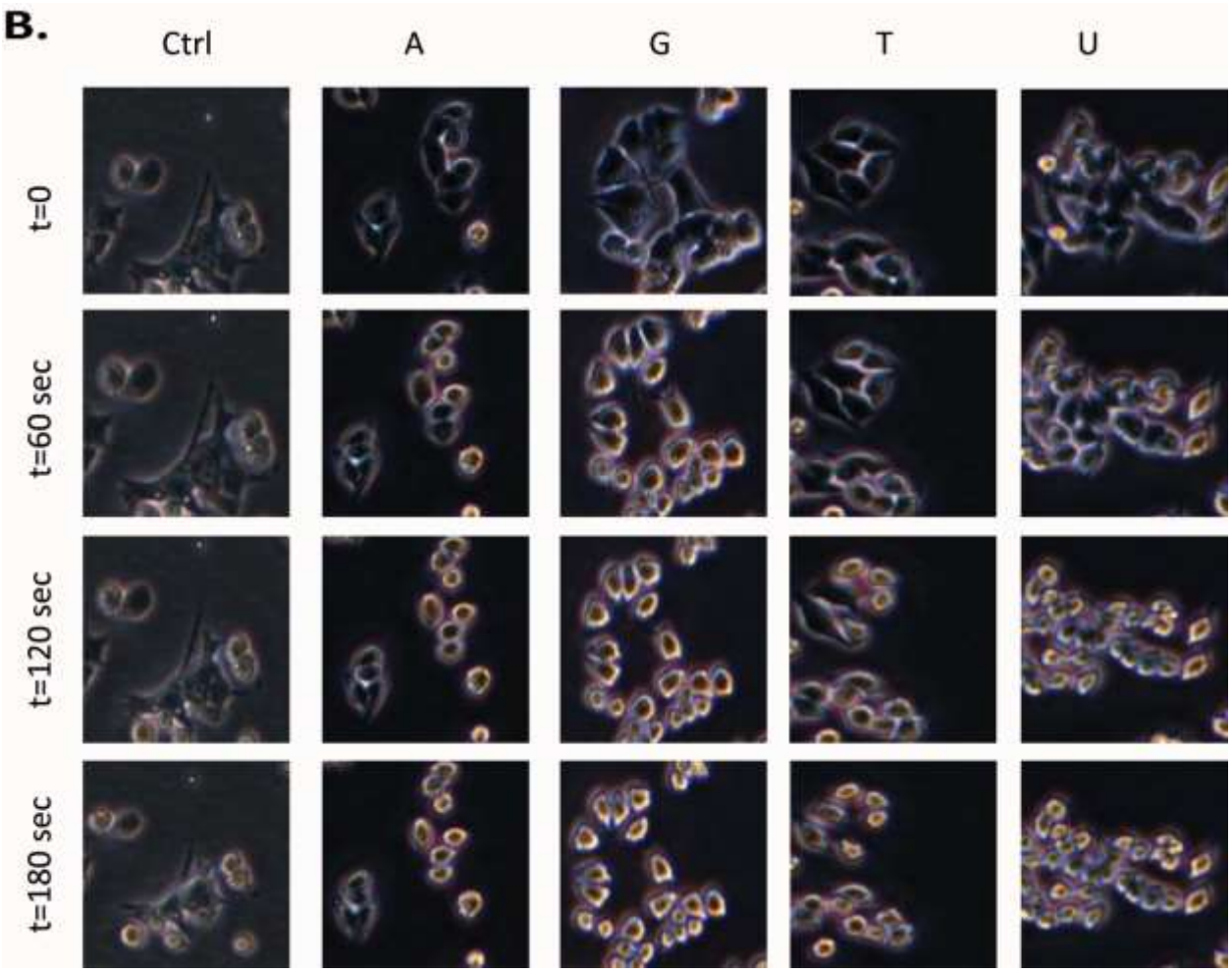

B.
Ctrl
A
G
T
U
t=0
t=60 sec
t=120 sec
t=180 sec

Figure 14: NAs increased the detachment rate of hct116 cells in both cell types.

Both cell types were treated with corresponding concentrations of NAs (0.1 μM) or 5-FU (10 μM) then warm trypsin were added for cell detachment. Microscopic pictures were taken every 20 seconds for the HCT116-5-FU-sensitive (A) and the 5-FU resistant cells (B).

