FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

# Anticancer activity of 4-N gemcitabine analogues

An Undergraduate Honors Thesis submitted in partial fulfillment of the

requirements for the degree of

UNDERGRADUATE HONORS

in

BIOLOGICAL SCIENCES

by

Alana Ming-Amede Van Dervort

2012

**To:** Dr. Timothy Collins, Chairperson

Department of Biological Sciences

This Undergraduate Honors Thesis in Biological Sciences, written by Alana Ming-Amede Van Dervort, and entitled "Anticancer activity of 4-N gemcitabine analogues", is submitted to you in partial fulfillment of the requirements for Undergraduate Honors in Biological Sciences. The Biological Sciences Undergraduate Honors Committee and the candidate's research supervisor(s) have read this thesis. We recommend that it be approved.

Dr. Stanislaw Wnuk,
Honors Research Supervisor

Dr. Ophelia Weeks,
Honors Research Supervisor

Dr. Walter Goldberg, Chairperson,
Undergraduate Honors Committee

Date of Honors Research Presentation: April 16, 2012

This thesis by Alana Van Dervort is approved.

Dr. Timothy Collins, Chairperson
Department of Biological Science

Acknowledgements

I would like to thank the Wnuk laboratory, and in particular Dr. Stanislaw Wnuk and Jesse Pulido for providing me formative mentorship integral to the actualization of my Honors Thesis and making my primary experience in research a positive one. I would also like to express my gratitude to the Barbieri and Roy laboratories at FIU for providing generous advice and resources, as well as Dr. Goldberg for his critical guidance during the final stages of my Honors Thesis development. Special thanks go to Professor Ramon Gomez, for his statistical insight regarding the data analysis. To the dedicated individuals who comprise FIU MARC U*STAR and QBIC program, in particular Bryan Dewsbury, Courtney Aiken, and Dr. Ophelia Weeks, I have the utmost gratitude, for they have enabled me to make my vision and certain trajectory toward biomedical research a reality.

ABSTRACT OF THE UNDERGRADUATE HONORS THESIS

IN BIOLOGICAL SCIENCES

Anticancer activity of 4-N gemcitabine analogues

By

Alana Ming-Amede Van Dervort

Florida International University

Miami, Florida

2012

Professor Stanislaw Wnuk, Major Professor

Gemcitabine (2', 2'-difluoro-2'-deoxycytidine or dFdC) has become a standard chemotherapeutic agent in the treatment of several cellular and solid tumor- related malignancies. Gemcitabine's anti-cancer activity has been attributed to its inhibitory effects on the cell's DNA synthetic machinery resulting in the induction of cell arrest and apoptosis. Despite its broad application, treatment capacity with this drug is limited due to complicated administration schedules stemming from low bioavailability and tumor resistance associated with its rampant intracellular enzymatic inactivation. The aim of this study is to characterize the anti-cancer activity of novel designed and synthesized gemcitabine analogues, that were modified with long alkyl chains at the 4-amino group of the cytosine ring. This study proposes the use of these alternative derivatives of gemcitabine that not only uphold current drug standards for potency, but additionally confer chemical stability against enzymatic inactivation. During screening conducted to

identify prospective gem-analogue candidates, I observed the potent anticancer properties of three 4-N modified compounds on MCF-7 breast adenocarcinoma cells. Experiments described here with these compounds referred to as LCO, LCAO, and Gvaldo, evaluate their cytotoxicity on MCF-7 cells at the concentrations of 25μM and 2.5μM , and assess their inhibitory effects on DNA synthesis and cell cycle progression using sulphorhodamine B and bromodeoxyuridine assays as well as flow cytometric analyses, respectively. Among the compounds tested, LCO was shown to be most active inhibitor of DNA synthesis ($\alpha$=.05; $p<.001$) as reflected as a distinct G0/G1 versus S-phase arrest in the 25μM and 2.5μM treatments, respectively. Together, these experiments provide preliminary evidence for the clinical application of LCO-like gemcitabine derivatives as a novel treatment for breast cancer.

.

Table of Contents

List of Figures

List of Tables

List of Acronyms

dFdC, Gemcitabine, 2', 2'-difluorodeoxycytidine

Ara-C, Cytosine Arabinoside

dcK, Deoxycytidine Kinase

dFdCMP, Gemcitabine Monophosphate

dFdCDP, Gemcitabine Diphosphate

dFdCTP, Gemcitabine Triphosphate

RNR, Ribonucleotide Reductase

CTP synthase, Cytosine Triphosphate Synthetase

dCDA, Deoxycytidine Deaminase

hNTs ,Nucleoside Transporters

dFdC-DNA, Gemcitabine incorporation into DNA

dFdC-RNA, Gemcitabine incorporation into RNA

LCO, Long Chain amide-linkage (acyl) Olefin

Gvaldo, Gemcitabine with 'Valproic acid' moiety

LCAO, Long Chain (Alkyl) Olefin with an amine linkage

BrdU, 5-Bromodeoxyuridine

SRB, Sulphorhodamine B

TCA, Trichloroacetic Acid

POD, Peroxidase

RT-PCR, Real Time Polymerase Chain Reaction

PARP-1, Poly Adenosine Disphosphate Polymerase 1

# Introduction

Gemcitabine, 2', 2'-difluorodeoxycytidine (dFdC), has emerged as one of the most significant nucleoside analogues employed toward the treatment of breast, colon, lung, and ovarian Gemcitabine remains the definitive chemotherapeutic strategy for patients afflicted with metastatic pancreatic cancer (Burris et al. 1997). Together with cytosine arabinoside (Ara-C), gemcitabine heads the successful implementation of an important class of anti-cancer molecules known as cytidine analogues; indeed, its structural similarity to cytidine is essential to its potent antitumor effects (Mini et al. 2006). Numerous *in vitro and in vivo* experimental models have contributed to an understanding of gemcitabine's metabolic and molecular interactions underlying its cytotoxicity (Mini et al. 2006).

The ability of gemcitabine to demote cancer cells to an anti-proliferative status is largely due to its inhibitory action on DNA synthesis (Hertel at al. 1990). To accomplish this feat, gemcitabine must first enter the cell via cell-membrane bound nucleoside transporters (Mackey et al. 1998). Once inside the cytoplasm, gemcitabine is phosphorylated by deoxycytidine kinase (dCK) to become gemcitabine monophosphate (dFdCMP). This molecule serves as the substrate for subsequent phosphorylation events resulting in di- and triphosphate (dFdCDP and dFdCTP) gemcitabine metabolites (Mini et al. 2006). In its triphosphate forms, gemcitabine exercises its most important functions: that is, the inhibition of DNA polymerase's S-phase replicative processes, via single-nucleotide-mediated chain termination (Plunkett et al. 1995). As a diphospophate metabolite, gemcitabine represses ribonucleotide reductase (RNR) and as well cytosine

triphosphate synthetase (CTP-Synthase), ultimately hampering the pool of available nucleotides for nucleic acid incorporation (Mini et al. 2006). Collectively, DNA polymerase and RNR attenuation may lead to apoptotic signaling. Gemcitabine metabolites are thought to induce stress on topoisomerase stability, leading to DNA cleavage (Pourquier et al. 2002).

Confirmation of gemcitabine's efficacy via its cytoplasmic turn pharmacological interactions has earned its place as one of the most hopeful candidates for the eradication of various types of malignancy (Figure 1). Unfortunately, its full actualization as a formative anti-cancer agent is been tempered by intracellular deactivation events. Gemcitabine is subject to intracellular enzymatic inactivation via deoxycytidine deaminase-mediated conversion of gemcitabine monophosphate to form difluorodeoxyuridine dFdU (Mini et al. 2006; Figure 1). The opposing action of cytoplasmic deaminase is largely responsible for gemcitabine's short intracellular half-life and hence complex dosage schedule (Reddy et al. 2009). Thus, separating gemcitabine's potent anti-cancer activity from toxicity profile by delaying or preventing enzymatic deactivation represents the 'holy grail' for gemcitabine's full clinical impact.

