Synthesis of Novel Pyrimidine Analog Gemcitabine-Decitabine Dimer

BY

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DEDICATION

I want to dedicate this work to my parents, who made many sacrifices so that I could be where I am today. Thank you for your unwavering love, encouragement, and belief in me.

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I would like to express my deepest gratitude to my mentor, Dr. William Gmeiner, for the opportunity to work in his laboratory over the past two years and for his invaluable guidance, encouragement, and support throughout my research journey. I am also grateful to Dr. Sezgin Kiren for generously allowing me to conduct the chemical synthesis in his laboratory at Winston-Salem State University and for his expertise and assistance in the synthesis process. My sincere thanks also go to my committee members, Dr. Timothy Pardee and Dr. Dawen Zhao, for dedicating their time, insight, and expertise to help shape and strengthen this project.

I am thankful to all members of the Gmeiner Lab—Jessica VonCannon, Rebekah Sikora, Erika Sudderth, Dr. Santu Sarkar, Dr. Upasana Das, Dr. Akanksha Behl, Bara'ah Khaled, Taylor Young, Charles Okechukwu, and Tyler Wilson—for their collaboration, camaraderie, and support. I am especially appreciative of Jessica VonCannon and Erika Sudderth for their assistance with cell culture work for this study, and of Izzy Dover for initiating the decitabine synthesis that formed an important part of this project.

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ABSTRACT

Nucleotide substitutions play a critical role in cancer development and therapeutic response, with antimetabolites like gemcitabine and decitabine commonly targeting DNA synthesis and epigenetic regulation. This study reports the synthesis and preliminary evaluation of a novel gemcitabine-decitabine dimer (Gem-Dec dimer) as a potential chemotherapeutic agent. The dimer was synthesized using a modified guadecitabine synthesis pathway that omits amine protection, improving efficiency and minimizing structural degradation. Its chemical integrity was confirmed via thin-layer chromatography, which demonstrated stability across various culture media. Cytotoxicity assays using colorectal cancer cell lines HCT116 and LS174T revealed that the dimer retains anticancer activity, particularly in HCT116 cells, though with slightly reduced potency compared to monomeric gemcitabine. Combination treatment studies showed synergistic cytotoxic effects in HCT116 cells, with up to 36% greater inhibition of cell viability compared to either drug alone, suggesting mechanistic complementarity. LS174T cells, however, demonstrated minimal responsiveness. These findings confirm that gemcitabine and decitabine can be chemically conjugated to form a stable, bioactive compound and suggest that the Gem-Dec dimer merits further investigation in cancer types where its constituent drugs have proven efficacy. This work establishes a foundation for developing novel, dual-action nucleoside analog-based therapies that target both DNA replication and epigenetic dysregulation in cancer.

ABBREVIATIONS

AcN – Acetonitrile

Ar - Argon

DCM - Dichloromethane

DMAP – 4-Dimethylaminopyridine

DMF – N, N-Dimethylformamide

Et_3N – Triethylamine

$Et_3N.3HF$ – Triethylamine trihydrofluoride

EtOAc – Ethyl Acetate

FBS – Fetal Bovine Serum

i-PrOH – Isopropyl Alcohol

MDS – Myelodysplastic syndromes

MeOH - Methanol

Na_2SO_4 – Sodium Perchlorate monohydrate

$NaHCO_3$ – Sodium Bicarbonate

NPEOC - *p*-nitrophenylethoxycarbonyl

RT – Room Temperature

THF – Tetrahydrofuran

TLC – Thin Layer Chromatography

INTRODUCTION

Cancer is the leading cause of death worldwide, accounting for nearly one in six deaths (World Health Organization, 2025). The substantial mortality associated with cancer has prompted researchers to explore innovative treatment modalities aimed at optimizing patient survival outcomes.

Nucleotide substitution is a vital process that drives genetic diversity and plays a major role in how cancer evolves within the body. In cancer, these changes in DNA—caused by internal processes, environmental factors, or errors in DNA repair—are key to both the start and spread of tumors (Stratton et al., 2009). Unlike inherited mutations, these somatic changes build up in body cells over a person's lifetime, leading to the growth of cell groups that gain an edge in multiplying faster.

In cancer, somatic nucleotide substitutions—changes in the DNA of body cells—can be split into two types. Driver mutations give cells a growth or survival edge, basically fueling the tumor's bad behavior. Passenger mutations, on the other hand, are like silent hitchhikers—they do not affect the cell's behavior but still tell us a lot about what's going on mutation-wise in the tumor (Vogelstein et al., 2013). Thanks to next-generation sequencing (NGS) and projects like The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC), a more comprehensive understanding of these patterns and rates has been possible.

These substitutions are classified into two main categories: transitions and transversions, each with distinct biochemical properties, mutation frequencies, and biological consequences (Freese, 1959; Li et al., 1984).

Transition Substitutions

Transition substitutions are a common type of DNA change where one purine (A or G) swaps with another purine, or one pyrimidine (C or T) swaps with another pyrimidine. These swaps are less disruptive because they involve bases with similar chemical properties, so they do not drastically alter the DNA's double helix structure. Because of this similarity, the body's DNA repair systems often miss these changes, making transitions more common in the mutations that build up in body cells over time (Kunkel & Erie, 2005).

Transversion Substitutions

Transversion substitutions involve the substitution of a purine for a pyrimidine or vice versa (e.g., A to T, A to C, G to T, G to C). Due to transversions resulting in a significant change in the size and structure of the base, these substitutions often lead to a more substantial loss of DNA stability and function (Guo et al., 2017). Transversions are typically observed at lower frequencies than transitions; however, they tend to have more severe functional consequences, such as amino acid changes with impaired function, including protein misfolding and loss of function (Wakeley, 1996; Loewe & Hill, 2010). Environmental mutagens can induce specific types of transversions. For example, polycyclic hydrocarbons found in cigarette smoke are known to cause cytosine to adenine transversions, which is a signature consistent with smoking-related lung cancers (Alexandrov et al., 2016).

Mechanisms of Nucleotide Substitution

Mechanisms of nucleotide substitution occur through spontaneous errors during DNA replication, chemical modifications, and environmental factors. This occurs when DNA polymerase inserts the wrong nucleotide into the new DNA strand. Normally, proofreading performed by mismatch repair (MMR) systems corrects these errors, but some errors may be missed, thus becoming a permanent mutation in the DNA.

Deficiencies in MMR genes play a critical role in causing changes to DNA nucleotides. For example, Lynch syndrome, which involves mutations in MMR genes, significantly increases single-nucleotide changes, especially in colorectal and endometrial cancers (Peltomäki, 2016). Likewise, errors in DNA polymerase proofreading, like those from POLE gene mutations, lead to tumors with a high number of mutations (Rayner et al., 2016). These discoveries highlight how crucial DNA repair pathways are for keeping our genome stable and preventing mutations that can cause cancer.

Patterns of Nucleotide Substitution in Cancer

Understanding the distribution and type of nucleotide substitutions across varying cancer types provides insight into the underlying mutational process, such as the endogenous mechanisms like oxidative stress and DNA replication errors, as well as exogenous exposures such as ultraviolet radiation or chemical mutagens (Helleday et al., 2014). For example, melanoma exhibits a high frequency of cytosine to thymine transitions due to the

DNA damage that ultraviolet (UV) light can induce (Hodis et al., 2012). This pattern is not random but shaped by the interplay of mutagenic agents and cellular repair mechanisms.

Whole-genome sequencing studies have revealed that nucleotide substitutions often cluster in specific genomic regions, such as regulatory elements or coding sequences of driver genes. Martincorena et al. (2017) demonstrated that positive selection for substitutions in genes like TP53 and KRAS drives tumor evolution. Conversely, passenger mutations—substitutions with no functional impact—accumulate neutrally, contributing to intratumor heterogeneity (Nik-Zainal et al., 2012). Understanding these patterns is critical for distinguishing driver from passenger mutations in cancer genomics.

Functional Consequences in Cancer

Nucleotide substitutions can have profound effects on cellular function. Missense mutations, which alter a single amino acid, can activate oncogenes (e.g., BRAF V600E in melanoma) or inactivate tumor suppressors (e.g., TP53 mutations in multiple cancers) (Olivier et al., 2010). Nonsense mutations, leading to truncated proteins, are particularly deleterious in tumor suppressor genes, as seen in APC mutations in colorectal cancer (Fearnhead et al., 2001). Additionally, substitutions in non-coding regions, such as promoter or enhancer regions, can dysregulate gene expression.

The functional impact of substitutions extends to therapeutic resistance. For instance, EGFR T790M mutations in non-small cell lung cancer confer resistance to first-generation tyrosine kinase inhibitors (Pao et al., 2005). Similarly, nucleotide substitutions in DNA

repair genes, such as BRCA1/2, can restore protein function in PARP inhibitor-resistant ovarian cancers (Patch et al., 2015). These findings highlight the dynamic role of nucleotide substitutions in tumor adaptation to therapeutic pressures.

Clinical Relevance

The study of nucleotide substitutions has transformative implications for precision oncology. Mutational signatures can guide diagnostic and therapeutic strategies. For example, tumors with high microsatellite instability (MSI) due to MMR deficiencies are responsive to immune checkpoint inhibitors, as demonstrated by Le et al. (2017).

Antimetabolite chemotherapies such as gemcitabine and decitabine are especially relevant in the context of nucleotide substitution because they interact directly with DNA synthesis and processes linked to nucleotide integrity and mutation.