8. NAs promote the homotypic aggregation of HCT116 cells in both cell lines

Cancer cell metastasis is associated with a decreased homotypic aggregation of the tumor cells. So, in order to evaluate whether our NAs could affect homotypic cell adherence, aggregation assay was performed. As shown in figure (15), our NAs, with 0.1 μM concentration, significantly promoted homotypic aggregation of the HCT116 cells, both 5-FU-sensitive and 5-FU resistant type, in a time dependent manner. Starting from 2 hours, the formation of the aggregated cells significantly began to appear in the treated groups of cells, as compared to the untreated ones (control). Furthermore, the size of the aggregate was bigger in A and G than in the other two synthesized NAs (T and U).

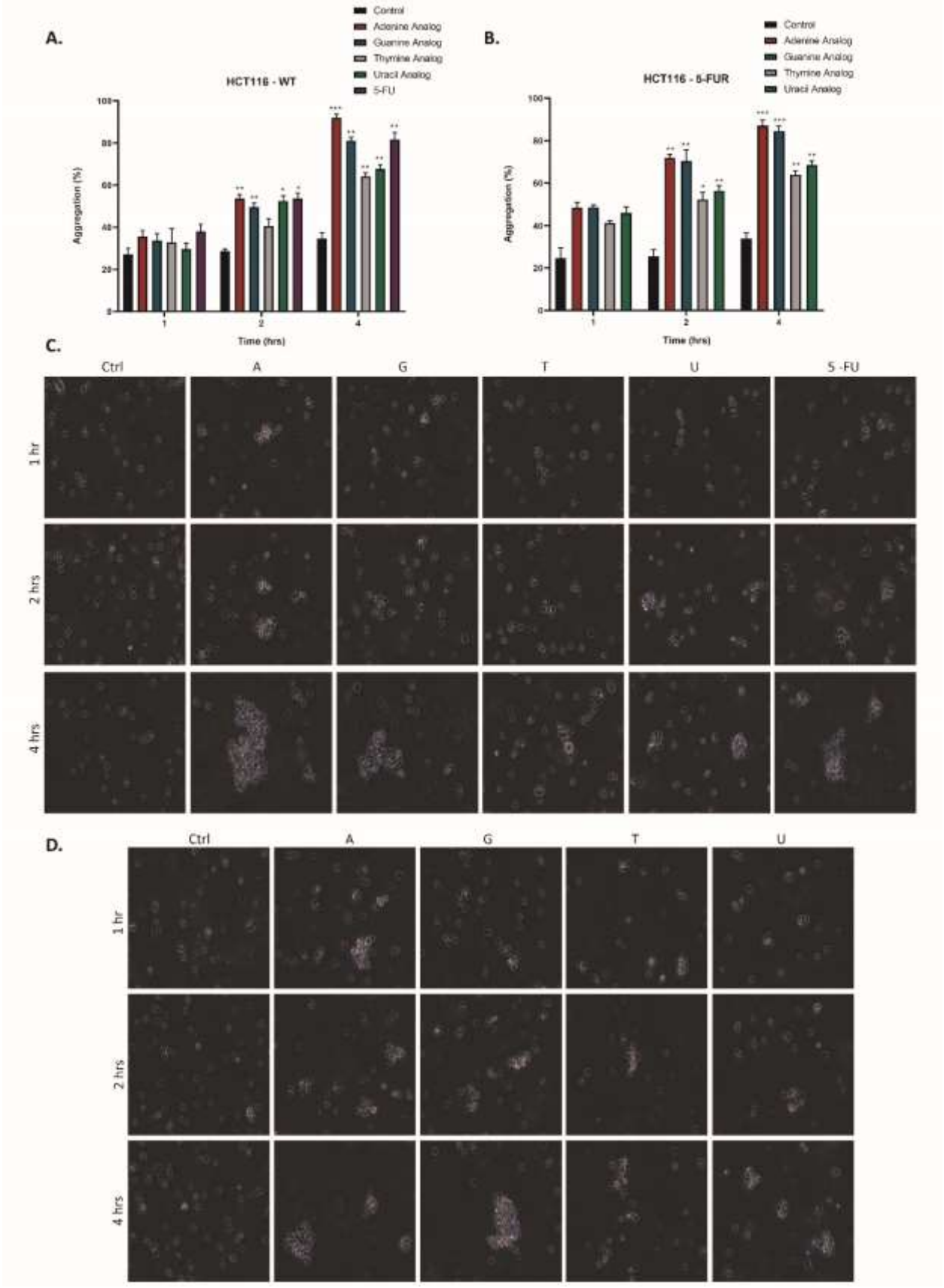

A.
HCT116 - WT
Control
Adenine Analog
Guanine Analog
Thymine Analog
Uracil Analog
5-FU
Aggregation (%)
Time (hrs)
B.
HCT116 - 5-FUR
Control
Adenine Analog
Guanine Analog
Thymine Analog
Uracil Analog
Aggregation (%)
Time (hrs)
C.
Ctrl
A
G
T
U
5 -FU
1 hr
2 hrs
4 hrs
D.
Ctrl
A
G
T
U
1 hr
2 hrs
4 hrs

