Continuation/Research Progression Projects Form (7)

Required for projects that are a continuation/progression in the same field of study as a previous project.
This form must be accompanied by the previous year's abstract and Research Plan/Project Summary.

Student's Name(s) Margarita Bogdanova-Shapkina

To be completed by Student Researcher: List all components of the current project that make it new and different from previous research. The information must be on the form; use an additional form for previous year and earlier projects.

Components	Current Research Project	Previous Research Project: Year: 18-19
1. Title	Endogenous Roles of NT5C2 Identified with Genetic Screening and Treatment Implications	Mechanisms and Effects of NT5C2 inhibition in Acute Lymphoblastic Leukemia
2. Change in goal/ purpose/objective	Identify full endogenous role of NT5C2, including any pathways it affects besides the purine biosynthesis process, and investigate treatment implications	Identify and investigate sources of aberrant NT5C2 activation other than NT5C2 gene mutation (such as post-translational modifications) and mechanisms by which pharmaceutical inhibition of NT5C2 can be achieved.
3. Changes in methodology	Performed whole-genome knockout mizoribine screening to identify impacted pathways, validating this screen, as well as whole-genome KO screening with pharmaceutical inhibition of NT5C2.	Performed proximity labelling of NT5C2 to identify its kinase(s); performed MTT and malachite green assays on different NT5C2 variants to identify effects
4. Variable studied	Gene expression of cells resistant and sesnsitive to mizoribine and NT5C2 inhbitor during screening	Cell viability of various NT5C2 variants in response to pharmaceutical inhibition; proteins responsive to proximity labelling
5. Additional changes		

Attached are:

☑ Abstract and Research Plan/Project Summary, Year 18-19

I hereby certify that the above information is correct and that the current year Abstract & Certification and project display board properly reflect work done only in the current year.

Margarita Bogdanova-Shapkina — Student's Printed Name(s)

M. Bogdanova-Shapkina — Signature

01/20/20 — Date of Signature (mm/dd/yy)

Abstract 18-19

Mechanisms and Effects of NT5C2 Inhibition in Acute Lymphoblastic Leukemia

Aberrant over-expression of *NT5C2* has been linked to chemoresistance in relapsed acute lymphoblastic leukemia. Multiple gain-of-function *NT5C2* mutations, each causing one of three distinct conformational changes, have been identified, but account for only some cases of *NT5C2* over-expression. Preliminary data supports HTP as a potential NT5C2 inhibitor. The purpose was threefold: to determine the efficacy and mechanism of action of HTP and its analogs, to explore how post-translational modifications regulate NT5C2 activity, and to establish the practicality of NT5C2 inhibition in treatment. HTP activity was analyzed using MTT and malachite green assays in cell lines representing both B- and T-cell ALL and a majority of naturally-occurring activating NT5C2 mutations. Activating phosphorylation of NT5C2 was confirmed through phosphomimetics, then kinase identification was attempted through library-based motif analysis and BioID screening. NT5C2 KO was performed using CRISPR-Cas9 editing. No HTP analog was cytotoxic at treatment levels, but reduced NT5C2 activity was detected - suggesting a need for combination therapy in treatment - except in cells with the L375F mutation. L375F negates the need for allosteric NT5C2 activation, suggesting that HTP binds to the allosteric site. Activating phosphorylation was confirmed, and found to be likely cell-cycle dependent with a MAPS-related kinase. An NT5C2 KO T-ALL model was generated without adverse effects, supporting the need for combination therapy and promoting NT5C2 inhibition as a safe therapeutic option. The discovery of the mechanism of an available NT5C2 inhibitor, a novel form of NT5C2 activation, and the feasibility of NT5C2 inhibition has important implications on ALL treatment.

Bahojwani, D., Yang, J. J., & Pui, C. H. (2015). Biology of childhood acute lymphoblastic leukemia. *Pediatric Clinics,* 62(1), 47-60.

Belver, L., & Ferrando, A. (2016). The genetics and mechanisms of T cell acute lymphoblastic leukaemia. Nature Reviews Cancer, 16(8), 494.

Bhatia, S., Landier, W., Hageman, L., Kim, H., Chen, Y., Crews, K. R., & Maloney, K. W. (2014). 6MP adherence in a multiracial cohort of children with acute lymphoblastic leukemia: a Children's Oncology Group study. *Blood*, *124*(15), 2345-2353.

Chu, Q., Rathore, A., Diedrich, J. K., Donaldson, C. J., Yates III, J. R., & Saghatelian, A. (2017). Identification of Microprotein–Protein Interactions via APEX Tagging. *Biochemistry*, *56*(26), 3299-3306.

Dephoure, N., Zhou, C., Villén, J., Beausoleil, S. A., Bakalarski, C. E., Elledge, S. J., & Gygi, S. P. (2008). A quantitative atlas of Mitotic phosphorylation. *Proceedings of the National Academy of Sciences*, *105*(31), 10762-10767.

Dieck, C. (2018a). [High-throughput screening of various compound libraries for NT5C2 inhibitor]. Unpublished raw data.