Gemcitabine (2′,2′-difluoro 2′-deoxycytidine) is a pyrimidine nucleoside analog structurally related to deoxycytidine. Upon cellular uptake, it is phosphorylated by deoxycytidine kinase (DCK) into its active diphosphate (dFdCDP) and triphosphate (dFdCTP) forms. dFdCTP is incorporated into DNA during replication, resulting in masked chain termination—allowing for one additional nucleotide to be added before DNA synthesis halts—thus interfering with replication fork progression and triggering apoptosis (Mini et al., 2006). dFdCDP, in parallel, inhibits ribonucleotide reductase (RNR), decreasing the pool of deoxynucleotides available for DNA synthesis. This dual action amplifies DNA stress and selectively targets rapidly proliferating tumor cells.

Gemcitabine is widely used in the treatment of pancreatic, lung, bladder, and breast cancers, and its efficacy can be heavily influenced by nucleotide substitutions in genes encoding enzymes critical for its metabolism and transport.

Decitabine (5-aza-2′-deoxycytidine) is a cytidine analog primarily used in hematologic malignancies, particularly myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). It functions as a DNA hypomethylating agent. After incorporation into DNA, decitabine covalently traps DNA methyltransferase 1 (DNMT1) during replication, leading to DNMT1 degradation and global DNA hypomethylation (Kantarjian et al., 2006). This reactivates previously silenced tumor suppressor genes and disrupts the epigenetic maintenance of malignant phenotypes. In contrast to traditional cytotoxic agents, decitabine acts more subtly by altering gene expression, often requiring multiple treatment cycles before clinical responses become evident.

Importantly, decitabine's efficacy may be affected by both epigenetic landscapes and nucleotide substitutions in genes involved in DNA damage recognition and repair. For example, mutations in TP53, frequently found in high-risk MDS and AML, have been associated with varied responses to decitabine therapy. Some studies suggest that TP53-mutant leukemias may be particularly vulnerable to hypomethylating agents due to their epigenetic instability (Welch et al., 2016). Additionally, resistance to decitabine may arise through increased expression of cytidine deaminase (CDA), which catabolizes the drug, or by mutations in transporters and kinases involved in its activation.

The objective of this work is to synthesize a novel gemcitabine-decitabine dimer (Gem-Dec dimer) and evaluate its potential as a chemotherapeutic agent. Both gemcitabine and

decitabine are well-established anticancer drugs, making them strong candidates for combination therapies. Although limited, previous studies exploring the combined use of these agents have demonstrated enhanced cytotoxicity and synergistic effects, particularly in lymphoma models (Lin et al., 2023). However, these investigations utilized co-administration of single agents rather than a covalently linked compound. In contrast, the present study aims to create and test a chemically linked Gem-Dec dimer and compare its cytotoxic efficacy to that of the individual drugs.

To achieve this, we adapted the synthetic strategy reported by Rohacova et al. (2019) for the preparation of guadecitabine. This methodology was selected for its use of mild protecting groups and its efficient conversion from starting materials to the final dimer. Notably, one key advantage of this approach is the omission of amine group protection on both gemcitabine and decitabine. This simplification permits the use of smaller, more labile protecting groups that are easier to remove during synthesis, thereby reducing the risk of damaging sensitive aromatic regions of the molecule. As a result, the synthetic process was streamlined, enhancing the overall yield and structural integrity of the final dimer.

METHODS

During the synthesis of the Gemcitabine–Decitabine dimer, we investigated multiple strategies, including the incorporation of a *p*-nitrophenylethoxycarbonyl (NPEOC)-protected phosphoramidite of decitabine at the N4 position. This strategy, previously described for oligonucleotide synthesis by Guimil García et al., intended to protect the amine group during the coupling process. Although the NPEOC-protected phosphoramidite of decitabine was successfully synthesized, all attempts to couple it with gemcitabine were unsuccessful. Furthermore, cleavage of the NPEOC protecting group resulted in degradation of the azacytosine ring, likely due to the acidic conditions required for deprotection—a well-recognized instability of decitabine. This degradation rendered the intermediate unsuitable for further synthetic steps.

Given the limitations we encountered with the NPEOC-protection strategy, we pursued the alternative synthetic route outlined in Figure 1. This approach was particularly notable because it did not require protection of the amine group, yet still allowed for the selective protection of the 3′- and 5′-hydroxyl groups on gemcitabine and decitabine.

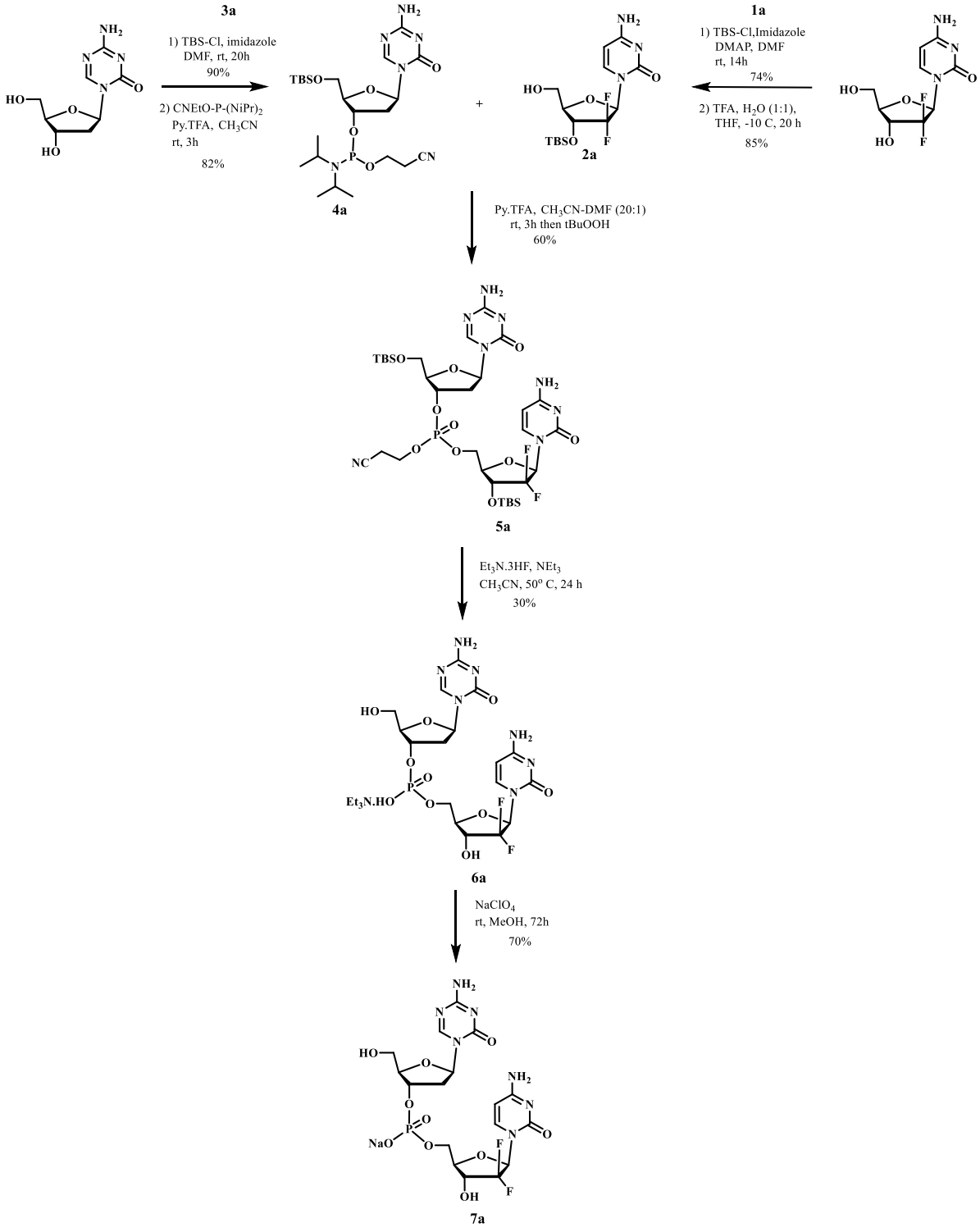

Figure 1: *Reaction scheme for Gem-Dec Dimer*

Example 1: Preparation of 3',5'-bis-O-(tert-butyldimethylsilyl)-2',2'-difluoro-2'-deoxycytidine (1a)

Gemcitabine (2.5 g, 9.49 mmol) was suspended in DMF (50 mL) with imidazole (3.88 g, 56.99 mmol), DMAP (116 mg, 949.8 µmol) and tert-Butyldimethylsilyl chloride (TBMS, 8.59 g, 56.99 mmol). The suspension was stirred overnight at RT. The suspension was then partitioned between ethyl acetate (EtOAc) and a saturated solution of $NaHCO_3$ and then brine. The organic layer was collected and dried over Na_2SO_4 and the solvents were evaporated. The compound was purified on a silica gel column using DCM:MeOH 100:1, and the gradient was increased until the pure product was eluted. After purification, 3.45 g (74%) was obtained. The obtained product was analyzed by NMR and the spectrum is shown in **Figure 2**.