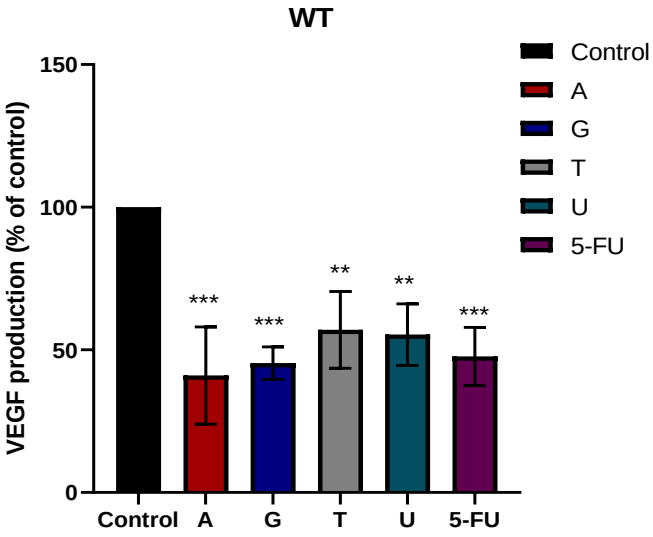
Figure 15: NAs promote the aggregation of both types of HCT116 cells in a time dependent manner.

Cells were treated with NAs (0.1 μM) or 5-FU (10 μM) for 1, 2, and 4 hours and % of cell aggregation was determined. (A): % of aggregation of HCT116-5-FU-sensitive cells was represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$. (B): % of aggregation of HCT116-5-FUR was represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$. (C): representative figures of HCT116-5-FU-sensitive cells for aggregation assay. (D): representative figures of HCT116-5-FUR cells for aggregation assay.

9. NAs decrease the levels of VEGF in both cell types.

In order to further investigate the effects of NAs on angiogenesis, we checked the levels of the major pro-angiogenic factor, vascular endothelial growth factor (VEGF). HCT116 of both cell types were treated with 0.1 μM NAs or 10 μM 5-FU. The levels of VEGF produced were then checked. As shown in figure (16), all NAs and 5-FU significantly decreased the VEGF production in both cell types as compared to the control (vehicle). Further, Adenine revealed a significant effect, like 5-FU, in reducing VEGF production. This effect of A was also observed in the 5-FU resistant cells.

A.


WT
VEGF production (% of control)
150
100
50
0
Control
A
G
T
U
5-FU
***
***
**
**
***

B.