The experiments described here report the evaluation of novel gemcitabine analogues defined by strategic modifications to overcome deaminase-mediated deactivation (Figure 1). It has previously hypothesized that the addition of 4-N alkyl and 4-N acyl to a gemcitabine structural skeleton may fulfill the following objectives: (1) cytoplasmic stability and resistance to enzymatic deamination, (2) maintenance anti-tumor pharmacologic activity and (3) the presence of chemical moieties that allow for the introduction of an $^{18}F$ radioligand for use in positron emission tomography (Mini et al.

2009; Laing et al. 2009; Bender et al. 2009). To that end, I have performed several biological assays on MCF-7 breast adenocarcinoma cells, described below, in order to test the capacity of the Wnuk group's originally conceived and synthesized analogues to induce anticancer effects. Figure 2 depicts the chemical structure of novel gem-analogues to be tested in this study.

**Figure 1: The Cellular Pharmacology of Gemcitabine (from Mini et al. 2009).**

Metabolism, mechanisms of action and self-potentiation of gemcitabine. 1, transport by nucleoside transporters (hNTs); 2, phosphorylation; 3 and 4, deamination; 5, dephosphorylation; 6, accumulation of the triphosphate; 7, incorporation into DNA; 8, incorporation into RNA; 9, inhibition of ribonucleotide reductase (RR); 10, inhibition of CTP-synthetase; 11, inhibition of thymidylate synthase (TS); 12, inhibition of deoxycytidine monophosphate deaminase (dCMPDA); inhibitory effect. Other abbreviations: dCK, deoxycytidine kinase; TK2, thymidine kinase 2; dCDA, deoxycytidine deaminase; 59-NT, 59-nucleotidase.

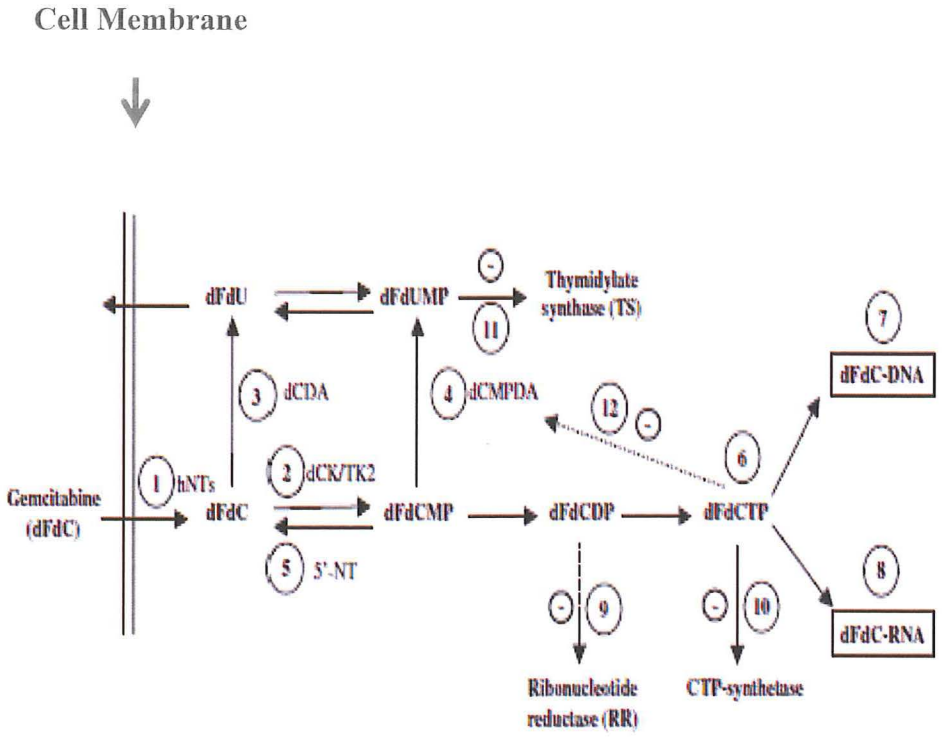
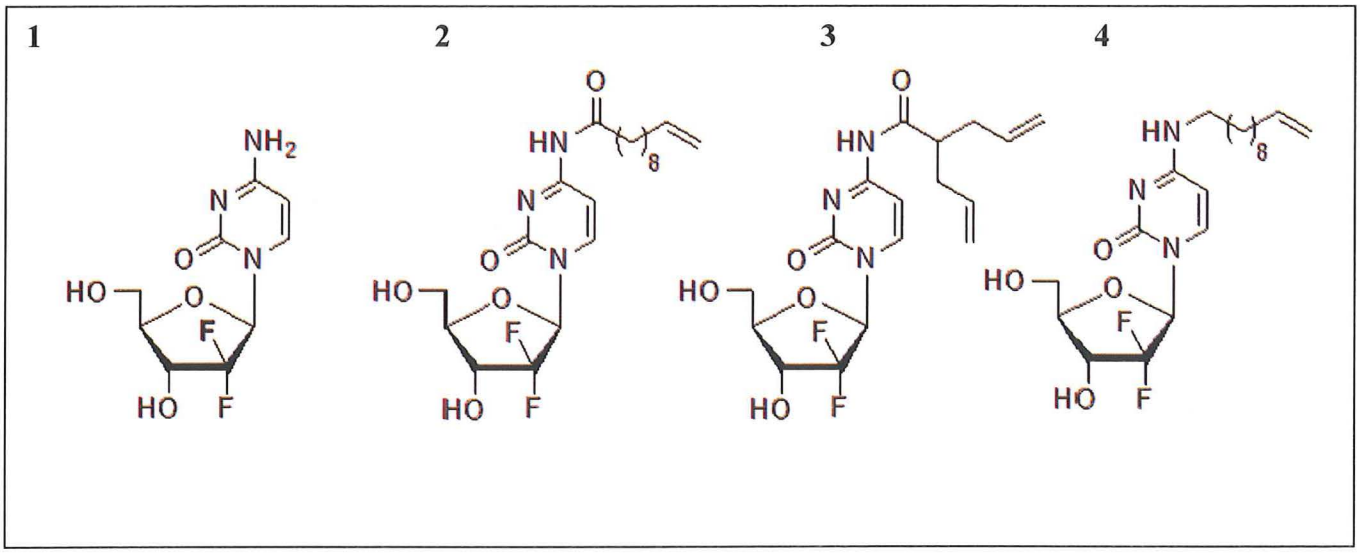


**Figure 2: Chemical Structure of Novel Synthesized 4-N Gemcitabine Analogues (gem-analogues):** (1) Gemcitabine, (2) LCO: long chain amide-linkage (acyl) olefin, (3) Gvaldo: gemcitabine with 'valproic acid' moiety and (3) LCAO: long chain (alkyl) olefin with an amine linkage. All compounds possess terminal olefins (alkenes) as a structural feature intended for the incorporation of $^{18}F$ radioligand for drug uptake monitoring.




## Materials and Methods

**Cell and Culture Conditions.** MCF-7 (human breast carcinoma) cells were procured from ATCC Cell Biology Collection (Manassas, VA). Culture conditions consisted of DMEM/F-12 media supplemented with 10% heat-inactivated fetal bovine serum, antibiotics ($1x10^4$ units/ml Penicillin, $1x10^4$µg/mL Streptomycin) and 1M HEPES buffer. Cells were maintained in a humidified 5% $CO_2$ chamber; sub-culturing and harvesting with trypsin occurred twice a week in accordance with cells achieving 70% confluence.