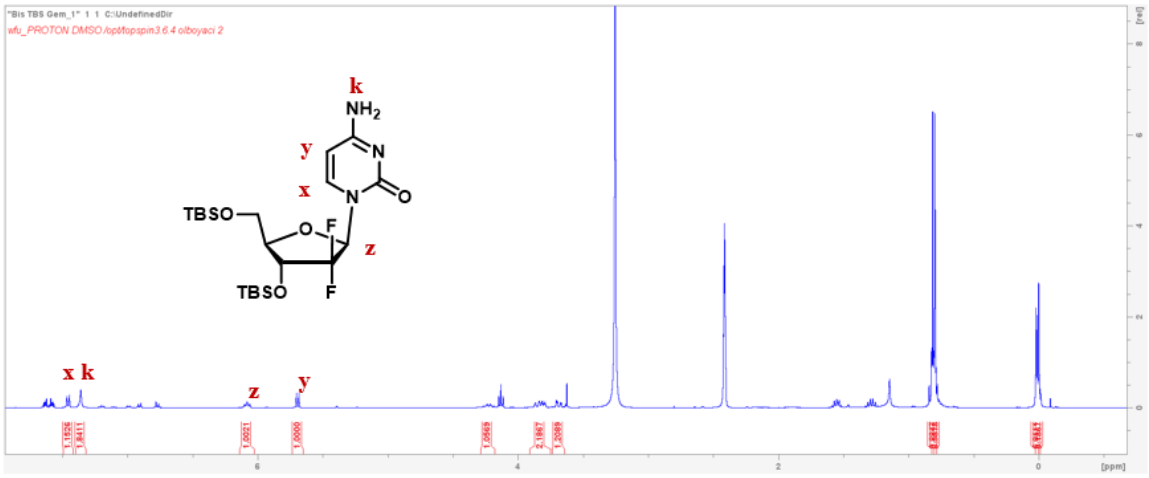

***Figure 2**: H NMR spectra of 3',5'-bis-O-(tert-butyldimethylsilyl)-2',2'-difluoro-2'-deoxycytidine (1a)*

Example 2: Preparation of 3'-O-(tert-butyldimethylsilyl)-2',2'-difluoro-2'-deoxycytidine (2a)

1a (4.66 g, 9.48 mmol) compound was suspended in THF (50 mL) and a 1:1 (v/v) solution of trifluoroacetic acid (TFA, 10 mL, 9.48 mmol) and water (10 mL). The solution was placed in the freezer at -10°C overnight. The cold solution was neutralized with a saturated solution of $NaCO_3$. The aqueous layer was extracted and washed three times with EtOAc. The organic extracts were then dried and concentrated under reduced pressure. The compound was purified by silica gel column using DCM:MeOH 100:1, and the gradient was increased until the pure product was eluted. After purification, 3.04 g (85%) was obtained. The obtained product was analyzed by NMR and the spectrum is shown in **Figure 3**.

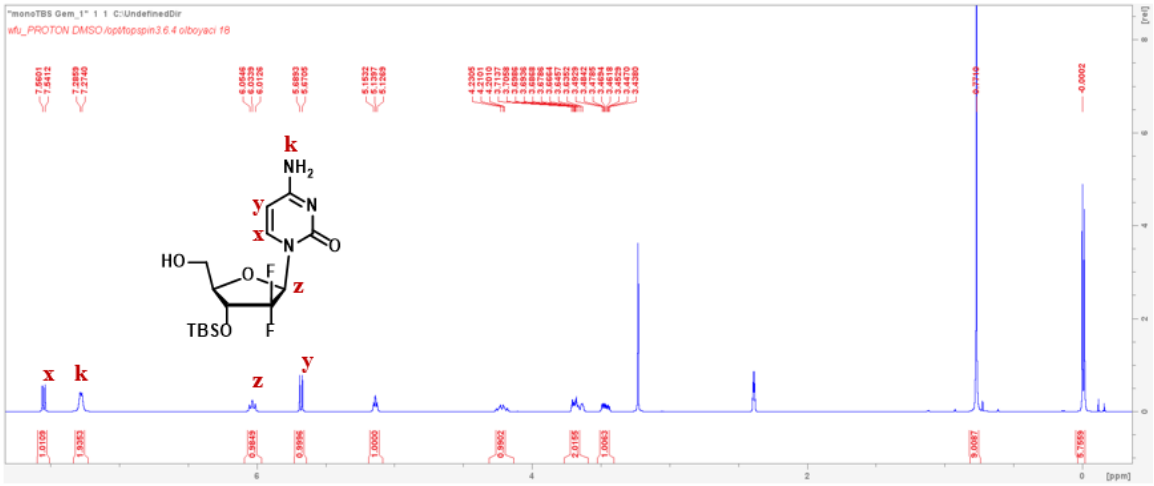

***Figure 3**:H NMR spectra of 3'-bis-O-(tert-butyldimethylsilyl)-2',2'-difluoro-2'-deoxycytidine (2a)*

Example 3: Preparation of 5'-O-tert-butyldimethylsilyl-2'-deoxy-5-azacytidine (3a)

Decitabine (2.0 g, 8.764 mmol) and imidazole (1.492 g, 21.91 mmol) were dried overnight at 40°C. They were then suspended in dry DMF (30 mL) under an Argon atmosphere. The mixture was heated to 50°C until the suspension was dissolved, then the solution was cooled to 0°C. tert-Butyldimethylsilyl chloride (TBMS, 1.453 g, 9.640 mmol) was added portion-wise during 30 minutes, so that the temperature of the reaction mixture did not exceed 5°C. Then the reaction mixture was stirred at 0°C for 2 hours. Water (150 mL) was added. The formed suspension was stirred for 20 minutes and filtered. Solids were washed with water (2 x 50 mL) and dried under vacuum (1 mBar, 24 hr).

The obtained product was dissolved in i-PrOH (30 mL) at 65°C then n-hexane (20 mL) was added during 30 minutes. The formed suspension was stirred for 2 hours at 0°C. The product was filtered, washed with n-hexane (50 mL) and dried under vacuum (1mBar, 16 hr).

The obtained product was dissolved in boiling i-PrOH (20 mL) then n-hexane (30 mL) was added during 30 minutes. The formed suspension was cooled to 20°C and stirred for 1 hour. Product was filtered, washed by n-hexane (10 mL) and dried under vacuum (1mBar, 16 hr). Yield 2.7 g (90%). The obtained product was analyzed by NMR and the spectrum is shown in **Figure 4**.

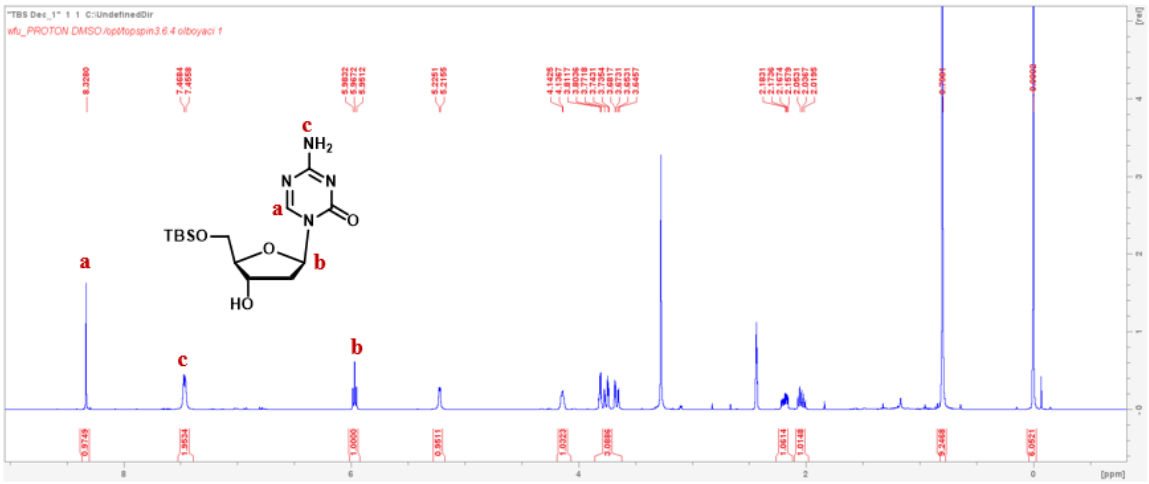

***Figure 4**:H NMR spectra of 5'-O-tert-butyldimethylsilyl-2'-deoxy-5-azacytidine (3a)*

Example 4: Preparation of 5'-O-tert-butyldimethylsilyl-2'-deoxy-5-azacytidine-3'-O-[(2-cyanoethyl)-N, N-diisopropylphosphoramidite] (4a)

3a (1 g, 2.92 mmol) was dried under vacuum (40°C, 1 mBar, 12 hr), then suspended in dry acetonitrile (25 mL). Pyridinium trifluoroacetate (Py. TFA, 338 mg, 1.75 mmol) was added in one portion, followed by the addition of cyanoethyltetraisopropyl phosphorodiamidite (1.11 mL, 3.50 mmol) in one portion. The mixture was stirred at RT for 3 hours, filtered, evaporated, and suspended in diisopropylether/n-heptane 9:1 (10 mL). The first crop of product was filtered off. Mother liquor was evaporated and treated again with DIPE/n-heptane 9:1 for a total of 3 treatments. Total yield 1.3 g (82%). The crude product was used directly in the next step.

Example 5: Preparation of 5'-O-tert-butyldimethylsilyl-P(O)-(2-cyanoethyl)-Pdeoxo-2'-deoxy-5-azacytidylyl-(3'→5')-3'-O-tert-butyldimethylsilyl-2',2'-difluoro-2'-deoxycytidine) and 5'-O-tert-butyldimethylsilyl-P-(2-cyanoethyl)-2'-deoxy-5-azacytidylyl-(3'→5')-3'-O-tert-butyldimethylsilyl-2',2'-difluoro-2'-deoxycytidine (5a)

2a (0.755 g, 2.00 mmol) and 4a (1.3 g, 2.40 mmol), and Py. TFA (641 mg, 3.32 mmol) were dried under vacuum (40°C, 1 mBar, 16 hr). Mixture was suspended in dry mixture AcN/DMF 20:1 v/v (13.6 mL), then N-methylimidazole (136 µL, 1.70 mmol) was added to the mixture. Suspension was stirred for 3 hours for completion. The formed product was then directly oxidized by the addition of tert-butylhydrogenperoxide (tBuOOH, 5.5 M in decane, 407 µL, 2.24 mmol). The mixture was stirred for 1 hour and then evaporated to yield 1g (60%) of 5a. The obtained product was analyzed by NMR and the spectrum is shown in **Figure 5**.