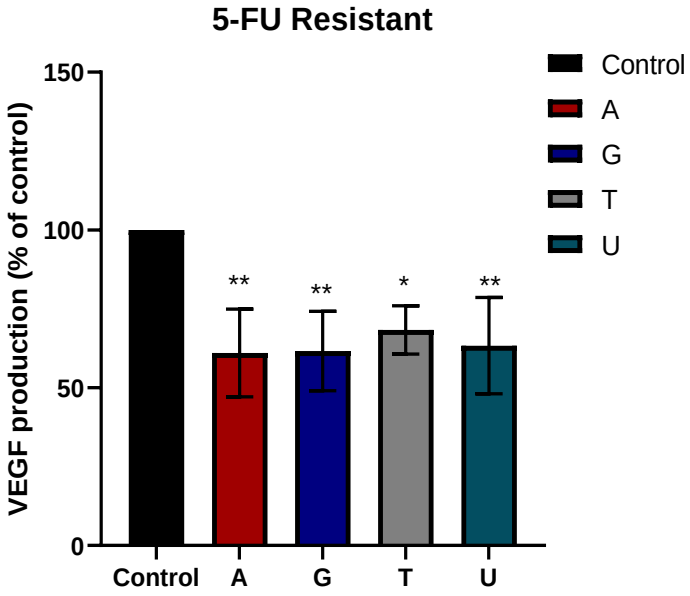
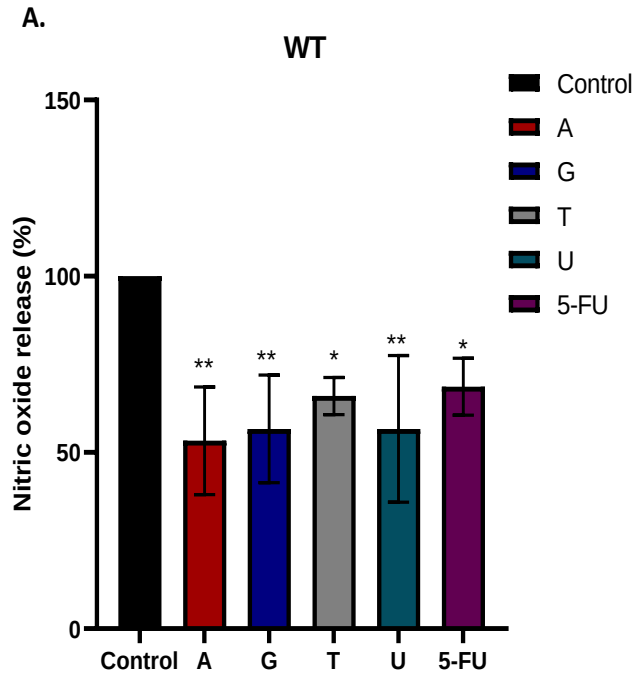

5-FU Resistant
VEGF production (% of control)
150
100
50
0
Control
A
G
T
U
**
**
*
**

Figure 16: NAs decrease the levels of VEGF in both cell types.

HCT116 cells, of both types, were treated with 0.1 μM of NAs or 10 μM 5-FU and VEGF production level was determined. (A): levels of VEGF in HCT116-5-FU-sensitive cells treated with NAs/5-FU as compared to control (vehicle). **$p < 0.01$ and ***$p < 0.005$. (B): levels of VEGF in 5-FU resistant HCT116 treated with NAs as compared to control (vehicle). *$p < 0.05$ and **$p < 0.01$.

10. NAs decrease the levels of Nitric Oxide in both cell types

Nitric oxide (NO) is a molecule that plays a key role in cellular communication. It can induce proliferation, migration and angiogenesis. Thus, we wanted to check the effect of our NAs on the NO production. Indeed, as shown in figure (17), in both types of HCT116, all NAs, when added in a concentration of 0.1 μM, significantly reduced the levels of NO production as compared to the control. In fact, A displayed a high efficacy in decreasing the cellular production of NO not only in the HCT116-5-FU-sensitive cells, but also in the 5-FU resistant one (50% decrease).


A.
WT
Control
A
G
T
U
5-FU
Nitric oxide release (%)
150
100
50
0
**
**
*
**
*
Control
A
G
T
U
5-FU

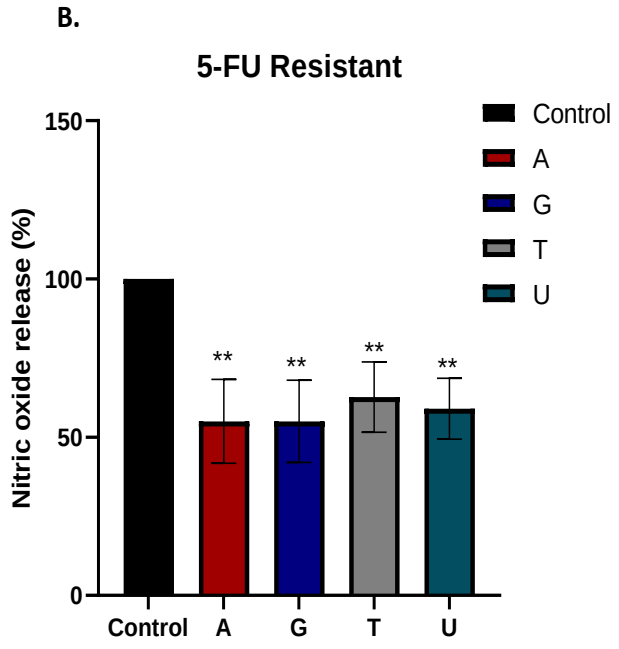

B.
5-FU Resistant
Control
A
G
T
U
Nitric oxide release (%)
150
100
50
0
**
**
**
**
Control
A
G
T
U

Figure 17: NAs decrease the levels of NO in both cell types.