Dieck, C. L., Tzoneva, G., Forouhar, F., Carpenter, Z., Ambesi-Impiombato, A., Sánchez-Martín, M., & Ferrando, A. A. (2018). Structure and mechanisms of NT5C2 mutations driving thiopurine resistance in relapsed lymphoblastic leukemia. Cancer cell, 34(1), 136-147.

Horiuchi, D., Collins, C. A., Bhat, P., Barkus, R. V., DiAntonio, A., & Saxton, W. M. (2007). Control of a kinesin-cargo linkage mechanism by JNK pathway kinases. *Current Biology*, *17*(15), 1313-1317.

Hunger, P.; Mullighan, G. (2015-10-14). "Acute Lymphoblastic Leukemia in Children". *New England Journal of Medicine. 373*(16): 1541–1552. doi:10.1056/nejmra1400972. PMID 26465987.

Kim, D. I., Jensen, S. C., Noble, K. A., KC, B., Roux, K. H., Motamedchaboki, K., & Roux, K. J. (2016). An improved smaller biotin ligase for BioID proximity labeling. *Molecular biology of the cell*, *27*(8), 1188-1196.

Tzoneva, G., Perez-Garcia, A., Carpenter, Z., Khiabanian, H., Tosello, V., Allegretta, M., ... & Paganin, M. (2013). Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL. *Nature medicine,* 19(3),368.

Tzoneva, G., Dieck, C. L., Oshima, K., Ambesi-Impiombato, A., Sánchez-Martín, M., Madubata, C. J., & Sulis, M. L. (2018). Clonal evolution mechanisms in NT5C2 mutant-relapsed acute lymphoblastic leukaemia. *Nature*, *553*(7689), 511.

Margarita Bogdanova-Shapkina 2018-2019

A. RATIONALE

Acute Lymphoblastic Leukemia (ALL), the most common cancer found in pediatric patients, is an aggressive tumor characterized by infiltration of the bone marrow by malignant T- or B-cell progenitors. 20% of pediatric and over 50% of adult ALL patients experience disease relapse despite intensive chemotherapy. Relapsed ALL has demonstrated a high degree of resistance to chemotherapy drugs, primarily purine analog 6-MP, during the course of renewed treatment. Activation mutations in *NT5C2*, a gene encoding cytosolic purine 5′ -nucleotidase, have been identified as a driver of chemotherapy resistance in T-ALL as NT5C2's aberrantly increased nucleotidase activity inactivates 6-MP. In addition, some NT5C2 mutants exhibit independence from the allosteric ATP signalling that regulates wild type NT5C2, and as such become constitutively active.

Preliminary drug discovery using high-throughput screening of synthesized-compound libraries has identified a potential inhibitor of NT5C2. This compound has poor efficacy compared to current standards of treatment, is insoluble in water, and remains generally unsuited for medical use, requiring the creation of improved analogues. It also remains untested in different variants of ALL as well as in conjunction with primary ALL treatments (like 6MP) to mirror the combination therapy effective in patients.

In addition, activating mutations of the *NT5C2* gene are found in less than 25% of T-cell ALL and 13% of B-cell ALL patients. As such, wild type NT5C2 nucleotidase may be activated via alternative mechanisms and remain a driver of chemoresistance in ALL. The allosteric activation of wild-type NT5C2 at through high ATP levels proportionally increased nucleotidase activity, indicating that NT5C2 activity does not plateau but continues to rise with increased signalling. A novel atlas of phosphorylated proteins compiled through mass spectrometry of confirmed that NT5C2 undergoes phosphorylation.

The effect of wild type NT5C2 inhibition in vivo, both in terms of cell viability and 6-MP resistance, must be determined to ensure clinical relevance.

B. HYPOTHESIS (ES), RESEARCH QUESTION(S), ENGINEERING GOAL(S), and/or EXPECTED OUTCOMES.

1. Analyze wild-type NT5C2 phosphorylation, including its effects on NT5C2 activity and the kinase involved
2. Identify allosteric or active site inhibitor of NT5C2 and determine efficacy under various conditions and for multiple variants of ALL
3. Ascertain effect of NT5C2 WT inhibition in ALL

C. RESEARCH METHODS

Describe in detail your RESEARCH METHODS:

- **Procedures:** Detail all procedures and experimental design including methods for data collection. Describe only your project. Do not include work done by mentor or others.

Please note: the following procedures may be expanded upon in later stages of experimentation depending on the results of earlier procedures.

