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The Anti-Proliferative Effects of Methotrexate and Novel UCP1162 on Acute Myeloid Leukemia Cell Lines

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Abstract

Cancer cells proliferate at rapid rates due to the aberrant activity of proteins involved in regulating the cell cycle. This characteristic allows mutated cancer cells to spread and metastasize, causing lesions to form throughout the body. Two treatment conditions, one classical antifolate methotrexate (MTX) and non-classical, novel antifolate UCP1162, were tested on a panel of acute myeloid leukemia (AML) cell lines to determine if UCP1162 has higher anti-proliferative activity. High dose MTX is used as a first line chemotherapy in common childhood malignancies such as acute lymphoid leukemia (ALL). Methotrexate is excluded from acute myeloid leukemia (AML) treatments based on clinical trials that suggest AML cells are intrinsically resistant. UCP1162, like MTX, targets dihydrofolate reductase (DHFR). UCP1162 does not appear to be limited by mutations of the reduced folate carrier (RFC) protein or folate polyglutamate synthase (FPGS) since it is not expected to use these mechanisms. UCP1162 is expected to accumulate better intracellularly and therefore, should provide more potent and longer-lasting anti-proliferative effects. Another main focus is how the anti-proliferative activity relates to the inhibition of nucleotide synthesis and DNA methylation. The comparison between these two treatments can be assessed by assays that provide information on the resulting cytotoxic effects. UCP1162 works with a lower EC50 than MTX, which may reduce cytotoxicity and side effects, allowing this treatment to be more tolerable. If UCP1162 is proven to have higher anti-proliferative effects than MTX, then it could play a key role in the arresting of cancer cells and improving patient care.

Introduction

Leukemia and lymphoma

Leukemia and lymphoma are "blood-related" cancers that affect cells of the hematopoietic lineage. These cancers of blood-forming cells differ from each other as well as from solid tumors. Leukemia is cancer of the bone marrow, where the transformed leukemic stem cells largely reside. Abnormal white blood cells are produced which no longer function properly and, in turn, causes weakening of the immune system [1]. This diminished immune response creates an opportunity for the cancer to spread through the human body faster and for other acquired diseases to flourish. The four main groups of leukemia are: acute myelogenous leukemia (AML), chronic, myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL) and chronic lymphoblastic leukemia (CLL). Leukemia is one of the most common childhood cancers, however, age ranges and symptoms have much overlap with lymphoma [2].

When compared with leukemia, lymphoma is any malignancy of the lymphocytes within lymphoid tissue. This includes cells such as white cells that can accumulate in the thymus, bone marrow, lymph nodes and spleen. The two groups of lymphoma are: Hodgkin Lymphoma (HL) and non-Hodgkin lymphoma (NHL) [1]. The most common treatments for these hematologic malignancies are: chemotherapy, targeted therapy, radiation therapy, stem cell transplantation and surgery.

Leukemia occurs when one cell within the bone marrow mutates and becomes malignant and then forms a cluster of cancerous cells and begins to grow and affect other parts of the body through the bloodstream. The number of white blood cells drastically increase and overcrowd the red blood cells and platelets that the body needs for physiological processes. The leukemia cells

migrate to lymphoid tissues and begin to affect the thymus, spleen and lymph nodes. As mentioned, there are four main types to consider when diagnosing leukemia. Acute lymphoblastic leukemia (ALL) is the most common childhood leukemia and has rapid progression that affects the lymph nodes and central nervous system. Acute myeloid leukemia (AML) is one of the most common forms of adult leukemia and also progresses rapidly, affecting myeloid cells within the blood and bone marrow. Chronic lymphoblastic leukemia (CLL) is the other most common form of adult leukemia, but grows much slower than ALL and AML, beginning in the lymphocytes and spreading to the blood. Lastly, chronic myeloid leukemia (CML) begins in the blood-forming cells, such as WBCs, and then spreads to blood from the bone marrow. Each of these types of leukemia can be differentiated through blood tests, such as a complete blood count (CBC), blood smears, bone marrow biopsies, spinal taps and imaging, such as CT, MRI and PET scans. The cause of leukemia has not been identified, but evidence points to genetic and environmental factors causing mutations in the DNA of blood cells [3].

Methotrexate: an anti-rheumatic and anti-neoplastic agent

Methotrexate is typically used to treat rheumatoid arthritis by preventing the anti-inflammatory effects of the immune system. MTX enters the cell through RFC and is then polyglutamated by FGPS. MTX-glutamate inhibits aminoimidazole carboxamide ribonucleotide formyltransferase (AICART) and prevents the conversion of AICAR to 5-formamido imidazole-4-carboxamide ribotide (FAICAR). This causes an overexpression of AICAR in the cell which downstream leads to overexpressed AMP. AMP is transported extracellularly before a phosphate group is lost to form adenosine. Extracellular adenosine signaling prevents excessive inflammation by suppressing proinflammatory cytokines, inhibiting leukocyte entry into tissues

and triggering the production of anti-inflammatory cytokines. MTX has also been shown to have anti-proliferative effects on AML cell lines due to inhibiting DHFR and downstream folate-dependent enzymes thymidine synthase (TS) and aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARDT). This later causes blocking of dTMP, DNA synthesis and ATP production [4]. However, AML cell lines are methotrexate-resistant and therefore, this should not be used as a long-term treatment for patients experiencing this form of leukemia. Novel UCP1162 is more hydrophobic than MTX and easily diffuses through the plasma membrane. This allows UCP1162 to enter the cell without a carrier protein or channel, unlike folates and MTX do through the RFC receptor. UCP1162 also does not require polyglutamation upon entering the cell, so the FPGS enzyme is also not necessary for this treatment mechanism. This allows UCP1162 to overcome the two resistance mechanisms that affect the MTX treatment pathway and is more likely to be effective over long use when compared to MTX.