HCT116 cells, of both types, were treated with 0.1 μM of NAs or 10 μM 5-FU and NO production levels were determined. (A): levels of NO in HCT116-5-FU-sensitive cells treated with NAs/5-FU as compared to control (vehicle). *$p < 0.05$ and **$p < 0.01$. (B): levels of NO in 5-FU resistant HCT116 treated with NAs as compared to control (vehicle). **$p < 0.01$.

11. NAs inhibit angiogenesis in the CAM assay

Angiogenesis, or the formation or new blood vessels from pre-existing ones, is a crucial mechanism for cancer development. Anti-angiogenic agents are believed to inhibit metastasis. Thus, we checked the effect of our NAs on angiogenesis by using the chick embryo model. NAs of 0.1 μM, 5-FU of 10 μM, and control (vehicle) were added on the chorioallantoic membrane (CAM) above the embryo. After 24 hours, all NAs and 5-FU significantly inhibited angiogenesis, as depicted by the decrease in the total length of blood vessels and the number of junctions. Compared to the control where there is a 10% increase in vessels length and 30% increase in the number of junctions, all NAs significantly decreased both the blood vessels length and the junction number (figure 18 A-B). Specifically, A decreased the blood vessels length by more than 45% and the number of junctions by about 56% as seen in figure 18 (A-B). Moreover, 5-FU was shown to be less efficacious than A in decreasing angiogenesis.