**Experimental Design.** In preparation for sulphorhodamine B, bromodeoxyuridine, flow cytometric assays, MCF-7 cells were harvested then plated onto 96-well, flat-bottom microtiter plates at a density of $5x10^3$ cells per 100µl/well. Cells were then set for 24hr incubation to allow for proper cell adherence; gemcitabine and 4-N gemcitabine analogues for testing were next applied at 2.5nM and 25µM concentrations to those maintained in 100µL µl of complete medium. Cells were divided into three experimental populations: plated MCF-7 cells incubated in the presence and absence (control) of gem-analogues for 24, 48,72, or 96 hours. After said co-culturing period, the experimental populations were further evaluated as described below.

**Sulphorhodamine B Staining (SRB Assay) for the Determination of Total Cell Protein Content.** Following 72 hour incubation with treatment, MCF-7 cells were fixed with 50µL of 50% trichloroacetic acid (TCA) and incubated at 4°C. TCA was removed after one hour using three water washes; 100µL of 0.4% SRB solution was then applied to water-washed cells. SRB treated cells were subsequently stained for 15 minutes at room temperature. Acetic acid (1%) washes were (200 µL) performed four times to remove SRB staining. Plates were then air dried overnight and solubilized with 200 µL of 10mM Tris base (pH= 10.5). After 30 minutes, absorbance was measured using microplate reader at a wavelength of 560 nm.

**Quantification of Cell Proliferation by Measurement of 5-Bromodeoxyuridine Incorporation (BrdU Assay)** MCF-7 treatment and control populations, incubated with compound for 24, 48, 72 and 96 hours, and were quantified for cell proliferation. Cells

were labeled (Cell Proliferation ELISA BrdU, Roche Diagnostics, Mannheim, Germany). BrdU was added to the plated cells at a volume of 20 µl/well and a final concentration 10 µM per well. The mixture was then incubated for 2 hours at 37°C, then 'flicked' to remove labeling solution then stored overnight at 2°C. After 24hrs, cells were treated with 200 µl/well of FixDenat solution and incubated for 30 minutes at 37°C. After fixing, cells were treated with 100µl/well of anti-BrdU–Peroxidase (POD) working solution and incubated at room temperature for 90 minutes. The antibody conjugate was then removed via 3X washes with DPBS buffer solution; 100µL of substrate solution was then added to each well for a duration of 30 minutes and absorbance was measured by a microplate reader at a wavelength of 370 nm.

**DNA Measurement and Cell Cycle Analysis by Flow Cytometry with Propidium Iodide (PI) Staining.** A commercially available kit (Cell Cycle Phase Determination Kit, Cayman Chemical, Michigan, USA) was used in accordance with standard protocol. MCF-7 Cells were incubated with compounds for 48 hours at a concentration of 25µM and 2.5µM. Cells were then trypsinized and suspended in wash buffer. The cell suspension was subsequently centrifuged for pellet formation and subjected to two washes with Assay Buffer. The washed pellet was then resuspended in Assay Buffer at 1 x $10^6$ cells/mL; after which 1 mL Assay Fixative was added. The resulting solution was incubated for 2 hours and centrifuged at 500 x g for five minutes. After removing the Assay Fixative, 500 µL of Staining Solution (20 µL/mL RNase and 20µg/mL in Assay Buffer) was added to the remaining pellet which was then left at room temperature, in the absence of light for 30 minutes. A flow cytometer (Accuri C6, BD Accuri Cytometers,

Michigan, USA) was then used to perform measurements of absorbance 488 nm; further analysis of cell cycle population distribution was executed on BD CFlow Plus software.

# Results

## SRB Assay Shows Gem-Analogue Mediated Growth Inhibition

In preliminary studies, the Wnuk group evaluated the cytotoxic activity of novel synthetic gem-analogues utilizing a Sulphorhodamine B Staining Assay (SRB) to evaluate cell proliferation of chemically treated and untreated MCF-7 breast adenocarcinoma cells (Pulido unpublished observation). All gem–analogues demonstrated dose-dependent inhibition of cell proliferation, as compared with the control (Data courtesy of Jesse Pulido, PhD; Table 1). Most notably, LCO exerted the greatest inhibitory effect on cell growth, shown by the $IC_{50}$ value obtained of 0.15 μM; in fact, LCO renders 3-fold difference in potency as compared with the parent drug gemcitabine (0.48 μM). Gvaldo and LCAO showed less cytotoxic activity than that of Gem and LCO; however IC values still equated to significant growth inhibition. To ask whether the cytotoxic effects of gem-analogues could be explained by a repression of DNA replication and hence, cell proliferation, a series of BrdU analyses were performed on compound treated and untreated MCF-7 cell populations.

**Table 1: SRB Cytotoxicity Assay of Gem-Analogues on MCF-7 line.** The table reflects $IC_{50}$ values of compound treated cells after 72 hrs. Data courtesy of Jesse Pulido, PhD Candidate.

| **Compound** | **$IC_{50}$(μM)** |
|---|---|
| Gemcitabine [dFdc] | 0.48 |
| LCO | 0.15 |
| Gvaldo | 6.5 |
| LCAO | 11.0 |

## BrdU Analysis shows Gem-Analogue inhibition of DNA Synthesis at 25 μM

Growth inhibition mediated by gem-analogues may occur via cessation of 'new' DNA synthesis. To investigate this hypothesis, BrdU assays were performed on MCF-7 cells treated with 25 μM of gem-analogue compounds for 24, 48 72, or 96 hours; absorbance measurements (taken at 370nm) of incorporated 5-bromo-2'-deoxyuridine were used to quantify amount of DNA synthesis per cell population. Data combined from several experiments repeated in triplicate were analyzed by a series of One-Way ANOVAs with interaction, to test for statistical significance (Table 1).

A One-Way ANOVA as well as a Tukey Pairwise Analysis (a test for sample homogeneity) was performed on the effects of Gem analogues at 25 μM concentrations to evaluate mean absorbance differences as a function of time (24, 48, 72, and 96 hrs; Table

2). LCO, Gvaldo, as well as the parent compound gemcitabine reported significant effects on total MCF-7 cell DNA synthesis respectively ($\alpha$=.05; $p<.001$ Table 2). LCAO, however, did not propagate an appreciable impact on MCF-7 proliferation relative to that of the control ($\alpha$=.05; $p<.001$; Table 2). Although Gem, Gvaldo and LCO showed inhibition of DNA synthesis, Tukey post-hoc tests confirmed that in their inhibition they did not significantly differ from one another, such that the gem-analogues and gemcitabine show similar inhibition across all time points (Figure 3A, Figure 3B).

**Table 2. One-Way ANOVA & Turkey Pairwise Analysis at the 25 μM concentration.** Absorbance of each treatment was compared to assess differences in BrdU incorporation as a proxy for DNA synthesis by One-Way ANOVA with Tukey post-hoc tests. * Denotes the mean difference in absorbance is significant at the .05 confidence level. Dunett t-tests delegate one group as the control and compare all other groups to the assigned control using t-tests.