Antifolates are antimetabolites that target the folate metabolism, playing an important role in treatment of malignant and chronic inflammatory diseases. The first antifolate studied was aminopterin which supported remission from ALL in children. The remissions did not last but this initiated optimism in the field because this disease was now known to be treatable. Aminopterin was soon after replaced by methotrexate (MTX) due to its erratic toxic effects. The mechanism of action under which MTX works was a slow discovery. Chemists were looking for a ubiquitous antimetabolite to disrupt folate metabolism and treat cancer. It was unexpected when the antifolate they designed became today's most well-known treatment for rheumatoid arthritis. Pemetrexed was the next antifolate to be tested clinically which targets thymidylate synthase and was used to treat patients with mesothelioma and non-small cell lung cancer. This was followed closely by pralatrexate, in order to treat cutaneous T-cell lymphoma by blocking

dihydrofolate reductase [5]. However, mechanisms of these antifolates have shown significant resistance and prevents these molecules from being considered an effective chemotherapeutic agent for various cancers.

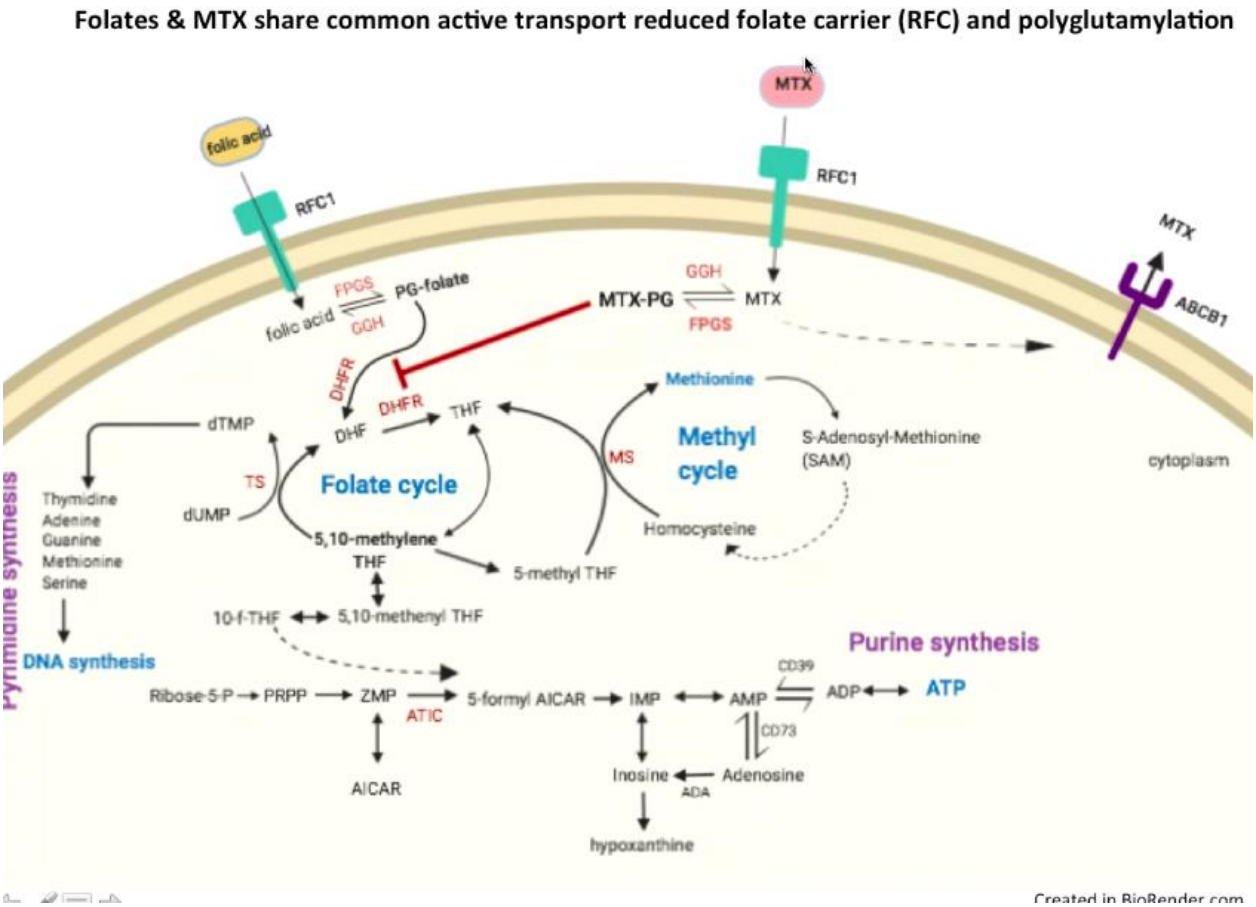

Figure 1. Folic acid and one-carbon metabolism.