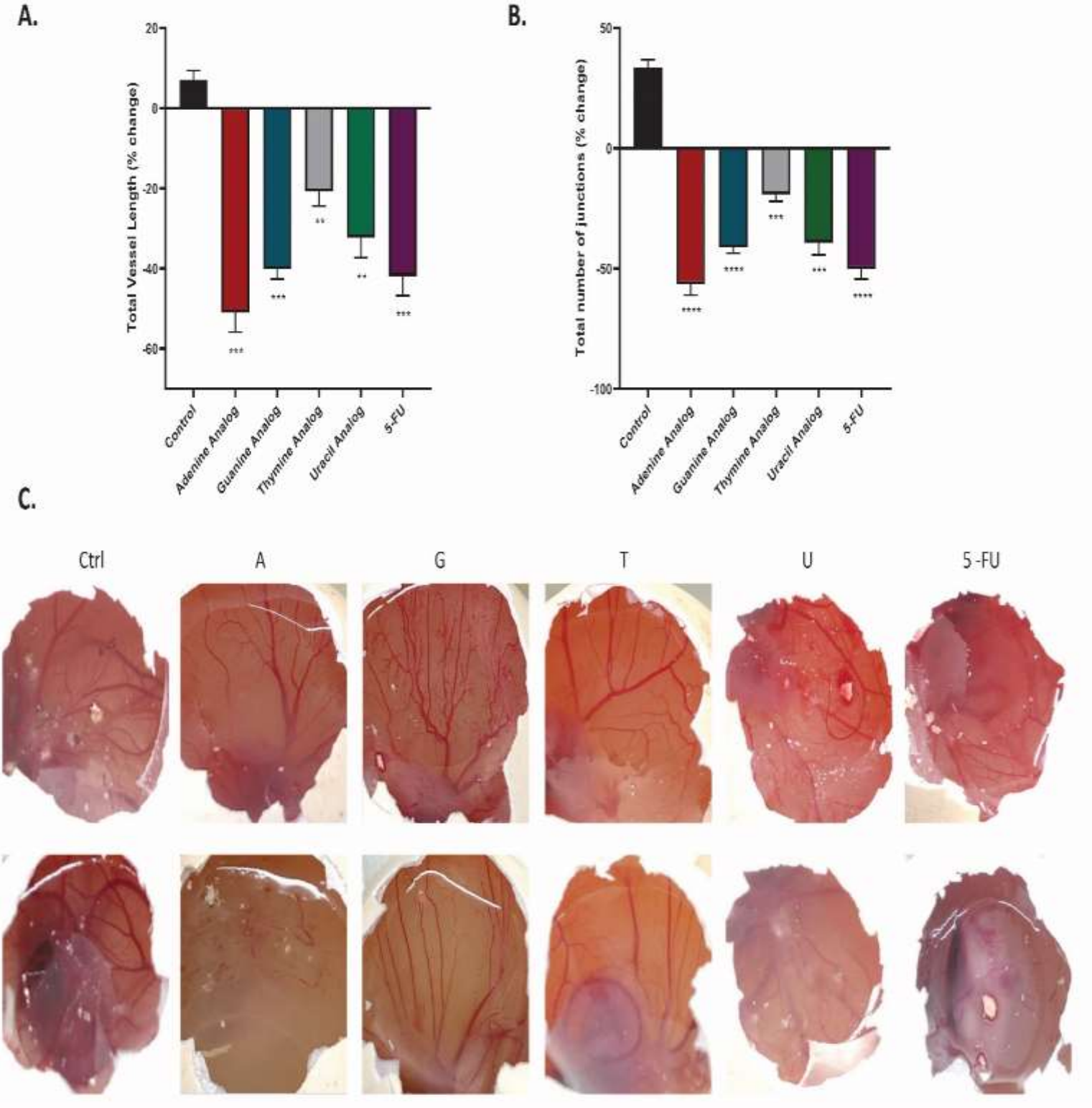

A.
Total Vessel Length (% change)
20
0
-20
-40
-60
***
***
**
**
***
Control
Adenine Analog
Guanine Analog
Thymine Analog
Uracil Analog
5-FU
B.
Total number of junctions (% change)
50
0
-50
-100
****
****
***
***
****
Control
Adenine Analog
Guanine Analog
Thymine Analog
Uracil Analog
5-FU
C.
Ctrl
A
G
T
U
5-FU
t=0
t= 24 hrs

Figure 18: NAs inhibit angiogenesis. This was demonstrated using the chick embryo chorioallantoic membrane assay.

On day 6, NAs (0.1 μM) or 5-FU (10 μM) were added on the CAM above the embryo. The change in total vessel length (A) and the total number of junctions (B) were assessed after 24 hours. Values are represented as mean ± SEM. **$p < 0.01$, ***$p < 0.005$, and ****$p < 0.0001$. (C): represented figures of the CAM at t=0 and t=24 hours.

CHAPTER V

DISCUSSION

CRC continues to be one of the leading causes of worldwide mortality in both genders. Despite the advances in the field of CRC management, 5-FU remains the most commonly used treatment for CRC. However, due to cellular resistance, 5FU may not always be effective. This explains in turn why new agents, that do not demonstrate chemo resistance, are needed to impel promissory advances in the treatment of CRC. Here, we synthesized four novel nucleoside analogs, two purines and two pyrimidines, with anti-cancer effects as indicated by the inhibition of various tumorigenic mechanisms that include proliferation, migration and adhesion of cells and angiogenesis. In both cell types, 5-FU sensitive and 5-FU resistant human CRC cells, our NAs revealed an anti-proliferative effect starting with 1 nM concentration. They were also shown to exert an anti-migratory effect, a decrease in cellular adhesion to collagen and an increase in homotypic cell-to-cell adhesion. In addition, they inhibited angiogenesis that was evident by the decrease in blood vessels in CAM.

Three main things make this study significant: the novelty of the synthesized nucleoside analogs, the potency of our synthesized NAs in inhibiting the hallmarks of malignancy in both types of HCT116 cells: 5-FU sensitive and 5-FU resistant and

finally the anti-angiogenic effect of our NAs not only in in-vitro experiments, but also in in-ovo (CAM) studies.

Resistance to chemotherapeutic drugs remains the main obstacle in CRC treatment. Current studies aim to overcome this resistance through several means that include replacement of monotherapy by combination therapy [128], optimization of drug delivery using nano-particles [129, 130], and development and utilization of novel compounds [131]. Here, we synthesized novel nucleoside analogs that had potential anti-cancer effect not only on the CRC cells, but also on the chemotherapeutic resistant cells.

To our knowledge, HCT116 cells are poorly differentiated [132, 133]. They tend to proliferate and migrate in a very aggressive way. It is documented that MAPK is over-activated in colorectal tumors [134, 135] and in drug-resistant HCT116 cells [136]. MAPK signaling pathway is responsible for many different cellular mechanisms including proliferation. ERK1/2, activated through phosphorylation, is the final mediator in this pathway. Indeed, Huang et al showed that ERK/MAPK inhibition through the specific inhibitor, PD98059, reversed the promotion of cellular proliferation in HCT116 cells [137]. Our results are consistent with other studies. Treatment of both cell types with NAs significantly decreased cell proliferation via the attenuation of the levels of ERK1/2 (phospho- to total). In this context, Adenine and Guanine analogues were found to be the most potent compounds. .