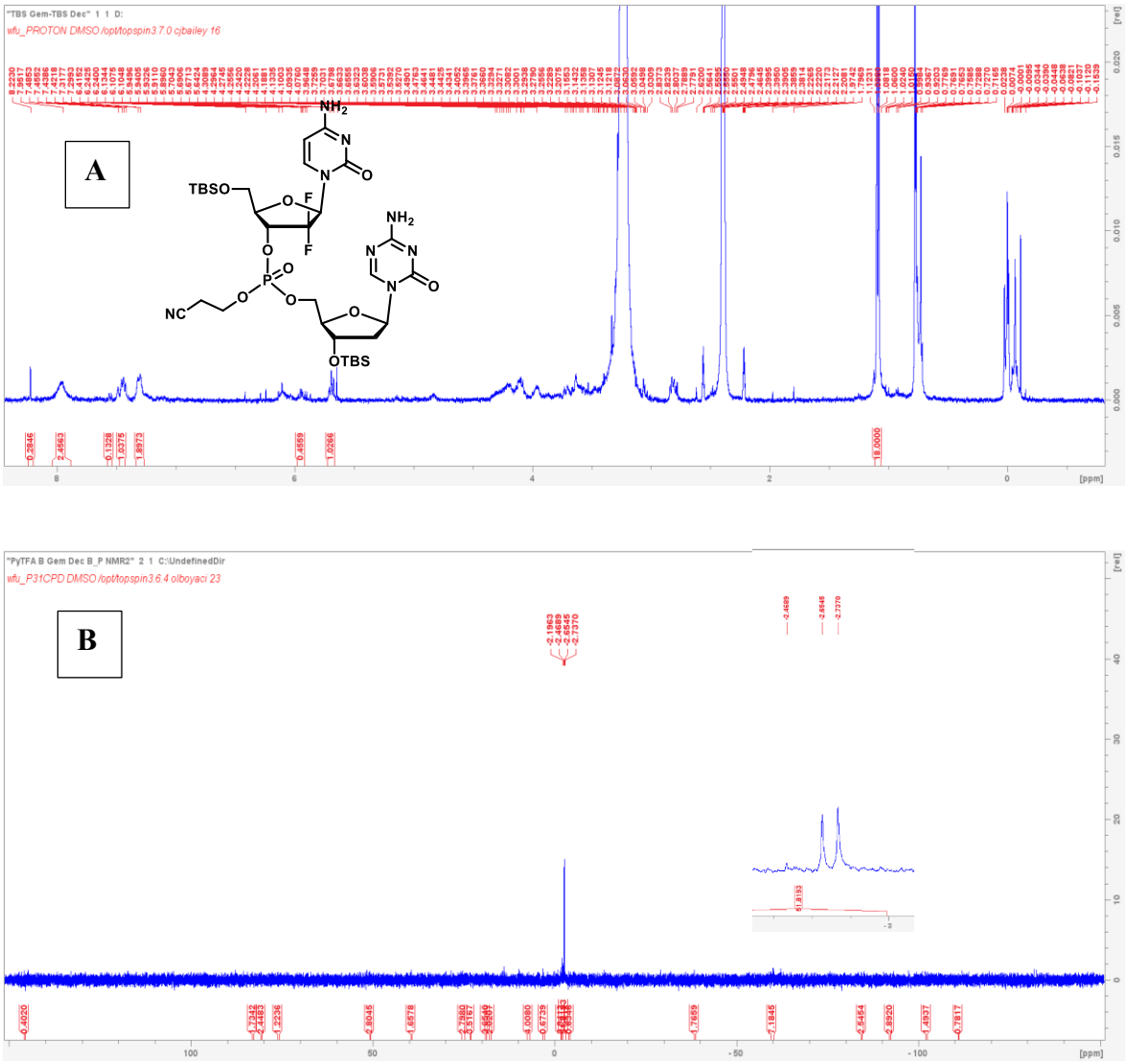

Figure 5:(A) *H NMR spectra of 5'-O-tert-butyldimethylsilyl-P-(2-cyanoethyl)-2'-deoxy-5-azacytidylyl-(3'→5')-3'-O-tert-butyldimethylsilyl-2',2'-difluoro-2'-deoxycytidine (5a).* ***(B)*** *P NMR spectra of 5'-O-tert-butyldimethylsilyl-P-(2-cyanoethyl)-2'-deoxy-5-azacytidine (5a)*

Example 6: Preparation of triethylammonium salt (6a)

The crude 5a (1.67 g, 2.00 mmol) was suspended in anhydrous acetonitrile (25 mL) then Et_3N (2.04 mL, 14.6 mmol) was added. Commercially available salt $Et_3N.3HF$ (2.0 mL, 12 mmol) was added, and the mixture was heated to 50°C under an Argon atmosphere for 24 hours. The suspension was cooled to RT and filtered off. Solids were washed with acetonitrile (5 mL), toluene (5 mL), DIPE (5 mL), n-heptane (5 mL), and dried under vacuum to yield 380 mg (30%). The obtained product was analyzed by NMR and the spectrum is shown in **Figure 6**.

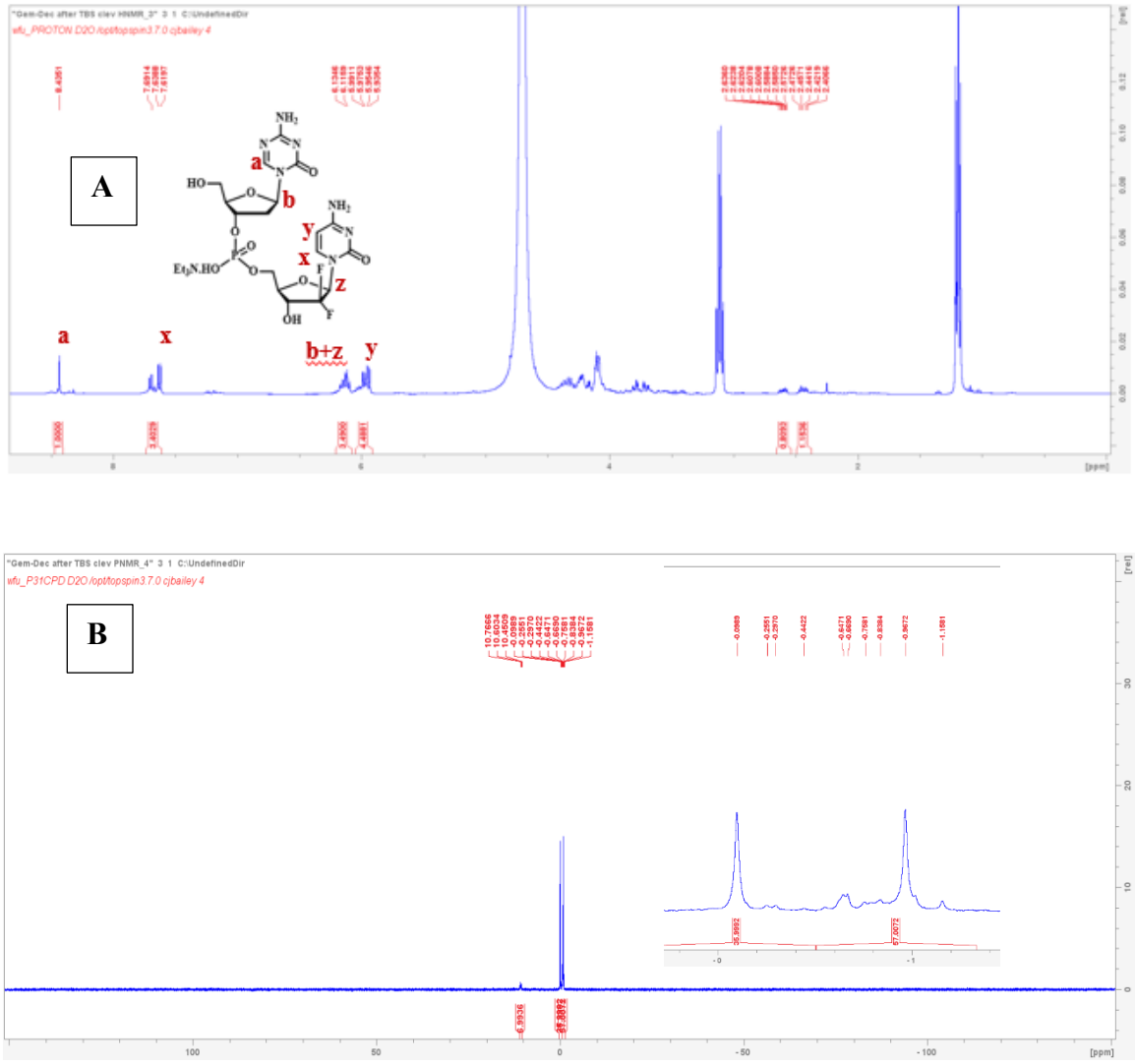

Figure 6:(A) *H NMR spectra of triethylammonium salt (6a).* ***(B)*** *P NMR spectra of triethylammonium salt (6a).*

Example 7: Preparation of sodium salt (7a)