The function of folate in one carbon metabolism, and the inhibition of one carbon metabolism by MTX is shown in Figure 1. Folate, also known as Vitamin B9, is not produced within the body so it must be acquired through dietary sources. It enters into the cell through the reduced folate carrier (RFC). Once inside the cell, folate is then reduced to dihydrofolate (DHF) by the enzyme dihydrofolate reductase through the oxidation of NADPH to NADP+. This redox

reaction occurs again to convert DHF to tetrahydrofolate (THF) through the same enzyme. The enzyme serine hydroxyl methyl transferase (SHMT) converts THF to N5, N10-methylene THF. This enzyme requires a serine that will donate a hydroxyl and methyl group, resulting in a glycine amino acid, and also pyridoxal phosphate, a cofactor and derivative of Vitamin B6. SHMT is a reversible enzyme therefore if glycine is readily available, then excess N5, N10-methylene THF can be converted back to THF until needed by the cell. N5, N10-methylene THF can now be recycled back to DHF by the enzyme thymidylate synthase. This enzyme is critical because it converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) through the reduction of an FADH2 to FAD and dTMP is utilized for DNA synthesis. However, N5, N10-methylene THF in excess can also be acted on by the enzyme methyl tetrahydrofolate dehydrogenase (MTHFD) through the oxidation of NADPH to NADP+ resulting in N5, N10-methenyl THF. This molecule is then converted to N10-Formyl THF by methenyl tetrahydrofolate cyclohydrolase (MTHFC) which requires a water molecule to function. The final N10-Formyl THF can then be utilized for purine synthesis and therefore, DNA replication [6]. When methotrexate is administered, it works as an inhibitor of DHFR, which prevents folate from becoming THF. This prevents all of the later pathways in the folate cycle and therefore, disrupts DNA synthesis and replication.

Advantages of UCP1162 as an anti-folate

Methotrexate can enter the cell the same way as folate, through its RFC receptors. It can also diffuse intracellularly by other active transporters, such as folate receptor alpha and proton coupled folate transporter, that work based on the cell's pH gradient. Once in the cytoplasm, the folylpolyglutamate synthase (FPGS) enzyme converts MTX to methotrexate polyglutamate

(MTX-PG). This is the activated form of methotrexate which can then go on to inhibit many cellular processes, such as purine synthesis, dTMP availability required for DNA synthesis, and all later transmethylation reactions by blocking the activity of the DHFR enzyme. Methotrexate has been used for decades as a therapeutic agent for multiple cancer types, however, its resistance mechanism proves it to be a less efficient treatment. The mechanism of resistance results in decreased cellular uptake of MTX, increased intracellular levels of the DHFR enzyme, down-regulation of RFC genes and an inconsistent rate of MTX polyglutamation [4]. Therefore, a new therapeutic treatment without resistance is a desired and novel UCP1162 has shown promising results as a replacement to MTX. UCP1162 overcomes resistance mechanism by passively diffusing across the membrane due to its high hydrophobicity, avoiding the RFC receptor pathway. It is also likely that it doesn't require poly-glutamation because it does not have a glutamate residue.

Molecular defects and treatments for AML

Some cases of AML have been shown to arise from rearrangements of the MLL gene. The most common MLL translocation partner proteins (AF4, AF9, ENL, and ELL) recruit the transcriptional activation complex, p-TEFb (cyclin T1 and CDK9), and initiates transcriptional elongation through polymerase II (Pol II). These MLL fusion proteins also interact with the histone H3K79 methyltransferase DOT1L which causes abnormal transcriptional activation of MLL target genes [7]. MLL translocation in AML cells causes expression of genes associated with stem cell-like phenotypes, which drives the aggressive growth of cancer cells. Therefore, UCP1162 is likely to suppress the aberrant methylation of MLL-target genes in AML cells due

to the reduction of the methyl donor pool [8]. This reduction in methylation may irreversibly differentiate the AML cells in order to stop their growth.

Current treatments for AML include: chemotherapy, targeted therapy, radiation therapy and/or stem cell transplants. Intensive chemotherapy is the most common treatment method for AML and is administered typically intravenously in order to enter the bloodstream directly. This treatment plan can be divided into three phases consisting of induction therapy, post-remission therapy and consolidation therapy. The adverse effects of chemotherapy can be devastating due to healthy cells and tissues also being affected. Targeted therapy is more complex and isolates the specific genes and proteins correlated with leukemia, in order to spare healthy tissue. An issue with this treatment is that not all cancers have a similar target gene or protein and it has been proven that many cancer cell lines affect a large number of these factors. AML affects the IDH1, IDH2 and FLT3 genes of leukocytes, however, it is believed that other genes are also being affected. Lastly, is radiation therapy, in which external beam radiation is used to stop the growth of cancer cells. The side effects are less debilitating than chemotherapy, but this treatment is typically only used when cancerous cells travel through the bloodstream and make their way to the brain [9]. There is currently no treatment for AML that is both effective and well tolerated.