Metastasis is a multistep process that includes enhanced cellular proliferation, migration and adhesion to the ECM, angiogenesis and attenuated homotypic cell-to-cell

adhesion as pre-requisite steps. As the carcinoma becomes advanced, cancer cells start to spread from the original site to a collateral organ [63, 64]. During this course, it is imperative for MMPs proteases, specifically MMP-2 (gelatinase A) and MMP-9 (gelatinase B), to achieve degradation of the extracellular matrix (ECM) [68]. Moreover, based on previous studies, the upregulation of MMP results in reinforced migration [138], whereas the downregulation of MMPs via TIMPs or MMP inhibitors predisposes to weakened migration [138]. Moreover, for the cells to migrate to new sites, they must adhere first to the extracellular matrix (ECM). A novel synthetic molecule, YH-306, is denoted by its inhibitory effect on the migration of HCT116 and HT-29 CRC cell lines [139]. This inhibitory effect is manifested when cell-to-ECM adhesions are minimized or coupled with random dispersion of paxillin. Ultimately, this is shown to prevent cellular attachment and formation of protrusions that are triggered by the activation of the ECM constituents: collagen type 1 or fibronectin [139]. Thus, both cell adhesion and migration are hindered; and subsequently metastasis is disrupted. Here, we showed that our NAs inhibited these hallmarks of metastasis. In agreement with the above studies, our NAs, with non-cytotoxic concentrations, significantly attenuated the adhesion of HCT116, of both types of cells, to collagen, one of the ECM components, in a time dependent manner. Thus, migration was likely inhibited by the attenuation of the MMP-2 and MMP-9 levels.

In addition, we showed that our NAs could induce cell-to-cell adhesions in a time dependent manner. Knowing that the loss of cell-to-cell adhesion is a remarkable hallmark for carcinogenesis, the induction of cellular aggregation is perceived as a potential mechanism that can be employed in cancer treatment. In healthy individuals,

homeostasis is upheld through adherence of cells to each other and to the ECM. This adherence involves physical attachment and exchanged signal transductions maintained through proteins known as adhesion molecules, cadherins and integrins in particular. Integrins are responsible for cell-to-ECM adhesions; whereas cadherins regulate homotypic cell-to-cell adhesions [79-81].In the light of this, the levels of cadherins and integrins should be further investigated in future studies.

It is well documented that drug resistance is associated with the expression of an epithelial–mesenchymal transition (EMT) phenotype [140-142]. During EMT, cancer cells gain migratory advantage via the disruption of cell-to-cell adhesion and cell-to-ECM adhesion [143]. Indeed, 5-FU resistant HCT116 cells were shown to have increased migration and potent invasiveness and morphological changes [140] which are all traits of EMT. In the present study, our NAs were able to overcome 5-FU resistance and also to reverse the EMT phenotype by attenuating 5-FU resistant cells migration and adhesion to ECM and also by promoting 5-FU resistant cell-to-cell homotypic adhesion. This is promising in sense that our NAs could potently reverse 5-FU resistance and induce cytotoxicity.

Angiogenesis is also a hallmark of cancer. It is defined as the development of new blood vessels through which oxygen and nutrients are provided for the tumor mass in order to support tumor growth and metastasis. It is a crucial mechanism for tumor initiation, progression and metastasis. Therefore, antiangiogenic agents should be considered for their anti-metastasis significance. One possible way to inhibit angiogenesis is by attenuating the levels of the pro-angiogenic molecules, VEGF. In fact, accumulating studies showed that advanced CRC stages are associated with

increased levels of VEGF, which is associated with metastasis [144, 145]. In addition, ERK/MAPK signaling is also involved in angiogenesis [146, 147]. When activated, it induces the production of VEGF [147]. Importantly, our results propose that the NAs significantly reduced VEGF production through ERK1/2 inhibition. This suggests that they inhibit CRC growth by blocking angiogenesis.

Nitric oxide is a molecule that plays a key role in cellular communication. Angiogenesis induces the release of NO from endothelial cells through a family of nitric oxide synthase enzymes including an inducible isoform called iNOS, which are in turn mediated by L-arginine [148]. The expression of NO implies its contribution to angiogenesis; thus, its repression may be a potential therapeutic to cancer metastasis. Gao et al showed that blocking the NO pathway by 1400W, a specific iNOS inhibitor, results in inhibition of angiogenesis in CRC in particular, which consequently antagonizes CRC metastasis [148]. In agreement with this, we showed that our NAs significantly decreased the levels of NO production in HCT116 cells. Taken together, we can suggest that our NAs might also inhibit CRC metastasis through the inhibition of the NO pathway.