In a round-bottom flask with a magnetic stirrer, 6a (120 mg, 183 μmol) was dissolved in 90% aq. MeOH (10 mL) at 47°C, then the solution was clarified by filtration. A solution of $NaClO_4$ hydrate (515 mg, 3.67 mmol) in MeOH was slowly added during 10 minutes into the stirred solution of 6a. The formed suspension was stirred and cooled to RT for 24 hours. Acetone (10 mL) was slowly added to the stirred mixture for 1 hour. The suspension was stirred and cooled to 5°C for 1 hour, then filtered through a sintered glass filter. The precipitate was thoroughly washed by acetone (2 x 25 mL) and dried under vacuum overnight. Yield 70 mg (67%). The solid was analyzed by NMR and is shown in **Figure 7.**

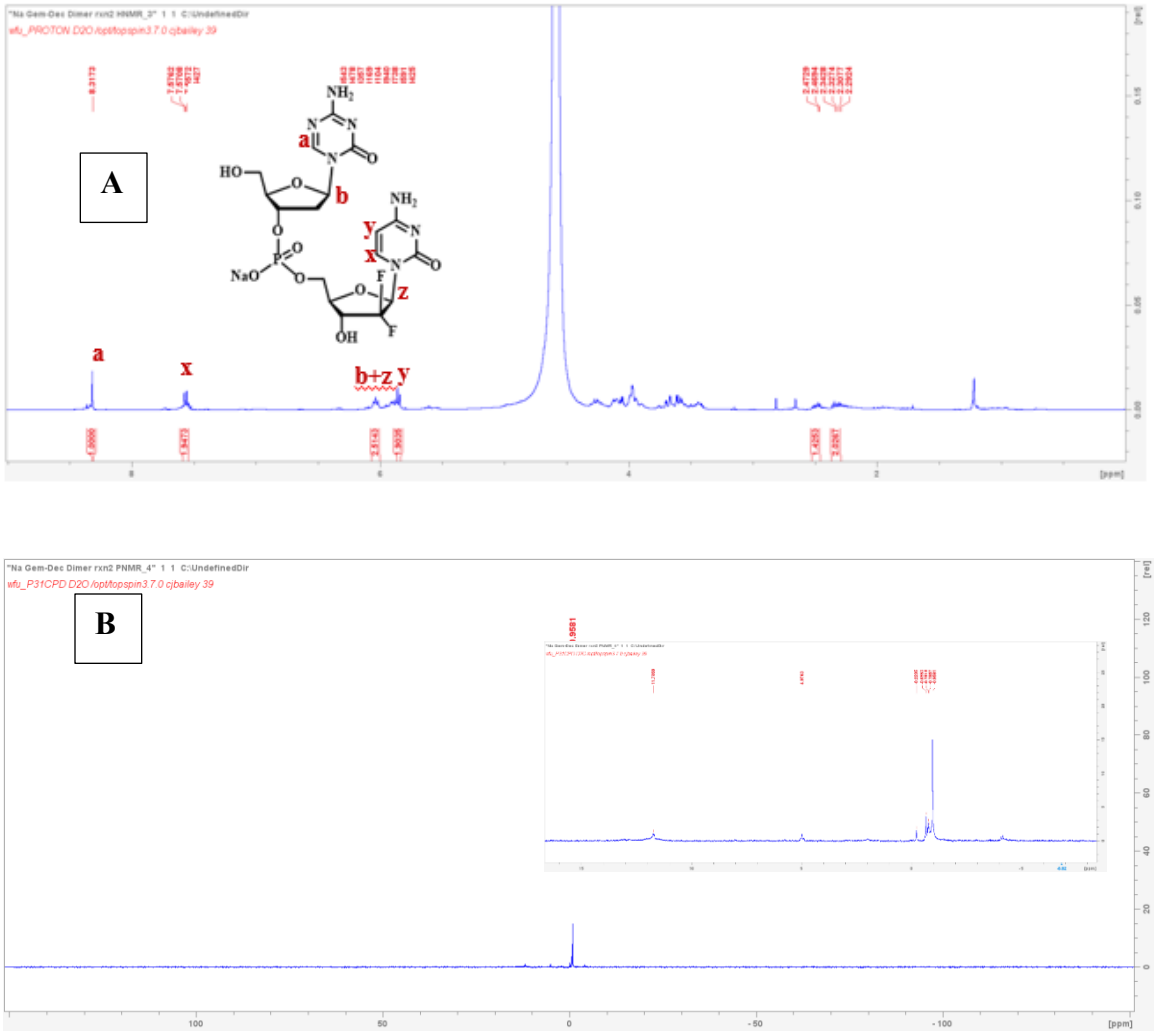

Figure 7: (A) *H NMR spectra of sodium salt (7a).* ***(B)*** *P NMR spectra of sodium salt (7a).*

Cell Lines and Culture Conditions

HCT116 and LS174T cells were used for this study. HCT116 cells were maintained in McCoy's 5A medium (ThermoFischer Scientific, Waltham, MA) with 10% FBS and 1% L-Glutamine and grown with 5% CO_2 in an incubator at 37°C. LS174T cells were maintained in MEM medium (ThermoFischer Scientific, Waltham, MA) with 10% FBS and 1% L-Glutamine and grown with 5% CO_2 in an incubator at 37°C.

Cytotoxicity Assay

Cell viability was measured using MTS assays. HCT116 and LS174T cells were counted and plated in 96-well plates with 100 µL of cell culture media (3,500 cells/well and 5,000 cells/well, respectively). After 24 hours, the cells were treated with Gemcitabine 0 µM to 0.6 µM by serial dilution of thirds, Decitabine 0 µM to 15 µM by serial dilution of thirds, or Gem-Dec dimer 0 µM to 0.6 µM by serial dilution of thirds. All concentrations of the drug were calculated using the molecular weight and volume. After treatment, the cells were incubated for 96 hours before assays were performed.

The Cell Titer 96 Aqueous One solution cell proliferation assay (MTS assay; Promega Corp., Madison, WI) was used to quantify relative cell viability. The MTS assay was performed according to the manufacturer's instructions.

Gemcitabine and Decitabine Synergy

The synergy of Gemcitabine and Decitabine was measured by using MTS assays and Combenefit software. HCT116 and LS174T cells were counted and plated in 96-well plates with 100 µL (3,500 cells/well and 5,000 cells/well, respectively). After 24 hours, rows 2 through 6 were treated with Decitabine 2 µM, 4 µM, 6 µM, 8 µM, and 10 µM for 24 hours. The media was then removed and replaced with 100 µL of fresh culture medium. All six rows were treated with Gemcitabine 0µM to 0.6 µM and incubated for 72 hours.

The Cell Titer 96 Aqueous One solution cell proliferation assay (MTS assay; Promega Corp., Madison, WI) was used to quantify relative cell viability. The MTS assay was performed according to the manufacturer's instructions. Three replicates were collected, and Combenefit software was used to determine the synergy.

Molecular Stability of Gem-Dec Dimer in Media

The Gem-Dec dimer was placed in FBS-free McCoy's and MEM media, as well as 10% FBS McCoy's and MEM media. A TLC was used to determine if the dimer was undergoing cleavage when introduced to the media using a DCM:MeOH 2:1 system on normal phase silica plates. The TLC plate was stained with anisaldehyde TLC stain and burned to visualize the organic compounds.

RESULTS

Cytotoxic Effects of Gem-Dec Dimer

First, the inhibition of cell viability of gemcitabine and decitabine on HCT116 and LS174T cells was found separately. Gemcitabine and decitabine are not used to treat colorectal cancer; however, due to their availability in the lab, they were used to determine the cytotoxic effects. Gemcitabine shows a cytotoxic effect in HCT116 cells; however, decitabine does not show a significant decrease in cell viability. Neither drug shows exceptional cytotoxic effects in the LS174T cells (**Figure 8**).

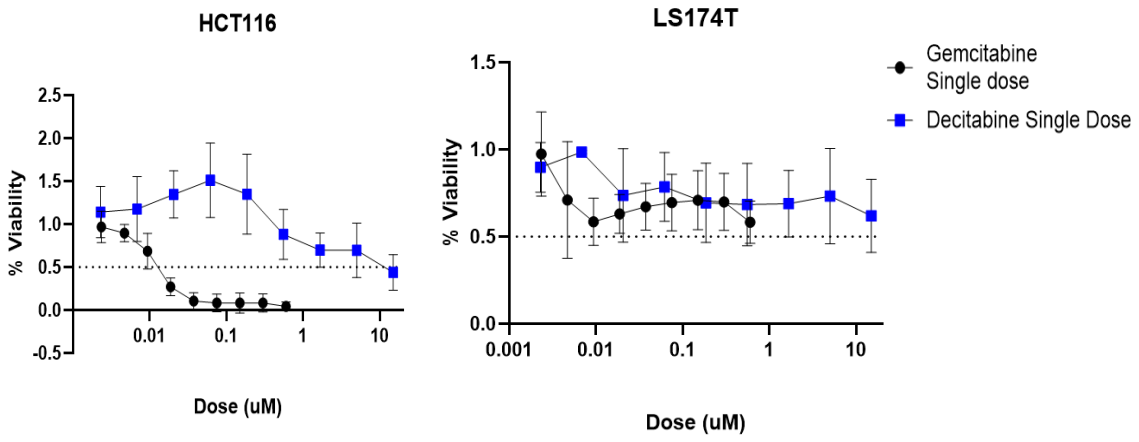

Figure 8*:Cytotoxic Effects of Gemcitabine and Decitabine in HCT116 and LS174T CRC cells*

Due to the low cytotoxic effects of decitabine, the doses for the Gem-Dec dimer were based on the gemcitabine doses. In HCT116, the dimer has similar effects to monomeric gemcitabine but is less effective (**Figure 9**). The IC50 of gemcitabine in HCT116 was found to be approximately 0.027 µM, whereas the IC50 of the Gem-Dec dimer was found to be 0.049 µM. Showing that it takes nearly twice as much of the dimer to see the same

results as monomeric gemcitabine. In the LS174T cells, the dimer proved to have an increased cytotoxic effect; however, it was not to the desired level.