Materials & Methods

Cell growth and treatment

Four different cell lines obtained from American Type Culture Collection (ATCC) were grown in specific media solutions. Media, PBS and TrypLE were purchased from ThermoFisher.

HCT116, a human colon cancer cell line, was grown in McCoy's media supplemented to a final concentration of 10% FBS, 1% anti anti and 1% non-essential amino acid. HCT116 is an epithelial cell line with adherent character. Therefore, a sterile PBS wash and TrypLE are required in order to pass these cells. CCRF-CEM, is a human T lymphoblast cell line that is grown in suspension in RPMI 1640 media supplemented to a final concentration of 10% FBS, 1% anti anti and 1% non-essential amino acid. These cells are acute lymphoblastic leukemia (ALL) cells. These cell lines were tested in order to see if UCP1162 is generally anti-proliferative to other types of cancer. The two cell lines categorized under acute myeloid leukemia (AML) and grown in suspension were THP-1 and MV-4-II. MV-4-II lymphoblasts were grown in RPMI 1640 media supplemented to a final concentration of 10% FBS, 1% anti anti and 1% non-essential amino acid. Lastly, THP-1 monocytes were grown in RPMI 1640 media supplemented to a final concentration of 10% FBS, 0.05 mM 2mercaptoethanol, 1% anti anti and 1% non-essential amino acid.

These cell lines were plated in T25 or T75 cell culture flasks. Each cell line was kept incubated at 37^{o}C and 5% CO_2 incubator. Cells were seeded at 2-3x10^5 cells/mL and grown up to 2-3X10^6 cells/mL. This required the flasks to be passed every 3-4 days. Cell counting was performed by a Countess II automated cell counter (ThermoFisher). The appropriate media in the fridge was warmed to 37^{o}C in the incubator for approximately 20 minutes before any procedure. The culture hood was turned on and sterilized with 70% ethanol before removing the flasks from the incubator and placing them in the hood. In the flasks, 4.5 milliliters of the appropriate fresh media were added, following with 0.5 milliliter of the cell suspension. These cells were passed every 3-4 days or when the cells looked confluent under a microscope.

When passing the cells lines, the old media was removed with a serological pipette and discarded into the liquid waste container. For the HCT116 cells, after all the media was removed, the flask was first washed with sterile PBS and then TrypLE was added to the flask. The TrypLE was moved around the flask mechanically for 1-2 minutes or until the bottom of the flask became clear. This step was required for the HCT116 cell line because they are adherent cells. To this, 4 mL of fresh media were added to the flask. The other three cell lines were grown in suspension so PBS wash and TrypLE were not used. Instead, the suspension cells were passed by adding suspension cells into a fresh, prewarmed media directly. The flasks were then returned to the incubator and the hood was sterilized after use.

Cell viability and ATP quantification

An ATP assay (ATP Determination Kit: A22066) and MTS viability were run in parallel to confirm that the given concentrations of MTX and UCP1162 were able to reduce the viability (anti-proliferative). The reduction of viability is directly proportional to the reduction of ATP levels. This reduction also confirms that the tested compound is functioning through the folate pathway and therefore, has antifolate action. For the ATP assay, cells were treated in 24-well plates overnight. The Mammalian Protein Extraction Reagent (MPER) extraction protocol was followed. Ninety microliters of the lysed cell extracts were added into the wells of the black 96-well plate. Ten microliters of ATP reaction solution were added on top of the sample extracts. Each well was mixed thoroughly with a multichannel pipette and then, the luminescence was read immediately using a plate reader. This bioluminescence assay quantifies ATP production through the use of firefly luciferase and its substrate D-luciferin. The resulting luminescence values were normalized to either nucleic acid or protein concentration of the samples measured

by a nanodrop instrument. A standard curve is created of luminescence versus ATP produced (picomoles) to find the concentration at which the greatest amount of ATP is produced.

The MTS assay is a colorimetric method used to determine the number of viable cells in cell culture. The assay is effective through dye reduction that provides information about cytotoxicity/anti-proliferative activity. The MTS reagent in the assay is reduced by the viable cells in the sample, which produces a colorful product that can be quantified at 490nm in a plate reader. For the MTS assay, a 96-well Falcon clear plate was used. A ten microliter sample of the flask was used to find the cell density. The target seeding density was 5x10^5 cells/mL. Using the formula, C1V1=C2V2, the appropriate volume was calculated and taken from the flask of cells and added to excess media in an aliquot tube in order to yield an optimal cell number in each well. Forty-eight wells were filled with one hundred microliters of this mixture in the aliquoted tube, one well with 100 microliters of the media that cell line was grown in and the rest with 100 microliters of distilled water. There were seven concentrations tested for each treatment and dmso was used as a control. Each concentration for each treatment and the controls were performed in triplets. The final concentrations of MTX were: 2.5 nM, 5 nM, 10 nM, 20 nM, 40 nM, 80 nM and 160 nM. The final concentrations of UCP1162 were: 0.3375 nM, 0.675 nM 1.25 nM, 2.5 nM, 5 nM, 10 nM and 20 nM. No treatment or dmso was added to the media control well. This plate was treated for approximately 48 hours before proceeding.