Finally, we know that in-vitro experiments are not sufficient for cancer treatment and translating our results to in-vivo studies or to clinics may not be accomplished easily or may need higher concentrations. On the other hand, we were able to show that our NAs are effective in in-ovo experiment using the CAM assay. This assay is a propitious technique widely used in studying the anti-angiogenic effect of cancer drugs. It is an alternative method for animal models and provides a natural environment for vessels development [149]. The chick chorioallantoic membrane is a

highly vascularized membrane that starts to develop at day 3 of incubation [150]. This is a very suitable mean for assessing the effects of our NAs on angiogenesis. Thus, we showed that our NAs could significantly inhibit angiogenesis evident by the decrease in the total vessel density in the chorioallantoic membrane as compared to the control. In addition, during metastasis, many secondary vessels start to branch from pre-existing ones [151]. Here, we find that these analogs decreased the total number of junctions, the main branching points in angiogenesis. Taken together, we can suggest that our NAs could potently inhibit angiogenesis which may result in anti-metastatic effect in CRC.

CONCLUSION

This study revealed the general scheme for synthesizing four nucleoside analogs and showed their biological activity in CRC treatment including the 5-FU resistant type. Our findings show that these NAs inhibit the hallmarks of cancer such as proliferation, migration adhesion, homotypic cell-cell aggregation and angiogenesis. Therefore, these NAs could act as a potent chemotherapeutic drug in CRC treatment.

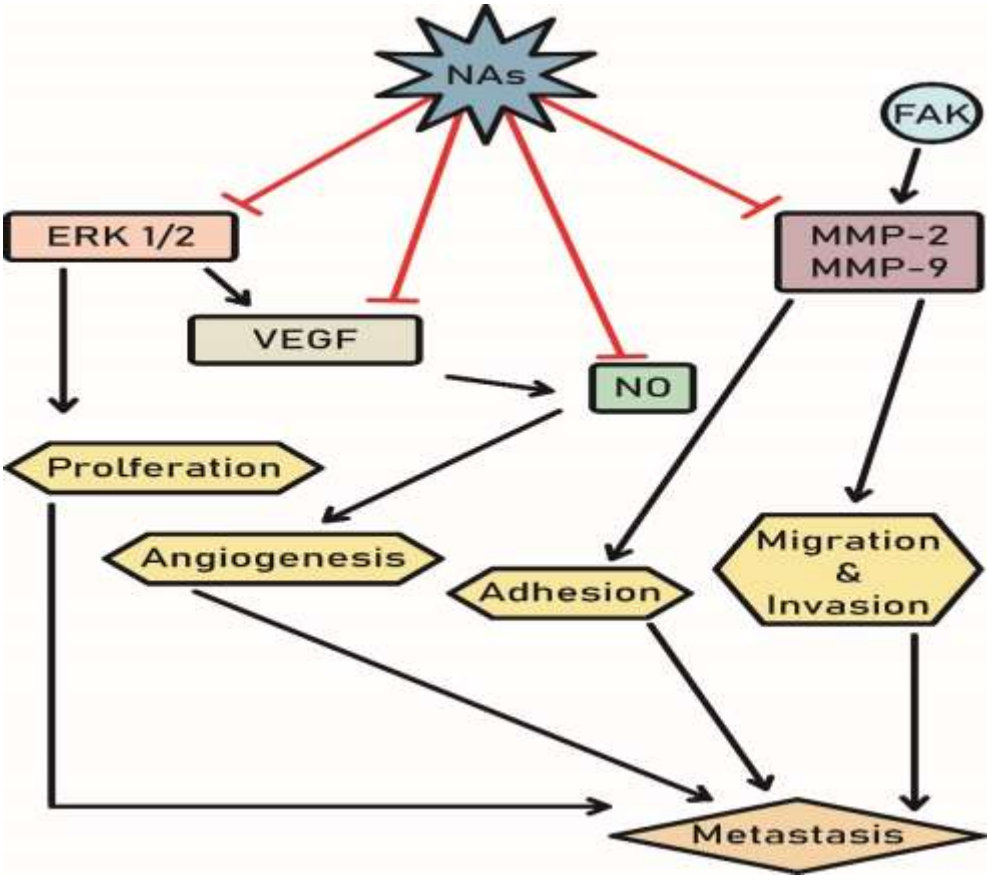


Figure 19: Schematic presentation of the suggested signaling pathway by which NAs inhibit the hallmarks of CRC.

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