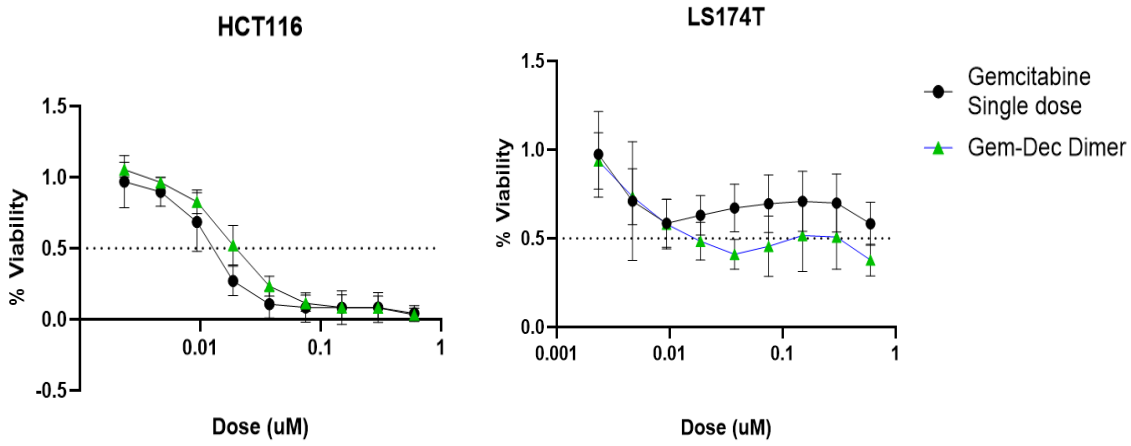

***Figure 9**: Cytotoxic effects of Gem-Dec dimer compared to gemcitabine*

Gemcitabine and Decitabine show synergy in CRC cell lines

Using the Highest Single Agent (HSA) model in the HCT116 cells, the study evaluated the combined cytotoxic effect of gemcitabine and decitabine across a dose matrix. The synergy scores were computed using the Combenefit software. The combination showed the most synergistic effects with 0.6 µM gemcitabine and 2 µM decitabine, with it inhibiting cell viability by 36% greater than either agent alone, as seen in **Figure 10**. Other synergistic effects, ranging from 18% - 33% greater inhibition, are found with varying concentrations of decitabine.

In the LS174T cells, we did not observe desirable synergistic effects (**Figure 11**). We believe that this is due to the low cytotoxic effects of the chemotherapeutic agents by themselves, that even once combined, there is not a strong enough effect within the cells.

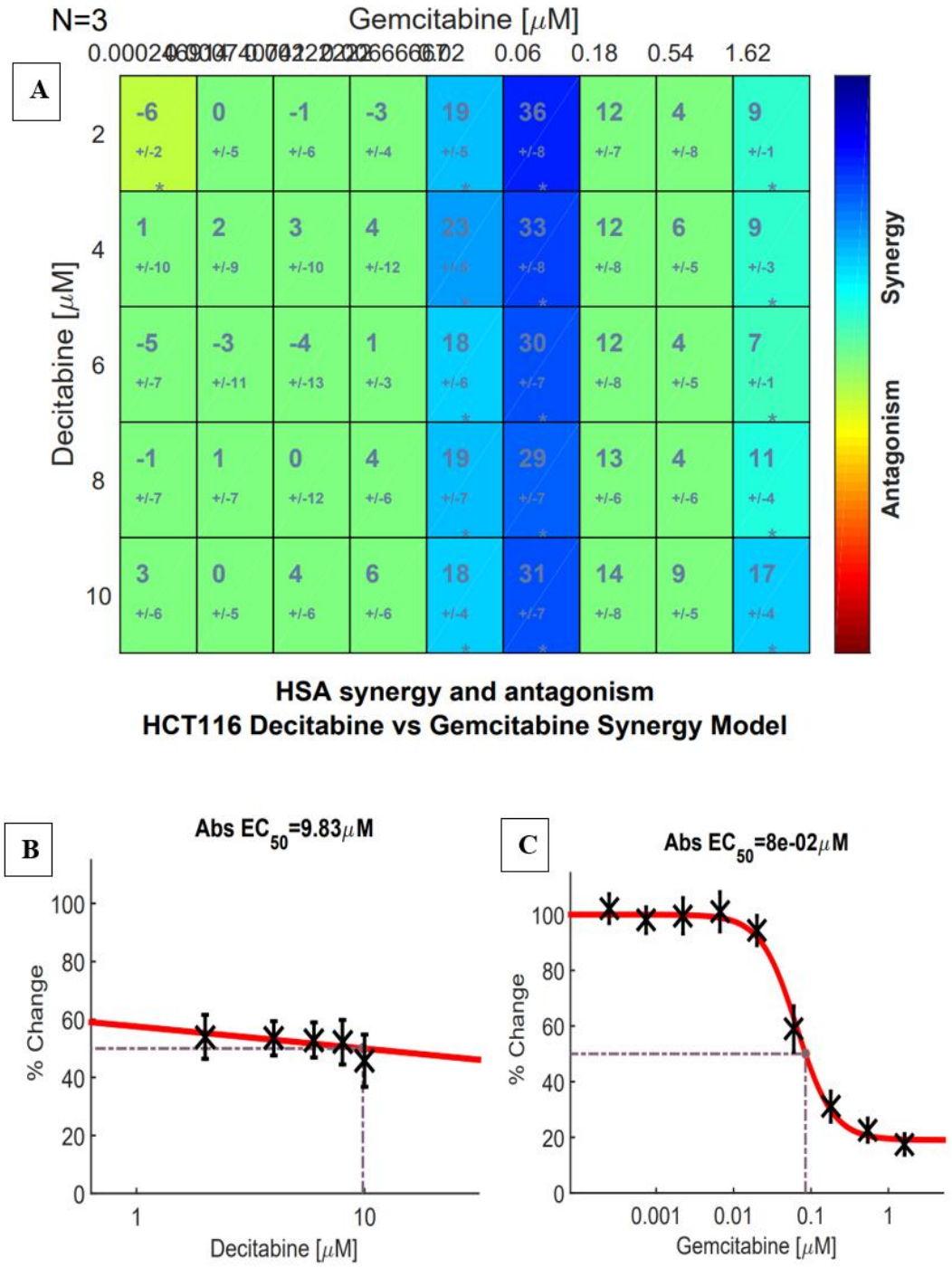

Figure 10: ***(A)*** *HSA synergy matrix for gemcitabine and decitabine in HCT116* ***(B)*** *Single agent dose response for decitabine* ***(C)*** *Single agent dose response for gemcitabine*

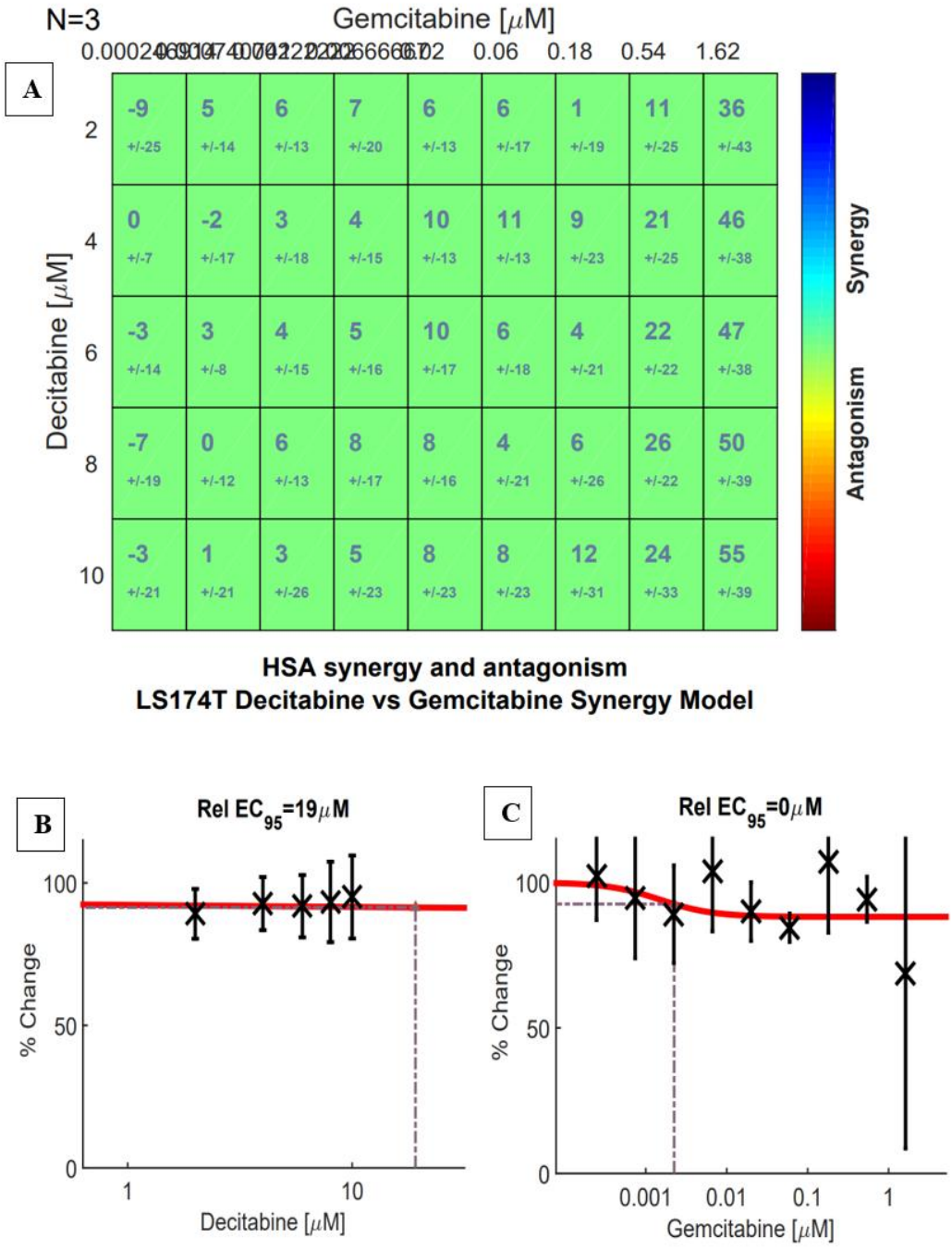

Figure 11: ***(A)*** *HSA synergy matrix for gemcitabine and decitabine in LS174T* ***(B)*** *Single agent dose response for decitabine* ***(C)*** *Single agent dose response for gemcitabine*