The CellTiter 96 AQ-One Solution Reagent was removed from the -20^{o}C refrigerator and thawed for 15 minutes in a 37^{o}C incubator. A multi-channel pipette was used to add 20 microliters of the CellTiter Solution to each of the wells, including the well that just contained culture medium. This is a colorimetric time-dependent assay so the multi-channel pipette was

used to produce uniform volumes in a shorter amount of time than an individual micropipette. The plate went back into the incubator for 1.5 hours or until the wells began to turn dark yellow in color. The absorbance was recorded at 490nm using a plate reader and the A490 values are directly proportional to the number of viable cells. The data was averaged for each treatment concentration and normalized to the media control before being graphed. From this data, we can determine the EC50 value of MTX and UCP1162 and therefore, compare the effectiveness of both using GraphPrism dose response curve fitting.

Cell cycle analysis

Flow cytometry is a technique used to express and determine both physical and chemical characteristics present within a population of cells. This method is one of the ways to do cell cycle analysis through the quantification of DNA content. DNA is stained by DNA binding dyes and they bind proportionally to the amount of DNA found in the cell and therefore fluoresce more brightly. This allows us to quantify the numbers and percentages of cells in the G1, S or G2/M phase. It is expected that the G2/M phase should fluoresce more brightly, followed by the S phase, since more DNA is found at these phases than the G1 phase. This enables us to characterize these cancer cell lines further and determine where each treatment is causing the cells to arrest their growth within the cell cycle and therefore slow tumor progression.

Results

Cell viability EC50 determination

The ATP response after a 24-hour treatment of MTX/UCP1162 can be seen in Figures 2-3. The ATP assay and MTS viability were run in parallel to confirm that the given concentrations

of MTX and UCP1162 were anti-proliferative and effective in resulting in a loss of viable cells. The cell viability per A490 nm versus the concentration of MTX/UCP1162 treatment can be seen in Figures 4-7. Using GraphPrism, these dose-response curves were generated and fitted using "dose-response-stimulation." The EC50 of each treatment was calculated and reported. Each graph represents the average cell viability in triplets from two separate MTS assay runs, except for Figure 7 in which MTS assay in triplets were run once. For Figure 4, when tested on the HCT116 cell line, the EC50 of MTX was 24.2nM while the EC50 for UCP1162 was 3.3nM. For Figure 5, when tested on the CCRF-CEM cell line, the EC50 of MTX was 5.6nM while the EC50 for UCP1162 was 1.2nM. For Figure 6, when tested on the THP-1 cell line, the EC50 of MTX was 21.9nM while the EC50 for UCP1162 was 1.6nM. For Figure 7, when tested on the MV-4-II cell line, the EC50 of MTX was 5.15nM while the EC50 for UCP1162 was 0.78nM. For all cell lines tested, including the adherent human colon cancer cell line and suspension ALL and AML cell lines, the EC50 for UCP1162 was significantly lower than that of MTX.

ATP assay

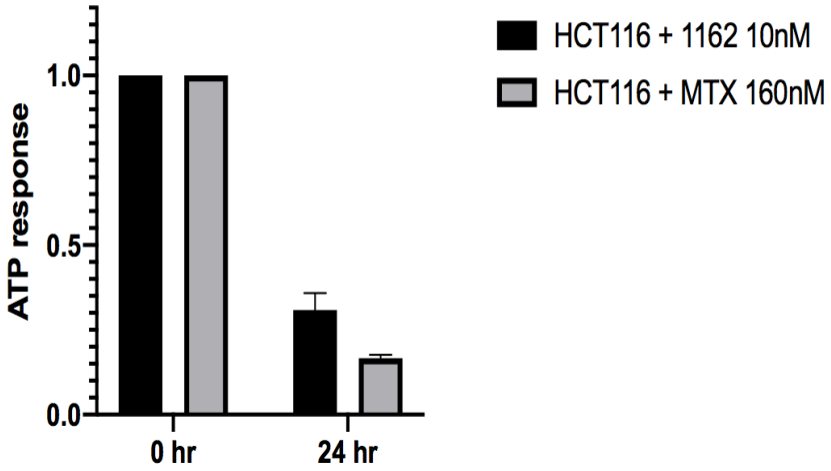

Figure 2. ATP assay showing decreased intracellular ATP levels for HCT116 cells when treated with MTX/UCP1162 for ~24 hours.

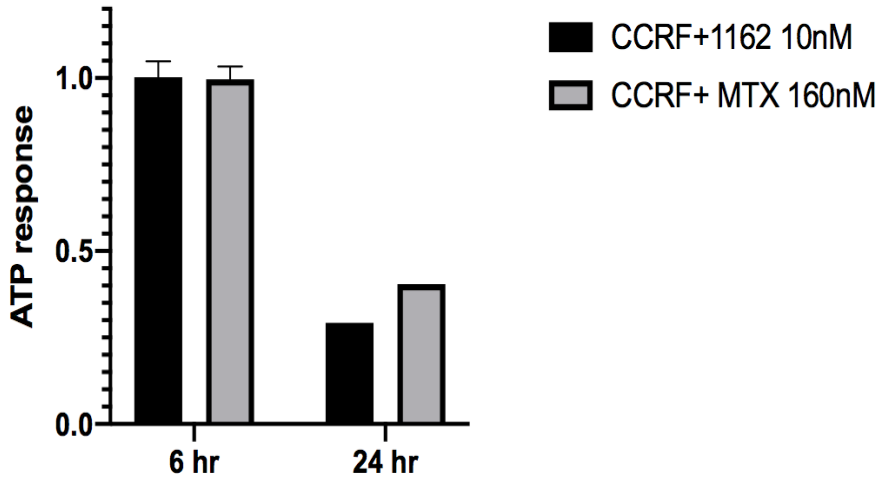

Figure 3. ATP assay showing decreased intracellular ATP levels for CCRF-CEM cells when treated with MTX/UCP1162 for ~24 hours.