**One-Way ANOVAs of Mean Absorbance Difference at the 25uM Concentration**

Dependent Variable:Absorbance

| | (I) Compound | (J) Compound | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | Control | Gem | 63.240* | 7.8513 | .000 | 41.054 | 85.426 |
| | | Gvaldo | 64.175* | 7.8513 | .000 | 41.989 | 86.361 |
| | | LCAO | 17.009 | 7.8513 | .209 | -5.177 | 39.195 |
| | | LCO | 62.429* | 7.8513 | .000 | 40.242 | 84.615 |
| | Gem | Control | -63.240* | 7.8513 | .000 | -85.426 | -41.054 |
| | | Gvaldo | .936 | 7.8513 | 1.000 | -21.250 | 23.122 |
| | | LCAO | -46.231* | 7.8513 | .000 | -68.417 | -24.045 |
| | | LCO | -.811 | 7.8513 | 1.000 | -22.997 | 21.375 |
| | Gvaldo | Control | -64.175* | 7.8513 | .000 | -86.361 | -41.989 |
| | | Gem | -.936 | 7.8513 | 1.000 | -23.122 | 21.250 |
| | | LCAO | -47.167* | 7.8513 | .000 | -69.353 | -24.980 |
| | | LCO | -1.747 | 7.8513 | .999 | -23.933 | 20.439 |
| | LCAO | Control | -17.009 | 7.8513 | .209 | -39.195 | 5.177 |
| | | Gem | 46.231* | 7.8513 | .000 | 24.045 | 68.417 |
| | | Gvaldo | 47.167* | 7.8513 | .000 | 24.980 | 69.353 |
| | | LCO | 45.420* | 7.8513 | .000 | 23.234 | 67.606 |
| | LCO | Control | -62.429* | 7.8513 | .000 | -84.615 | -40.242 |
| | | Gem | .811 | 7.8513 | 1.000 | -21.375 | 22.997 |
| | | Gvaldo | 1.747 | 7.8513 | .999 | -20.439 | 23.933 |
| | | LCAO | -45.420* | 7.8513 | .000 | -67.606 | -23.234 |
| Dunnett t (2-sided)[a] | Gem | Control | -63.240* | 7.8513 | .000 | -83.017 | -43.463 |
| | Gvaldo | Control | -64.175* | 7.8513 | .000 | -83.952 | -44.399 |
| | LCAO | Control | -17.009 | 7.8513 | .111 | -36.786 | 2.768 |
| | LCO | Control | -62.429* | 7.8513 | .000 | -82.205 | -42.652 |

Based on observed means.
The error term is Mean Square(Error) = 369.855.

*. The mean difference is significant at the .05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

**Figure 3A: Effect of 25 μM compounds treatment on DNA synthesis as denoted by mean absorbance of brdU incorporation.** Effect of Duration of Compound Application on DNA. Intervals occurred every 24 hours for a total of four time-points (24, 48, 72, and 96 hr).

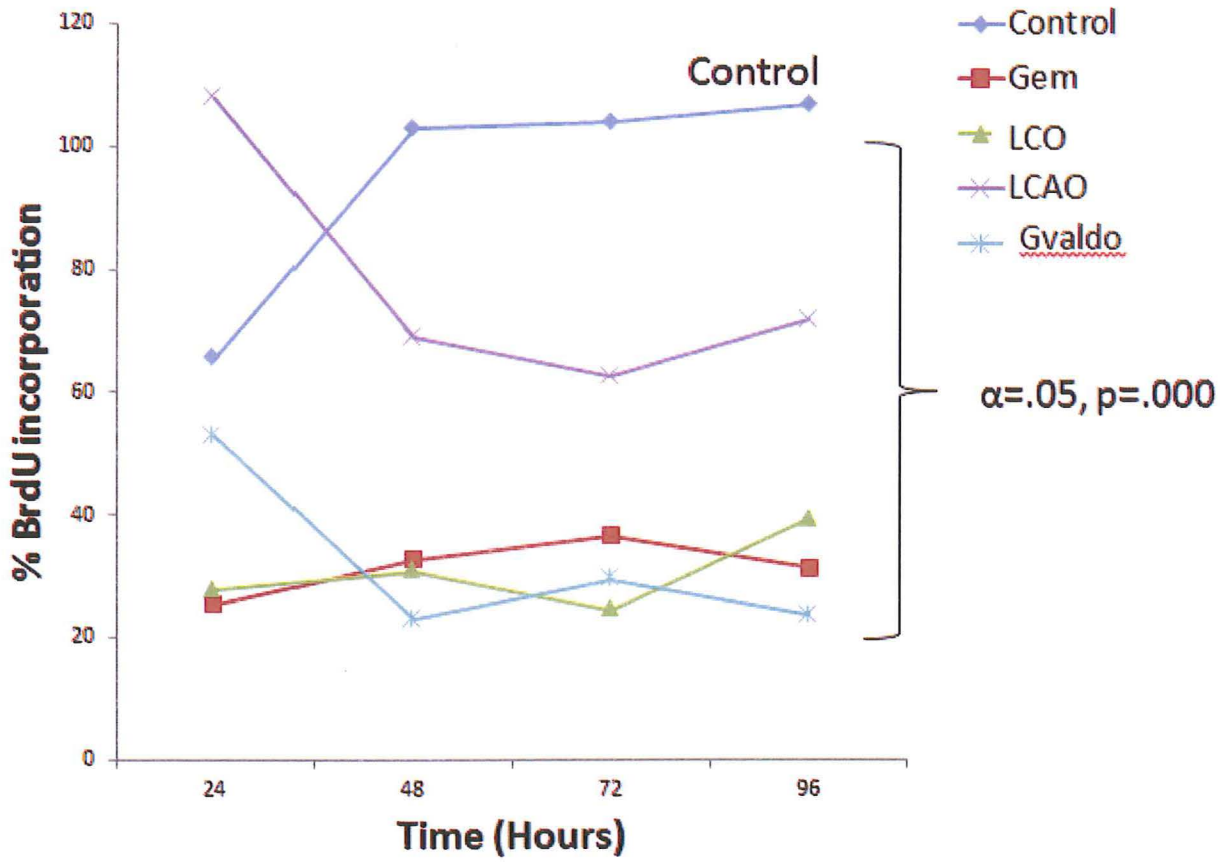
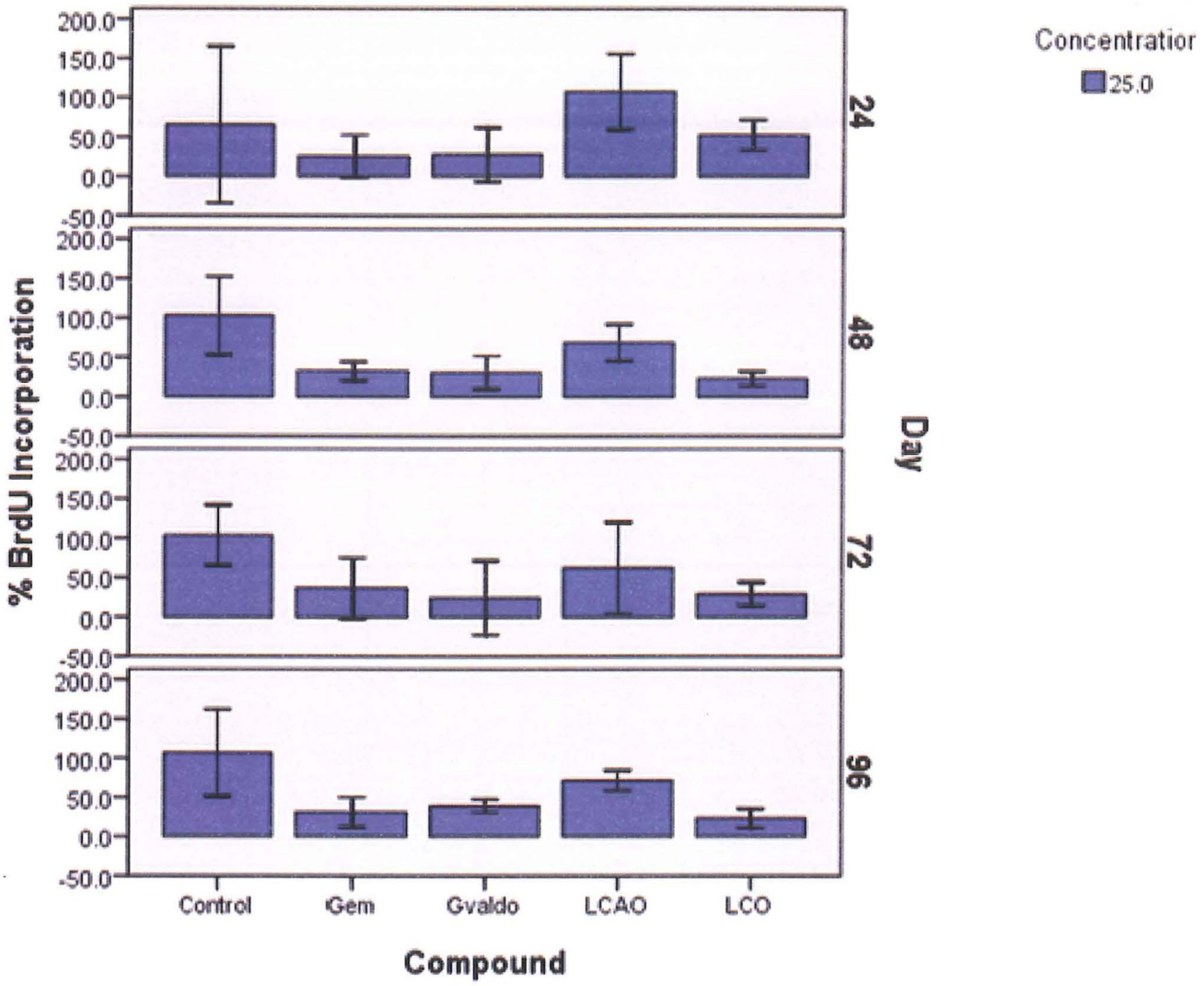