Gem-Dec Dimer shows no cleavage in cell culture media

A thin-layer chromatography (TLC) analysis was conducted to assess the chemical stability of the Gem-Dec dimer in cell culture media. The dimer was placed in FBS-free McCoy's and MEM, as well as McCoy's and MEM containing FBS. The TLC was run using normal phase silica plates in a 2:1 DCM: MeOH system. The TLC plate, seen in **Figure 12,** shows the spots for the dimer in water and media can be seen with no additional spots. In addition, the retention factor (Rf) values match across the different samples, with the Rf value being approximately 0.33. Having equal Rf values indicates that the Gem-Dec dimer is not cleaving when introduced to culture media and entering the cells intact.

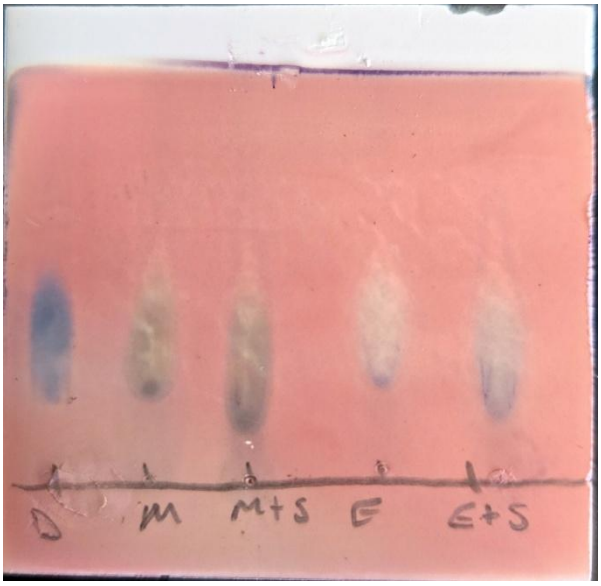

Figure 12*: TLC of Gem-Dec dimer in media:* ***D*** *– Gem-Dec dimer in water,* ***M*** *– FBS free McCoy's,* ***M+S*** *– McCoy's media with FBS,* ***E*** *- FBS free MEM,* ***E+S*** *- MEM with FBS*

The absence of secondary spots suggests that the dimer remains intact when introduced to cell culture medium. This is significant for determining the dimer's chemical stability to be used in drug formulations.

DISCUSSION

The cytotoxic effects of gemcitabine, decitabine, and their combined formulation as a Gem-Dec dimer were evaluated in two colorectal cancer cell lines, HCT116 and LS174T. Although neither gemcitabine nor decitabine is used in colorectal cancer therapy, they were selected based on their availability within the lab. The individual drug treatments revealed that gemcitabine exhibits notable cytotoxicity in HCT116 cells, whereas decitabine showed limited cytotoxicity in both cell lines.

Given the minimal cytotoxicity of decitabine, the dose selection for the Gem-Dec dimer was primarily guided by the effective gemcitabine concentrations. Interestingly, in HCT116 cells, the Gem-Dec dimer exhibited a similar dose-response trend to decitabine, albeit with slightly reduced potency. The IC50 of the dimer (0.049 μM) was nearly double that of gemcitabine alone (0.0247 μM), indicating that while the dimer retains biological activity, its efficiency is lower, possibly due to altered cellular uptake or intracellular activation. In contrast, the LS174T cells exhibited a modest increase in cytotoxic response to the dimer compared to the monotherapies, though the overall effect remained suboptimal. Further studies in different cancers, such as lymphoma, may yield more positive results regarding the cytotoxic effects of the dimer.

A synergy analysis using the HSA model further explored the interaction between gemcitabine and decitabine. In HCT116 cells, combination therapy demonstrated a clear synergy at specific concentrations, with up to a 36% greater inhibition of cell viability than the single agents alone. This suggests that decitabine may sensitize the colorectal cancer cells to gemcitabine. This could be due to the complementary mechanism -decitabine's

epigenetic modulation helping to modulate gemcitabine's DNA-damaging effects. Conversely, in LS174Tcells, little to no synergy was observed. This likely reflects the low baseline cytotoxicity of both drugs in the cell line, highlighting the importance of intrinsic cellular context in drug responsiveness.

Importantly, the chemical stability of the Gem-Dec dimer was confirmed via TLC. When incubated in various culture media, with and without FBS, no additional spots were observed on the TLC plate, and all samples showed consistent Rf values (~0.33). This indicates that the dimer does not undergo spontaneous cleavage in physiological media and is likely to enter the cells in its intact form. Further stability studies, such as time-dependency and temperature, would be beneficial for further development for therapeutic evaluation.

CONCLUSIONS

This study demonstrated, for the first time, that gemcitabine and decitabine can be successfully conjugated to form a novel chemotherapeutic agent. This represents a promising advancement in drug development, offering the potential for a new therapeutic strategy that leverages the combined properties of both nucleoside analogs.

The cytotoxic and synergistic effects of the gemcitabine-decitabine (Gem-Dec) dimer were evaluated in colorectal cancer models. Although colorectal cancer is not a primary target for either drug, the observed cytotoxicity—particularly in HCT116 cells—provides encouraging preliminary data. These results suggest the dimer may have therapeutic potential in other cancers where gemcitabine and decitabine are already clinically relevant. Further investigation into the intracellular delivery and uptake of the dimer is warranted to better understand its activity and optimize dosing strategies.

Synergy analysis revealed that gemcitabine and decitabine exhibit significant synergistic effects in HCT116 cells at specific concentrations, indicating potential mechanistic complementarity. Elucidating these underlying interactions could provide important insights into how the dimer enhances cytotoxicity and guide future therapeutic design. Collectively, these findings support continued preclinical development of the Gem-Dec dimer and lay the groundwork for exploring its utility in broader oncologic contexts.

REFERENCES

Alexandrov, L. B., Nik-Zainal, S., Wedge, D. C., Aparicio, S. A., Behjati, S., Biankin, A. V., Stratton, M. R. (2013). Signatures of mutational processes in human cancer. Nature, 500(7463), 415-421. https://doi.org/10.1038/nature12477

Fearnhead, N. S., Britton, M. P., & Bodmer, W. F. (2001). The ABC of APC. Hum Mol Genet, 10(7), 721-733. https://doi.org/10.1093/hmg/10.7.721

Fearon, E. R., & Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell, 61(5), 759-767. https://doi.org/10.1016/0092-8674(90)90186-i

Freese, E. (1959). The specific mutagenic effect of base analogues on Phage T4. Journal of Molecular Biology, 1(2), 87-105. https://doi.org/https://doi.org/10.1016/S0022-2836(59)80038-3

García, R. G., Brank, A. S., Christman, J. K., Marquez, V. E., & Eritja, R. (2001). Synthesis of Oligonucleotide Inhibitors of DNA (Cytosine-C5) Methyltransferase Containing 5-Azacytosine Residues at Specific Sites. Antisense and Nucleic Acid Drug Development, 11(6), 369-378. https://doi.org/10.1089/108729001753411335

Guo, C., McDowell, I. C., Nodzenski, M., Scholtens, D. M., Allen, A. S., Lowe, W. L., & Reddy, T. E. (2017). Transversions have larger regulatory effects than transitions. BMC Genomics, 18(1), 394. https://doi.org/10.1186/s12864-017-3785-4

Helleday, T., Eshtad, S., & Nik-Zainal, S. (2014). Mechanisms underlying mutational signatures in human cancers. Nature Reviews Genetics, 15(9), 585-598. https://doi.org/10.1038/nrg3729

Hodis, E., Watson, Ian R., Kryukov, Gregory V., Arold, Stefan T., Imielinski, M., Theurillat, J.-P., Chin, L. (2012). A Landscape of Driver Mutations in Melanoma. Cell, 150(2), 251-263. https://doi.org/10.1016/j.cell.2012.06.024

Jones, S., Chen, W. D., Parmigiani, G., Diehl, F., Beerenwinkel, N., Antal, T., Markowitz, S. D. (2008). Comparative lesion sequencing provides insights into tumor evolution. Proc Natl Acad Sci U S A, 105(11), 4283-4288. https://doi.org/10.1073/pnas.0712345105

Kantarjian, H., Issa, J. P., Rosenfeld, C. S., Bennett, J. M., Albitar, M., DiPersio, J., Saba, H. (2006). Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. Cancer, 106(8), 1794-1803. https://doi.org/10.1002/cncr.21792