MTS viability

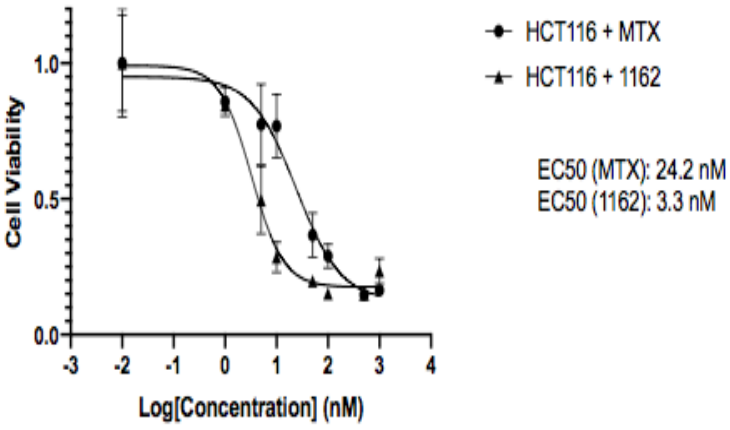

Figure 4. MTS cell viability assay performed on HCT116 cells treated with serial concentrations of MTX or UCP1162 for ~48 hours.

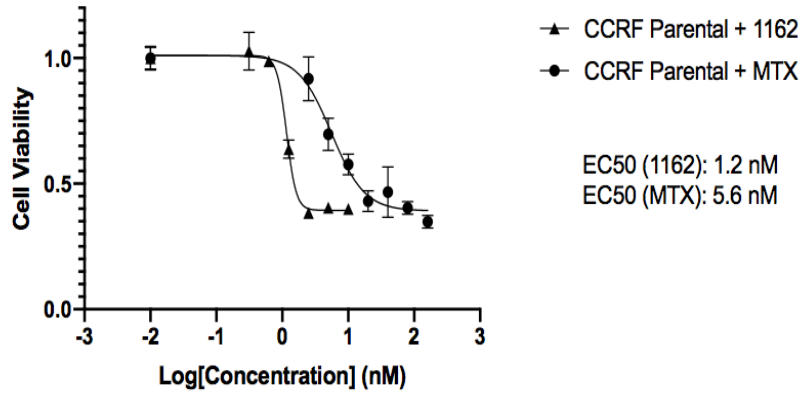

Figure 5. MTS cell viability assay performed on CCRF-CEM cells treated with serial concentrations of MTX or UCP1162 for ~48 hours.

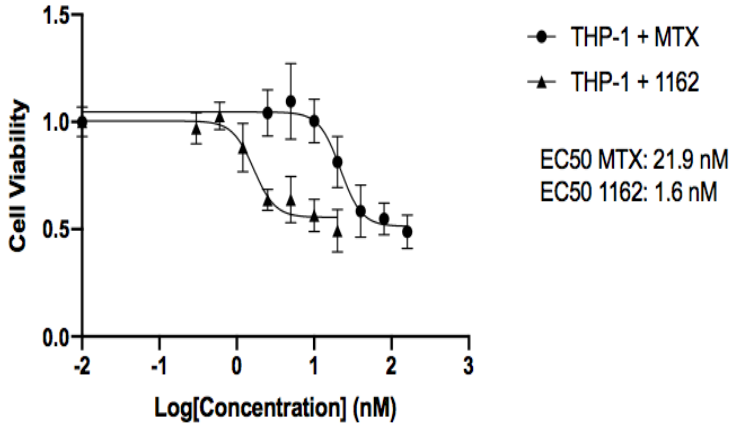

Figure 6. MTS cell viability assay performed on THP-1 cells treated with serial concentrations of MTX or UCP1162 for ~48 hours.

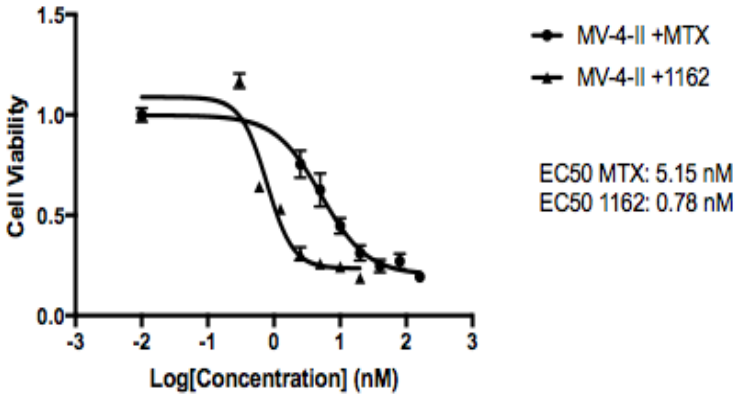

Figure 7. MTS cell viability assay performed on MV-4-II cells treated with serial concentrations of MTX or UCP1162 for ~48 hours.