**Figure 3B: Effect of 25 µM compound treatment on DNA synthesis as denoted by mean absorbance of brdU incorporation.** Histogram of Compound and control effects with 95% Confidence Interval.



## Gem-Analogues maintain DNA Synthesis inhibition at 2.5 μM

The results obtained at 25 μM warranted additional testing of gem-analogues at a lesser concentration (2.5 μM), in order to examine threshold effects of inhibition (Figure 4). As assessed by One-Way ANOVA testing and Tukey's test for homogeneity, effects on DNA synthesis were generally consistent with those observed at the 25 μM concentration, in that all gem-analogues continued to report significant inhibition of DNA synthesis ($\alpha$=.05; p<.001; Gvaldo $\alpha$=.05; p<.001; Table 3). However, LCO showed significant down-regulation of DNA synthesis at the 2.5 μM level ($\alpha$=.05; p<.000; Table 3), as did gemcitabine, although at 24 hr only (Figure 5C). Likewise, LCAO and Gvaldo also impaired DNA synthesis as suggested by a substantial decrease in mean BrdU absorbance at hour 48 (Figure 4A; Figure 4B). LCO proved to be distinctive from other gem-analogues, by being able to propagate its activity at both concentrations, as well as showing greater 2.5 μM inhibition at all time-points, than its gem-analogue counterparts. ($\alpha$=.05; p<.000; Table 3).

**Table 3. One-Way ANOVA & Tukey Pairwise Analysis at 2.5 μM Concentration.**

A One-Way ANOVAs were applied to determine significant interaction between the treatment and absorbance and/or hours. * Denotes that the mean difference in absorbance is significant at the .05 confidence level. Dunett t-tests delegate one group as the control and compare all other groups to the assigned control using t-tests.

**One- Way ANOVA of Mean Absorbance Difference at the 2.5uM Concentation**

Dependent Variable:Absorbance

| | (I) Compound | (J) Compound | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | Control | Gem | 30.220* | 4.7362 | .000 | 16.836 | 43.603 |
| | | Gvaldo | 20.582* | 4.7362 | .001 | 7.198 | 33.965 |
| | | LCAO | 21.547* | 4.7362 | .000 | 8.164 | 34.931 |
| | | LCO | 62.961* | 4.7362 | .000 | 49.578 | 76.345 |
| | Gem | Control | -30.220* | 4.7362 | .000 | -43.603 | -16.836 |
| | | Gvaldo | -9.638 | 4.7362 | .264 | -23.021 | 3.746 |
| | | LCAO | -8.672 | 4.7362 | .367 | -22.056 | 4.711 |
| | | LCO | 32.742* | 4.7362 | .000 | 19.358 | 46.125 |
| | Gvaldo | Control | -20.582* | 4.7362 | .001 | -33.965 | -7.198 |
| | | Gem | 9.638 | 4.7362 | .264 | -3.746 | 23.021 |
| | | LCAO | .965 | 4.7362 | 1.000 | -12.418 | 14.349 |
| | | LCO | 42.379* | 4.7362 | .000 | 28.996 | 55.763 |
| | LCAO | Control | -21.547* | 4.7362 | .000 | -34.931 | -8.164 |
| | | Gem | 8.672 | 4.7362 | .367 | -4.711 | 22.056 |
| | | Gvaldo | -.965 | 4.7362 | 1.000 | -14.349 | 12.418 |
| | | LCO | 41.414* | 4.7362 | .000 | 28.030 | 54.798 |
| | LCO | Control | -62.961* | 4.7362 | .000 | -76.345 | -49.578 |
| | | Gem | -32.742* | 4.7362 | .000 | -46.125 | -19.358 |
| | | Gvaldo | -42.379* | 4.7362 | .000 | -55.763 | -28.996 |
| | | LCAO | -41.414* | 4.7362 | .000 | -54.798 | -28.030 |
| Dunnett t (2-sided)[a] | Gem | Control | -30.220* | 4.7362 | .000 | -42.150 | -18.289 |
| | Gvaldo | Control | -20.582* | 4.7362 | .000 | -32.512 | -8.652 |
| | LCAO | Control | -21.547* | 4.7362 | .000 | -33.478 | -9.617 |
| | LCO | Control | -62.961* | 4.7362 | .000 | -74.891 | -51.031 |

Based on observed means.
The error term is Mean Square(Error) = 134.592.

*. The mean difference is significant at the .05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

**Figure 4A: Effect of 2.5 µM Gemcitabine analogues**. Effect of Duration of Compound Application on DNA synthesis as a function of time. Tumor cells were co-cultured with and without compound for 24, 48 72 or 96 hours; DNA synthesis determined by BrdU incorporation.