Kinzler, K. W., & Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. Nature, 386(6627), 761, 763. https://doi.org/10.1038/386761a0

Kunkel, T. A., & Erie, D. A. (2005). DNA mismatch repair. Annu Rev Biochem, 74, 681-710. https://doi.org/10.1146/annurev.biochem.74.082803.133243

Le, D. T., Durham, J. N., Smith, K. N., Wang, H., Bartlett, B. R., Aulakh, L. K., Diaz, L. A., Jr. (2017). Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science, 357(6349), 409-413. https://doi.org/10.1126/science.aan6733

Li, W. H., Wu, C. I., & Luo, C. C. (1985). A new method for estimating synonymous and nonsynonymous rates of nucleotide substitution considering the relative likelihood

of nucleotide and codon changes. Mol Biol Evol, 2(2), 150-174. https://doi.org/10.1093/oxfordjournals.molbev.a040343

Lin, L., Liu, X., Yu, H., Deng, H., Peng, K., Chen, J., Liu, X. (2023). Inhibitory effect and related mechanism of decitabine combined with gemcitabine on proliferation of NK/T cell lymphoma cells [Original Research]. Frontiers in Pharmacology, Volume 14 - 2023. https://doi.org/10.3389/fphar.2023.1134895

Loewe, L., & Hill, W. G. (2010). The population genetics of mutations: good, bad and indifferent. Philosophical Transactions of the Royal Society B: Biological Sciences, 365(1544), 1153-1167. https://doi.org/doi:10.1098/rstb.2009.0317

Martincorena, I., Raine, K. M., Gerstung, M., Dawson, K. J., Haase, K., Van Loo, P., Campbell, P. J. (2017). Universal Patterns of Selection in Cancer and Somatic Tissues. Cell, 171(5), 1029-1041.e1021. https://doi.org/10.1016/j.cell.2017.09.042

Mini, E., Nobili, S., Caciagli, B., Landini, I., & Mazzei, T. (2006). Cellular pharmacology of gemcitabine. Ann Oncol, 17 Suppl 5, v7-12. https://doi.org/10.1093/annonc/mdj941

Nik-Zainal, S., Van Loo, P., Wedge, David C., Alexandrov, Ludmil B., Greenman, Christopher D., Lau, King W., Campbell, Peter J. (2012). The Life History of 21 Breast Cancers. Cell, 149(5), 994-1007. https://doi.org/10.1016/j.cell.2012.04.023

Nowell, P. C. (1976). The clonal evolution of tumor cell populations. Science, 194(4260), 23-28. https://doi.org/10.1126/science.959840

Olivier, M., Hollstein, M., & Hainaut, P. (2010). TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harb Perspect Biol, 2(1), a001008. https://doi.org/10.1101/cshperspect.a001008

Organization, W. H. (2025). Cancer. Retrieved June 6 from https://www.who.int/news-room/fact-sheets/detail/cancer

Pao, W., Miller, V. A., Politi, K. A., Riely, G. J., Somwar, R., Zakowski, M. F.,Varmus, H. (2005). Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med, 2(3), e73. https://doi.org/10.1371/journal.pmed.0020073

Patch, A.-M., Christie, E. L., Etemadmoghadam, D., Garsed, D. W., George, J., Fereday, S., The Australian Ovarian Cancer Study, G. (2015). Whole–genome characterization of chemoresistant ovarian cancer. Nature, 521(7553), 489-494. https://doi.org/10.1038/nature14410

Peltomäki, P. (2016). Update on Lynch syndrome genomics. Fam Cancer, 15(3), 385-393. https://doi.org/10.1007/s10689-016-9882-8

Rayner, E., van Gool, I. C., Palles, C., Kearsey, S. E., Bosse, T., Tomlinson, I., & Church, D. N. (2016). A panoply of errors: polymerase proofreading domain mutations in cancer. Nature Reviews Cancer, 16(2), 71-81. https://doi.org/10.1038/nrc.2015.12

Rohacova, J., Zurek, J., Kantor, H., & Husek, A. (2019). Improved Processes For The Preperation of Guadecitabine and Intermediates Thereof. W. I. P. Organization.

Stratton, M. R., Campbell, P. J., & Futreal, P. A. (2009). The cancer genome. Nature, 458(7239), 719-724. https://doi.org/10.1038/nature07943

Tomasetti, C., Vogelstein, B., & Parmigiani, G. (2013). Half or more of the somatic mutations in cancers of self-renewing tissues originate prior to tumor initiation. Proc Natl Acad Sci U S A, 110(6), 1999-2004. https://doi.org/10.1073/pnas.1221068110

Vogelstein, B., Papadopoulos, N., Velculescu, V. E., Zhou, S., Diaz, L. A., Jr., & Kinzler, K. W. (2013). Cancer genome landscapes. Science, 339(6127), 1546-1558. https://doi.org/10.1126/science.1235122

Wakeley, J. (1996). The excess of transitions among nucleotide substitutions: new methods of estimating transition bias underscore its significance. Trends Ecol Evol, 11(4), 158-162. https://doi.org/10.1016/0169-5347(96)10009-4

Welch, J. S., Petti, A. A., Miller, C. A., Fronick, C. C., O'Laughlin, M., Fulton, R. S., Ley, T. J. (2016). TP53 and Decitabine in Acute Myeloid Leukemia and Myelodysplastic Syndromes. New England Journal of Medicine, 375(21), 2023-2036. https://doi.org/doi:10.1056/NEJMoa1605949

CURRICULUM VITAE

C. Jarrett Bailey

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OBJECTIVE

Pursue my passion for Biomedical Engineering via top flight training in a highly competitive graduate program.

EDUCATION

Rochester Institute of Technology Rochester, NY

B.S./M.S. in Biomedical Engineering and Science, Technology, and Public Policy, with a minor in Chemistry. Graduated: May 2023
GPA: 3.42 - Dean's List: Fall 2018, Spring 2019, Fall 2019

Wake Forest School of Medicine Winston-Salem, NC

M.S. in Biomedical Engineering
Graduated: September 2025
GPA: 3.61

RELEVANT COURSES

Undergraduate (Biomedical Engineering, minor in Chemistry)

Practical Methods of Tissue Engineering	Cellular and Molecular Biology I & II
Organic Chemistry and Lab I & II	Intro to Biomaterials Science
Systems Physiology I & II	Calculus I & II
Multivariable & Vector Calculus	Probability and Statistics I

Graduate (Science, Technology, and Public Policy)

Evaluation and Research Design	Graduate Decision Analysis

Special Topics – Biochemical Engineering (Spring 2023)

SKILLS

Equipment: Biosafety Cabinet (hood), micropipette, microscope, aseptic techniques
Processes: Titration, bacterial cell culture, pH meter, tissue culture, distillation, PCR
Programming: Microsoft Office, MATLAB, VBA, SAS
Certifications: CITI Program Biomedical Research – 3/10/2022, Lean Six Sigma Yellow Belt – Spring 2023, Environmental Enrichment for Laboratory Animals – Fall 2023, WFU – Working with the IACUC – Fall 2023

PROJECTS

Does Propensity for Transmigration (3D) Correlate with or Promote Increased Lateral (2D) Migration? – The goal of this project was to observe the effects of cell transmigration through narrow pores on post-migration behavior.
Cognitive Health Tracking System – The goals of this project was to create a working software system that integrated multiple input methods to help measure and track the cognitive health of individuals who have been diagnosed with diseases such as ALS.
Synthesis of Novel Pyrimidine Analog Gemcitabine-Decitabine Dimer – The goal of this project was to synthesize a novel Gemcitabine-Decitabine dimer and test the cytotoxic and synergistic effects *in vitro.*

WORK EXPERIENCE

ViewFlow (previously Scinovia**)** Raleigh, NC **August 2020 – January 2021**
May 2021 – August 2021
May 2022 – July 2022

Biomedical Engineering Co-op

- Collected data for analysis to build state of the art computer vision algorithms for medical imaging technology.
- Helped assemble medical imaging prototypes, including wiring, optics, LEDs, lasers, computers, controller boards, installed computer OS, software, and firmware.
- Worked with porcine hearts, vessels, and blood to calibrate and measure blood flow speeds and test new computer vision algorithms.
- Used MATLAB and Excel for data analytics including 2D and 3D plots.

Independent Contractor North Carolina **September 2014 – May 2018**

Certified Grade 8 Referee (United States Soccer Federation)

- Executed and enforced the Laws of the Game (FIFA, USSF, NCYSA) for U8 – U18 competitive matches for both genders across single game and multiple day tournament environments.
- Used my position as a referee to provide real-time feedback and learning experiences to young and novice players to help build their skills and to help them understand the correct way to play the game.

Deep Creek Pharma Winston-Salem, NC **May 2024 – February 2025**

Research Assistant

- Synthesized a novel Gemcitabine–Decitabine dimer with potential applications in cancer therapy.
- Performed and interpreted ^{1}H NMR and ^{31}P NMR spectroscopy to confirm molecular structure and purity of synthesized compounds.

LEADERSHIP AND ACTIVITIES

WFU Graduate School Honor Council, Member August 2024 – August 2025
National Society of Leadership and Success, Member April 2019 – Present

RIT Club Soccer Team, Member August 2018 – May 2023
National Honor Society, Member
May 2016 – June 2018
Team Captain for Varsity High School Soccer team August 2018 – November 2018

AWARDS AND RECOGNITION

RIT BS/MS Scholarship 2022 – 2023
Blue Cross and Blue Shield of North Carolina Family Scholars Scholarship 2019 – 2023
RIT Founder's Scholarship 2018 – 2022