Cell cycle analysis

The fluorescence measured as a result of DNA content is shown in Figures 8-10. These graphs were analyzed by a flow cytometry software in order to calculate the percentage of viable cells in each phase of the cell cycle. Flow analysis was not performed for the MV-4-II cell line. In Figure 8, the untreated HCT116 cell line shows 47.5% of viable cells in the G1 phase, 32.9% in the S phase and 16.5% in the G2 phase. When treated with 160nM of MTX, the HCT116 cells expressed 51.0% of viable cells in the G1 phase, 42.2% in the S phase and 3.97% in the G2 phase. When treated with 20nM of UCP1162, the HCT116 cells expressed 47.1% of viable cells in the G1 phase, 44.4% in the S phase and 6.0% in the G2 phase. In Figure 9, the untreated CCRF-CEM cell line shows 17% of viable cells in the G1 phase, 11% in the S phase and 38% in the G2 phase. When treated with 160nM of MTX, the CCRF-CEM cells expressed 23% of viable cells in the G1 phase, 11% in the S phase and 41% in the G2 phase. When treated with 20nM of UCP1162, the CCRF-CEM cells expressed 20% of viable cells in the G1 phase, 11% in the S

phase and 42% in the G2 phase. In Figure 10, the untreated THP-1 cell line shows 65% of viable cells in the G1 phase, 17% in the S phase and 17% in the G2 phase. When treated with 160nM of MTX, the THP-1 cells expressed 65% of viable cells in the G1 phase, 24% in the S phase and 10% in the G2 phase. When treated with 20nM of UCP1162, the THP-1 cells expressed 64% of viable cells in the G1 phase, 27% in the S phase and 9% in the G2 phase. Overall, these data point to an S-phase arrest by both UCP1162 and MTX for HCT116 and THP-1 cells. Data for the CCRF cells is more difficult to interpret, as this cell line appeared to be composed of both diploid and stable tetraploid lines.

Flow cytometry

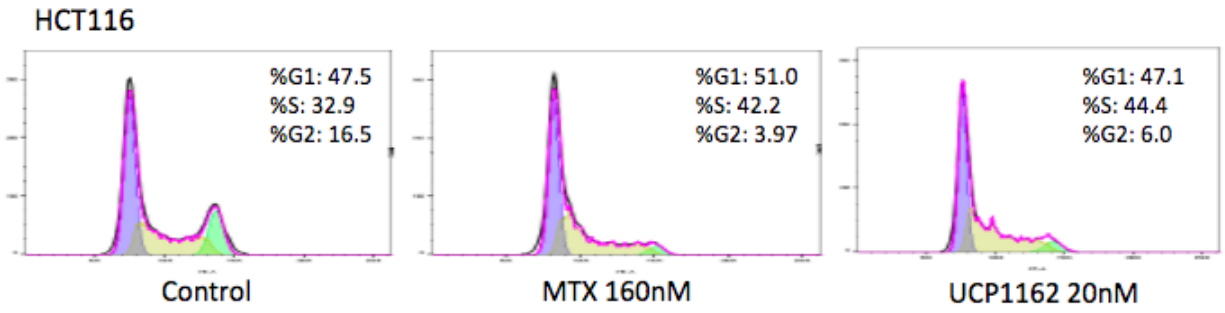

Figure 8. Cell cycle analysis by PI staining followed by flow cytometry run. The experiment performed on HCT116 cells treated with a single concentration of MTX or UCP1162 for ~ 24 hours.

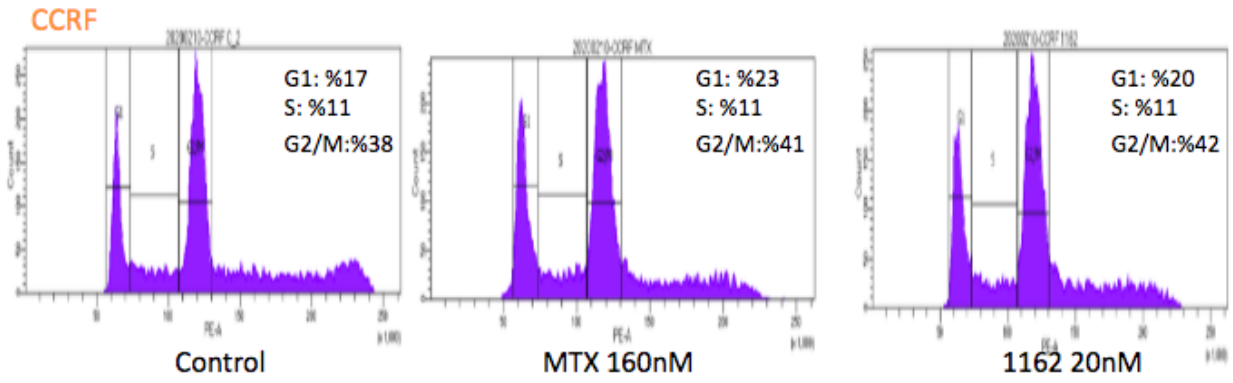

Figure 9. Cell cycle analysis by PI staining followed by flow cytometry run. The experiment performed on CCRF-CEM cells treated with a single concentration of MTX or UCP1162 for ~ 24 hours.