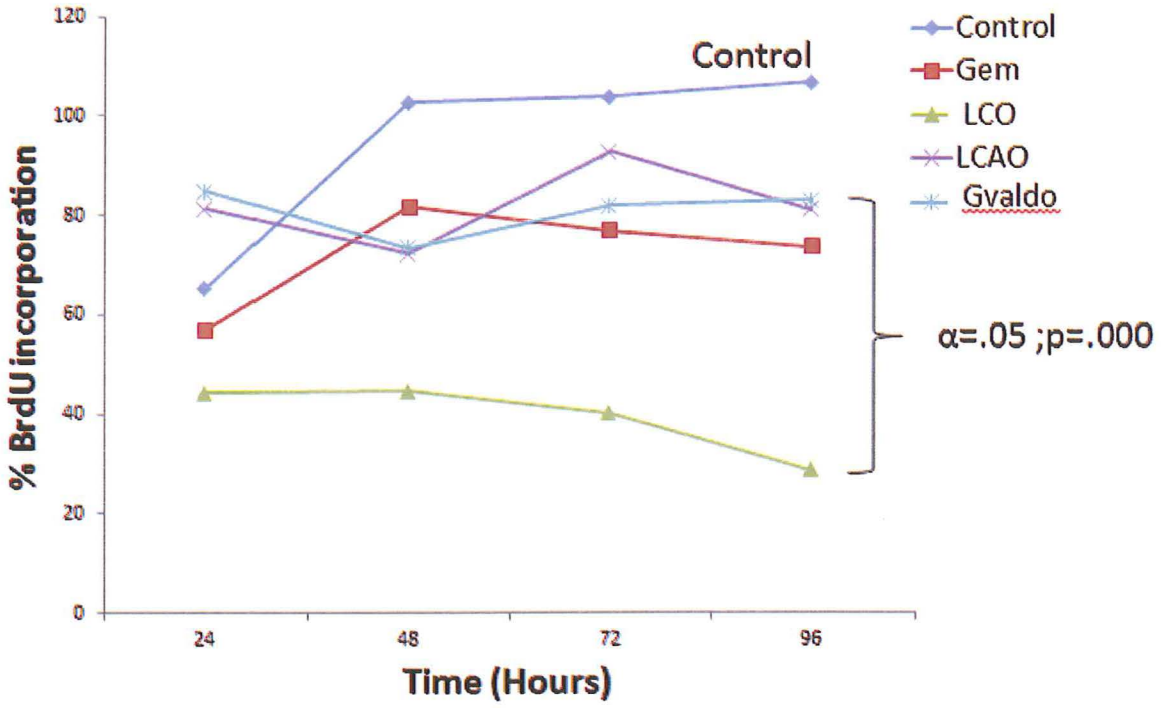
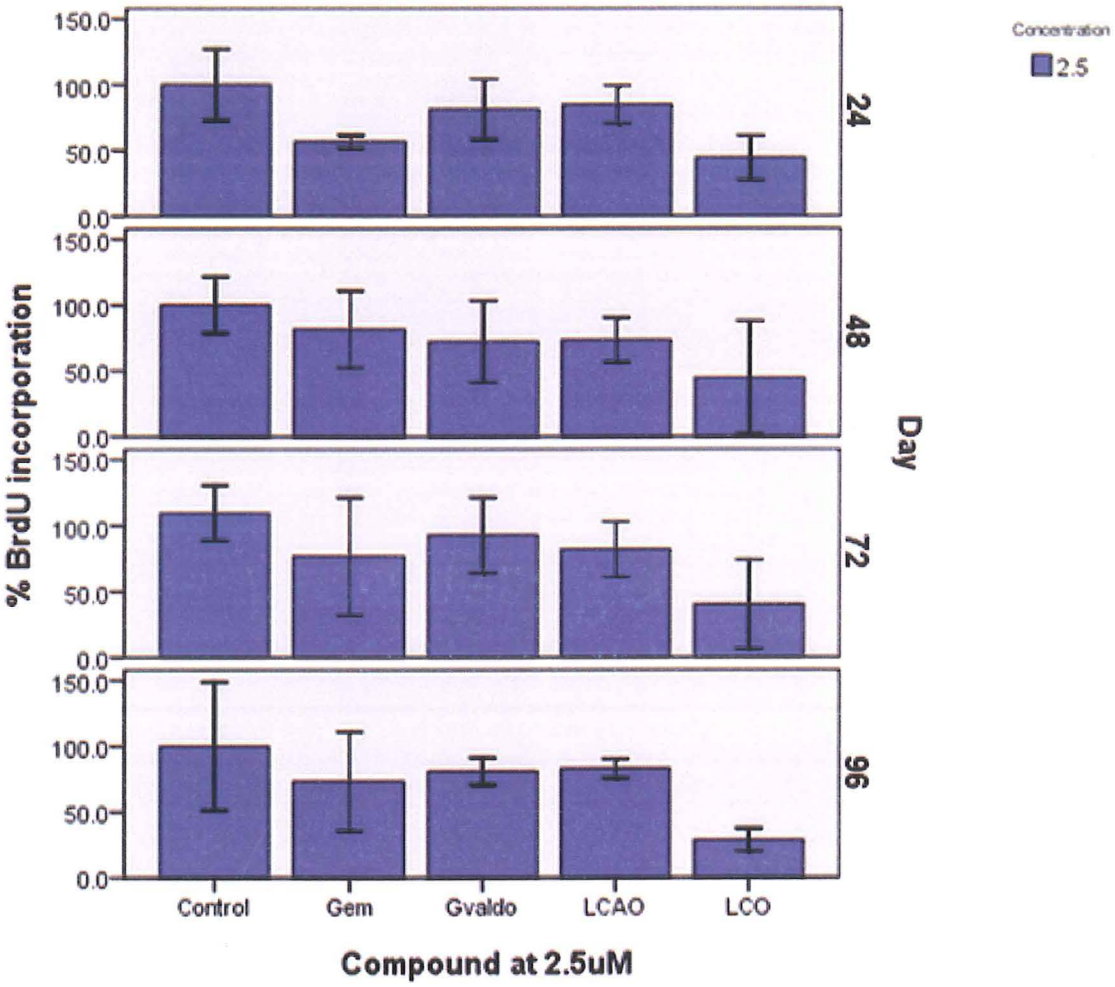


**Figure 4B: Effect of 2.5 µM Gemcitabine analogues**. Histogram showing effects of Compound and control with 95% Confidence Interval.



## Gem-Analogues induce $G_0/G_1$ arrest at the 25 μM concentration

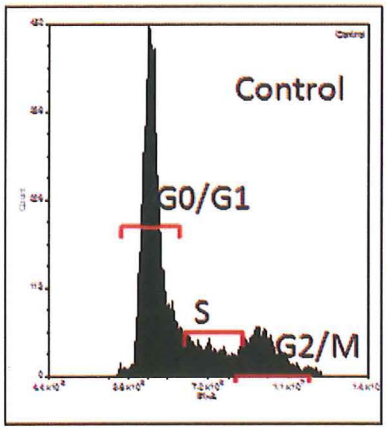
Results obtained from the SRB and BrdU assays provided confirmation that gem-analogue application induces cell cytotoxity as well as inhibition of DNA synthesis. To test whether gem-analogue potency was further manifested as a change in cell cycle dynamics and specifically an induction of cell cycle arrest, MCF-7 cells were treated with 25μM of compound for 48 hours and subsequently assessed for preferential accumulation in cell cycle phase(s) ($G_0$, $G_1$, S, $G_2$ and M). What emerges both visually and numerically from the data is a distinct reduction of cells in the S and $G_2$/M phases (Figure 5A). This effect is accompanied by an aggregation of treatment population in $G_0$/ $G_1$. Thus, at the 25μM concentration, the gem-analogue administration results in a $G_0$/ $G_1$ arrest.

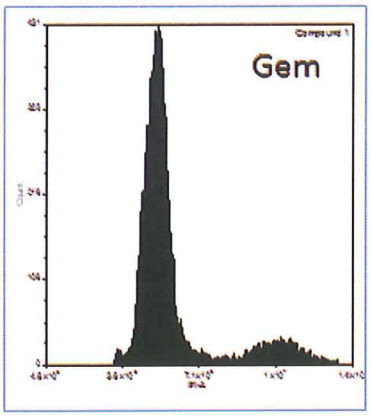
## Gem-Analogues induced arrest is partitioned between $G_0/G_1$ and S-phase arrest at the 2.5 μM concentration

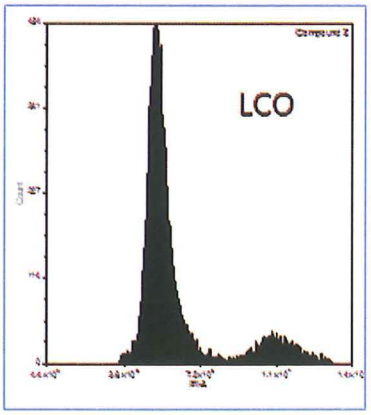
Flow cytometric testing was also executed at the 2.5 μM concentration to provide a complete picture of cell cycle specificity induced by gem-analogue co-incubation. At the lower concentration cell population treated with compound are preferentially accumulated in the $G_0$/ G phase; however, what is also observed is upregulation of cells in the S-phase as well (Figure 5B). Consistent with expectations of gem-analogue mediated interference DNA replicative capacity, is a reduction of cells in $G_2$/M, compared to the control (Figure 5B).

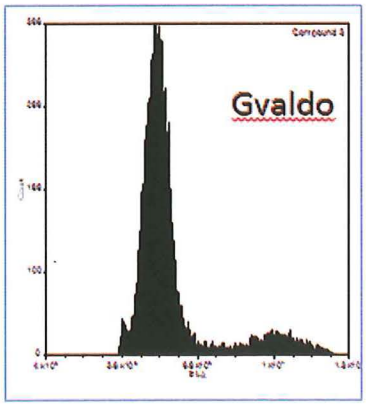
**Figure 5: Cell Cycle Analysis.** Flow Cytometric plots of cell count per cell cycle phase. The x-axis represents absorbance as a proxy for nucleic acid available for propidium iodide staining. The y-axis represents cell count as a quantification of cell density per respective cell cycle phase ($G_0$, $G_1$, S, $G_2$ and M). Data in Figure A represents cell cycle analysis at the 25 μM concentration, while data in Figure B depicts analysis performed at the 2.5μM concentration.