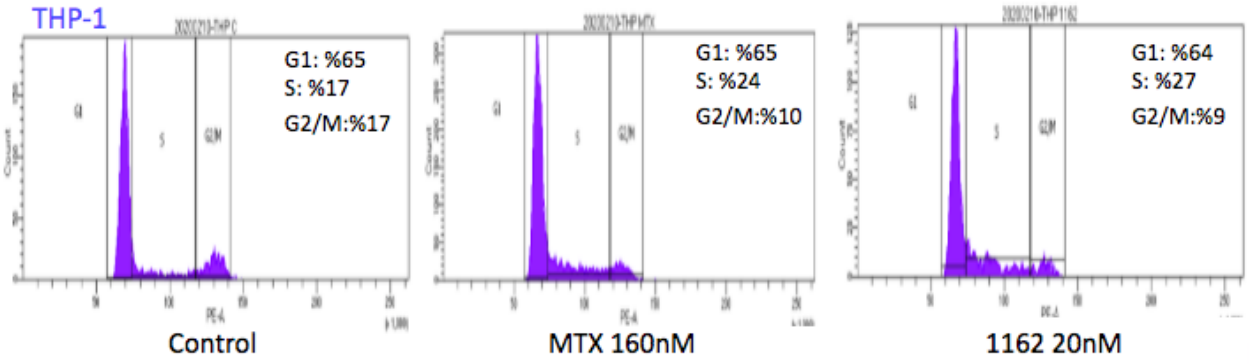

Figure 10. Cell cycle analysis by PI staining followed by flow cytometry run. The experiment performed on THP-1 cells treated with a single concentration of MTX or UCP1162 for ~ 24 hours.

Discussion

MTX has been used for decades as an antineoplastic and anti-inflammatory agent. However, resistance to MTX is an important concern that limits its clinical use. In the studies described here, MTX is compared to a newly developed antifolate, UCP1162, focusing on cell

proliferation and ATP synthesis. The ATP assay and MTS viability assay were run in parallel to confirm that the given concentrations reduced cellular viability (anti-proliferative) concomitant with the reduction of ATP levels. The reduction of viability is directly proportional to the reduction of ATP levels. Both UCP1162 and MTX decreased cellular ATP levels with similar dose-response profiles, consistent with an antifolate activity of UCP1162.

The EC50 is the dose required to reach 50% of the drug's maximum effect. From a pharmacological and clinical perspective, it is more desirable to administer a drug with a lower EC50 since a more potent compound is less likely to cause undesirable off-target side-effects. For all cell lines tested, the EC50 for UCP1162 was significantly lower than MTX, indicating UCP1162 is a stronger anti-proliferative. The lower EC50 should allow a lower dose of treatment for UCP1162 relative to MTX. This data indicates that UCP1162 may be a more desirable treatment than MTX to treat various forms of leukemia.

Flow cytometry was used to find the percentage of viable cells arrested in each phase of the cell cycle by MTX and UCP1162. With the exception of the CCRF-CEM cell line, both UCP1162 and MTX treatment caused cells to accumulate in S-phase. This is consistent with the depletion of dNTP pools resulting from inhibited nucleotide synthesis, resulting in cells running out of nucleotides causing an S-phase blockage to occur. This shows that UCP1162 and MTX work through a similar growth inhibition mechanism, and provides additional evidence that UCP1162 works through an antifolate mechanism.

UCP1162 follows a similar mechanism to MTX by inhibiting DHFR and downstream prevention of DNA replication. However, UCP1162 is expected to not be affected by the many resistance mechanisms that limit MTX due to not being involved in FPGS modifications. It is also likely to have fewer side effects due to a lower EC50, which also points to it being more

effective. Moving forward, the focus of UCP1162 development is to provide therapeutic relief to patients suffering with AML and possibly other forms of cancer. The next step is to determine the safe maximum UCP1162 concentration and stability in vivo. Following this we would like to see whether UCP1162 can drive MLL mutant AML cells to terminally differentiate with UCP1162 since aberrant histone methylation is thought to be critical for maintaining MLL mutant leukemias in a stem cell state. Another direction might be use of a DNA methylation antibody with a confocal microscope to determine how UCP1162 affects DNA methylation. Novel UCP1162 has proven to be a promising therapeutic with distinct chemical properties that may ultimately serve as a useful tool for patient treatment.

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This thesis and the research behind it would not have been possible without the support of my principle investigator, Dr. Charles Giardina, and graduate teaching assistant, Didem Ozcan. I would like to thank Dr. Giardina for allowing me to continue to expand my knowledge of cancer research under his supervision and project idea. I would also like to thank Didem Ozcan for providing me with the ATP assays, MTS assays and flow analysis' performed on the HCT116 and CCRF-CEM cells (shown in Figures 2-5 & 8-9) as well as the folate one-carbon metabolism diagram (shown in Figure 1). Dr. Giardina and Didem have each spent countless time mentoring me throughout my undergraduate laboratory career and have provided me the confidence necessary to pursue other research projects in my future.

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