**A. 25uM Concentration**



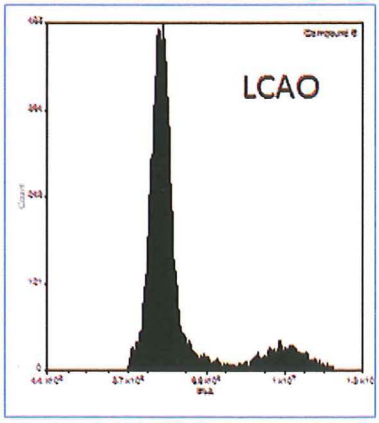



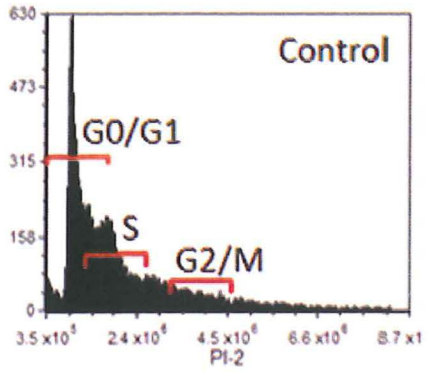



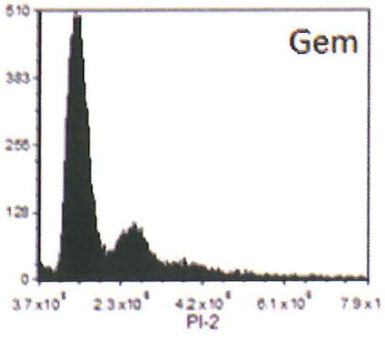

Cell cycle analysis of dFdC analogues.

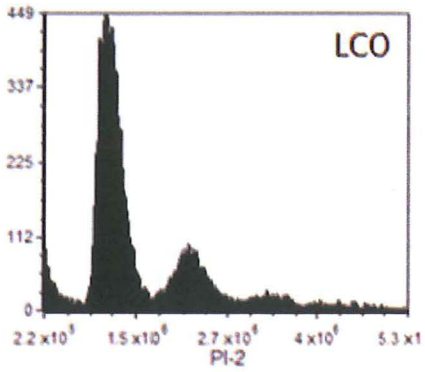
| | % DNA Content | | |
|---|---|---|---|
| Compound | G0/G1 | S | G2/M |
| Control | 51.78 | 33.17 | 15.05 |
| Gem | 78.89 | 9.29 | 11.82 |
| LCO | 80.76 | 8.39 | 10.85 |
| Gvaldo | 81.8 | 8.02 | 10.18 |
| LCAO | 77.64 | 10.34 | 12.03 |

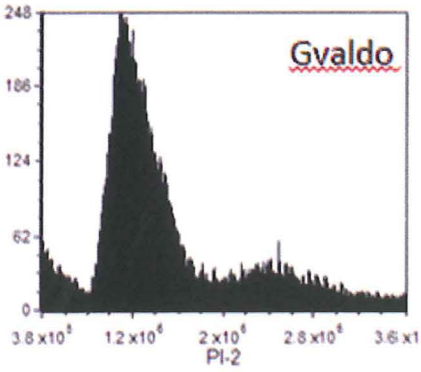


**B. 2.5μM Concentation**



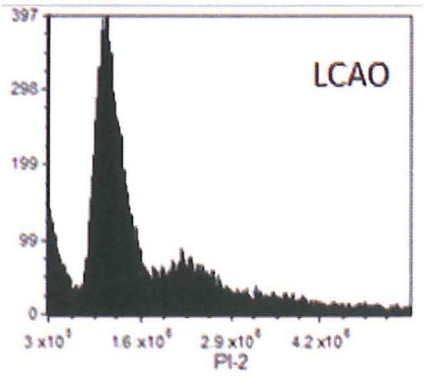




Cell cycle analysis of dFdC analogues.

| | % DNA Content | | |
|---|---|---|---|
| Compound | G0/G1 | S | G2/M |
| Control | 35.91 | 23.27 | 40.82 |
| Gem | 56.87 | 33.67 | 9.47 |
| LCO | 54.9 | 43.06 | 2.06 |
| Gvaldo | 57.25 | 34.28 | 8.28 |
| LCAO | 59.29 | 27.46 | 13.25 |



# Discussion

In the experiments reported here, novel conjugates of Gemcitabine previously synthesized by the Wnuk group were evaluated for anti-cancer activity. First, a preliminary SRB screening was performed; among the compounds tested LCO showed the most noteworthy cytotoxic activity, exhibition $IC_{50}$ values that were 3-fold greater than that rendered with Gemcitabine (Table 1). This result is consistent with a study performed by Stella et al. (2007), where 4-N lipophilic gemcitabine derivatives (4-N steroylgemcitabine) exhibited greater cancer cell toxicity on multiple cell lines compared to gemcitabine. The validation of gem-analogues cell toxicity led to the selection BrdU assays as an appropriate means to illuminate any interplay between cytotoxicity and DNA synthesis inhibition (Cappella et al. 2001). At the 25 μM concentration, all gem-analogues appear to propagate time-independent effects on DNA synthesis (Figure 3B). Figure 3A further illustrates this point in depicting inhibitory trends that are consistent amongst all experimental compounds (Figure 3A). To contrast, at the lower dosage of 2.5 μM, inhibitory effects become more disparate, with LCO inhibition increasing in magnitude up until hour 96 hr (Figure 4A and 4B). To contrast, gemcitabine shows its most potent inhibitory effects at hour 24 (Figure 4B). These results seem to diverge from observations made by others in which homogeneity of replicative inhibition was observed at the lower concentration of 1 μM, whereas differential repression was observed at the higher dosages of 5, 10 and 50 μM (Pignatello et al. 2010). Despite the emergent differences, the data acquired in this experiment speaks toward specific effects occurring at the lower concentration due to differential compound moieties at the 4-N position. At the higher concentrations, it can be hypothesized that the gem-analogues potentiate

saturating effects, such that there appears to be homogeneity amongst the activity profiles and the analogues do not appear appreciably different from that of gemcitabine (Figure 4A).

It is apparent that LCO, shows potency in its anti-cancer effects that put it statistically in a class of its own; this is evident at the 2.5μM concentration, where LCO differs appreciably not only from the control but from that of all other gem-analogues ($\alpha$=.05; $p<.001$; Table 3). Interestingly, LCO shows two waves of inhibitory activity at the lowest dosage (2.5 μM) at 24 hr and 96 hr, while gemcitabine is most effective at 24 hrs (Figure 5A and 5B). These observations suggest that long chain acyl derivations display slow cleavage of the amide linkage as they are hydrolyzed to gemcitabine. Thus, 4-N modified gemcitabine analogues, although possessing inherent inhibitory activities in their intact form, may also exert secondary anti-tumor effects due to intracellular conversion to gemcitabine (Pignatello et al. 2010). Quite possibly, the enzymatic conversion of LCO to gemcitabine may confer lasting *in vitro* effects; future studies should aim to characterize the hydrolytic stability of LCO by evaluating the preservation of the amide bond (Pignatello et al. 2010). Likewise, the inability of LCAO to assert inhibition comparable to that of LCO and Gvaldo counterparts is perhaps due conferred intracellular stability of the 4-N amine linkage that is not susceptible to hydrolytic attack and thus fails to 'free' gemcitabine for additional cytotoxic impact.

Given the chemical structure of LCO that may confer intracellular stability and, the hypothesis that its acyl confers resistance to inactivation and therefore, a bivalent potency is certainly a plausible one (Hajdo et al. 2010). Additionally, LCO reaching enzymatically saturating concentrations due to greater extracellular uptake may also

account for its inhibition. Indeed, the incorporation of lipophilic residues within the gemcitabine skeleton makes the gem-analogues an intrinsically amphipathic molecule, potentially facilitating direct membrane interaction and transfer without the protein membrane carrier (Castelli et al. 2007). Future studies focusing on the intracellular entry and cytoplasmic accumulation of LCO and other successful gemcitabine derivatives may clarify the mechanism of action and active functionalities of these compounds.

The results acquired for flow cytometric analysis provide an interesting platform for speculation. Previously, gemcitabine has been regarded as an S-phase inhibitor by means of its incorporation into DNA and subsequent termination of DNA synthesis (Huang and Plunkett 1995). Accordingly, *in vitro* studies involving gemcitabine showed treated cells adopting S-phase arrest phenotype (Shi et al. 2001). Despite these characterizations, the results yielded in this study show that while S-phase inhibition and may partly describe gem-analogue effects at the lower concentration of 2.5 μM, it is not entirely adequate; in fact, $G_0/ G_1$ accumulation was also observed at the of 2.5 μM compound administration, and $G_0/ G_1$ arrest was solely sufficient to describe the effects propagated at the 25 μM concentration. The dichotomy of effects exhibited at the 2.5 μM versus 25 μM concentrations suggests that at higher dosages, the mechanisms that are upstream of replicative processes involving self-potentiated inhibition (i.e thymidylate synthase, CTP-synthase and ribonucleotide reductase) are critical to the potency of the gem-analogues. Consistent with this observation is the finding that the exhaustion of nucleotide pools via ribonucleotide reductase inhibition has been implicated as an activator of p53-mediated $G_0/ G_1$ arrest (Johnson and Walker 1999). At the lower concentration of gem-analogue administration however, the susceptibility of treated cells

to S-phase arrest suggests that sensitivity may conferred to the DNA synthetic machinery; in literature this is depicted as a destabilization of DNA polymerase leading to the termination of replication (Mini et al. 2006).

Although the interaction of gem-analogues with DNA replicative machinery may account for the results garnered, there may be an additional temporal and spatial context to be investigated; hence, additional studies with molecular targets involved cell cycle regulation may offer a more complete picture of the spatiotemporal dynamics gem-analogue repression (Capella et al. 2001). This approach in synergy with the exploration of gem-analogue impact on oncogenic and cell-death signaling pathways may be best captured by Quantitative-PCR, 'Chip-Seq' or fluorescent imaging studies.

Taken together, the data suggests the potential utility of alkyl and acyl-modified gemcitabine analogues; these compounds offer powerful alternatives with enduring anti-cancer properties through circumventing the short intracellular half-life of gemcitabine itself (Mini et al. 2006). In order for LCO to achieve full clinical utility as an accepted complement or replacement to Gemcitabine, however, additional experimental models must be conducted further to characterize its molecular interactions in an *in vivo* system. Molecular interactions and in the context of DNA damage repair response mechanisms leading to apoptosis also provide intriguing and informative avenues for investigation. To that end, the quantification of mRNA expression of caspase-3, poly-ADP ribose polymerase-1 (PARP-1), p53 signaling via RT-PCR, may represent targets of interest. Mouse models also offer a hopeful means of testing for LCO's *in vivo* effects, as LCO's

efficacy at lower dosage of 2.5μM may mitigate the systemic toxicity sometimes associated with gemcitabine (Couvreur et al. 2006; Koolen et al. 2011). Knowledge of LCO's mechanisms of inhibition also makes it a hopeful candidate for radioligand addition, allowing for the visualization and monitoring of drug-uptake and metabolism and ultimately, its effective translation to clinical practice.

# References

Bender DM, Bao J, Dantzig, AH, et al. 2009. Synthesis, crystallization, and biological evaluation of an orally active prodrug of Gemcitabine. *Journal of Medicinal Chemistry 52*: 6958–6961.

Burris H III, Moore MJ, Anderson J, et al. 1997. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *Journal of Clinical Oncology, 15*:2403-2413.

Cappella P, Tomasoni D, Farerra M, et al. 2001. Cell cycle effects of gemcitabine. *International Journal of Cancer.* 93: 401-408.

Castelli F, Sarpietro MG, Rocco F, Ceruti M, et al. 2007. Interaction of lipophilic gemcitabine prodrugs with biomembrane models studied by Langmuir-Blodgett technique. *Journal of Colloid and Interface Science*. 313: 363-368.

Couvreur P, Stella B, Reddy LH, et al. 2006. Squalenoyl nanomedicines as potential therapeutics. *Nano Letters.* 6: 2544-2548.

Hadjo L, Szulc AB, Klajnert B, et al. 2010. Metabolic limitations of the use of nucleoside analogs in cancer therapy may be overcome by application of nanoparticles as drug carriers: a review. *Drug Development Research.*71: 383-394.

Hertel LW, Boder GB, Kroin JS, et al. 1990. Evaluation of the antitumor activity of gemcitabine (2′,2′-difluoro-2′-deoxycytidine). *Cancer Research.* 50: 4417-4422.

Huang P and Plunkett W, 1995. Fludarabine and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event. *Cancer Chemotherapy and Pharmacology.* 36: 181-188.

Koolen, SLW, Witteveen PO, Jansen RS, et al. 2011. Phase I study of oral gemcitabine prodrug (LY2334737) alone and in combination with erlotinib in patients with advanced solid tumors *Clinical Cancer Research.* 17: 6071-6082.

Johnson SE and Walker CL. 1999. Cyclins and Cell Cycle Checkpoints. *Annual Review of Pharmacology and Toxicoloy.* 39: 295-312.

Laing RE, Walter MA, Campbell DO, et al. 2009. Noninvasive prediction of tumor responses to gemcitabine using positron emission tomography. *Proceedings of the National Academy of Sciences.* 106: 2847-2852.

Mackey JR, Mani RS, Selner M, et al. 1998. Functional Nucleoside Transporters Are Required for Gemcitabine Influx and Manifestation of Toxicity in Cancer Cell Lines. *Cancer Research.* 58: 4349-4357.

Mini E, Nobili S, Caciagli B, et al. 2006. Cellular pharmacology of gemcitabine. *Annals of Oncology.* 17: 7-12.

Pignatello R, Vicari L, Pistara V, Musumeci T, et al. 2010. Synthesis and in vitro cytotoxic activity on human anaplastic thyroid cancer cells of lipoamino acid conjugates of Gemcitabine. *Drug Development Research.* 71: 294-302.

Plunkett W, Huang P, Xu YZ, et al. 1995. Gemcitabine: metabolism, mechanisms of action, and self-potentiation. *Seminars in Oncology.* 22: 3-10.

Pourquier P, Gioffre C, Kohlhagen G, et al. 2002. Gemcitabine (2′,2′-difluoro-2′-deoxycytidine), an antimetabolite that poisons topoisomerase I. Clinical Cancer Research. 8: 2499-2504.

Reddy LH, Renoir JM, Marsaud V, et al. 2008. Anticancer efficacy of squalenoyl gemcitabine nanomedicine on 60 human tumor cell panel and on experimental tumor. *Molecular Pharmaceuticals*. 6:1526-1535.

Shi Z, Azuma A, Sampath D, et al. 2001. S-Phase Arrest by Nucleoside Analogues and Abrogation of Survival without Cell Cycle Progression by 7-Hydroxystaurosporine. *Cancer Research*. 61: 1065-1072.

Stella B, Arpicco S, Rocco F, et al. 2007.Encapsulation of gemcitabine lipophilic derivatives into polycyanoacrylate nanospheres and nanocapsules *International Journal of Pharmaceuticals*. 344: 71-77.