1. PTM/kinase identification Procedures
    a. preliminary experimentation with phosphomimetics pointed to S502 as the likely site of phosphorylation and suggested that phosphorylation of NT5C2 drives 6-MP resistance.
    b. Performed kinase motif analysis to identify likely kinase candidates
    c. Screen for protein-protein interaction using BioID2 protocol
        i. Create lentiviral expression plasmids for NT5C2-biotin ligase-myc (experimental) and biotin ligase-myc (empty vector control) protein complexes
        ii. Transfect plasmids into 293T packaging cells
            1. Add 3 ug DNA of interest (a mixture for each NT5C2/EV complex expression plasmids), 2.7 ug GagPol combined lentiviral packaging plasmid, .3ug VSV-G lentiviral envelope plasmid, 18uL jetPEI transfection reagent, and 250 uL NaCl to 24 well plate well. Suspend jetPEI in an additional 250 uL NaCl prior to adding it to the DNA solution.
            2. Vortex 30 seconds and incubate at 37° for 30 minutes
            3. Drip each of the solutions (one each for NT5C2 and EV complexes) onto individual plate of >1,000,000 293T cells
            4. Incubate for 72 hours at 37°
        iii. Infect human T-ALL cells with produced lentiviral vector
            1. Prepare 2 24 well plates (for NT5C2 and EV complexes), adding 1 x 10^6 target cells per well
            2. Centrifuge contents of packaging cell plates (500 x g for 5 minutes).
            3. Extract supernatant containing virus, and filter to remove any remaining cells
            4. Add 1ug/mL polybrene infection reagent to viral supernatant, for final concentration of 8ug/mL
            5. Add 500 viral supernatant to each well for final 1:1 cell:virus ratio by volume
            6. Centrifuge cells with viral vector for 90 minutes at 30°C and 1000 x g
            7. Incubate cells 4-6 hours
            8. Add 1 mL RPMI
            9. Incubate 4-6 hours, then change media (removing viral vector remnants)
            10. Add 1ug/mL puromycin to select for infected cells
        iv. Add biotin for 50uL final concentration once cell count exceeds 75 x 10^6. Incubate 24 hours at 37°
        v. Perform cell lysis and extract proteins
            1. Centrifuge cells (5 minutes, 500 x g) and aspirate out media
            2. Wash twice with 5 mL PBS to remove residual free biotin. Resuspend after second wash
            3. Add 600uL lysis buffer and transfer lysed cells
            4. Add 240 uL of Triton 100X for final concentration of 2%
        vi. Perform Western blotting to confirm biotin ligase complex expression
            1. Run Western blots in accordance with standard protocol; on two separate membranes, screen NT5C2-complex sample and EV-complex sample for biotin (primary antibodies from mouse, fluorescent

secondary antibodies) and MYC (primary antibodies from rabbit, fluorescent secondary)
2. Membranes agitated overnight after application of each antibody, and agitated 5 times in 10-minute PBST wash cycles
3. Observe membrane using Odyssey imaging system. If there are distinct bands of different molecular weights for NT5 hC2 and EV on the myc test membrane, the NT5C2/empty vector-myc-biotin ligase complexes were successfully expressed.

vii. Perform pulldown of biotinylated proteins
1. Add 20% Triton X100 to lysed cells
2. Sonicate for two 4-minute sessions (30 pulses, 30% duty cycle, output level 3) to fully denature contents. If sample remains viscous, cloudy and/or clumped sonicate further until resolution resolution. Add 50 mM Tris-Cl (pH 7.4) and sonicate further for improved affinity capture
3. Aliquot into 3 pre-chilled 2 mL tubes per sample (e.g. 3 tubes each for EV and NT5C2 groups) and spin down at 4° (16500 xg, 10 minutes)
4. Equilibrate streptavidin beads by adding them to 1:1 lysis buffer/Triton solution that mimics sample conditions. Place tubes (equal in quantity to number of tubes with sample) containing beads in magnetic stand such that the magnetic beads accumulate to one tube wall. Wait 3 minutes and remove supernatant from bead tubes via pipetting
5. Resuspend beads with supernatant from centrifugation of sample in step 3 such that all sample groups have 3 tubes also containing streptavidin beads
6. Place all tubes on 4°C rotator overnight
7. Remove supernatant and wash 4 times (with wash buffers 1, 2, 3 as defined in protocol and Tris-Cl, consecutively), rotating for 8 minutes at room temperature between washes, to remove last remaining detergents and contaminants.
8. Pool the 3 tubes of each sample type together, spin down (5 min 2000 x g), and resuspend in 50 mM ammonium bicarbonate

viii. Perform Western blotting to identify kinase candidates
1. Run Western blots in accordance with standard protocol; on two separate membranes, screen NT5C2-complex sample and EV-complex sample for biotin (primary antibodies from mouse, fluorescent secondary antibodies) and MYC (primary antibodies from rabbit, fluorescent secondary)
2. Membranes agitated overnight after application of each antibody, and agitated 5 times in 10-minute PBST wash cycles
3. Observe membrane using Odyssey imaging system. If myc screen bands appear distinctly at level of NT5C2/EV, integrity of procedure is confirmed. If biotin screen bands appear exclusively in NT5C2 column, (a) protein(s) biotinylated only by biotin ligase-myc-NT5C2 construct, and therefore interacted with NT5C2 specifically, are present. Said proteins are potential kinases and their bands' presence encourages proceeding with mass spectroscopy

ix. Analyze final sample via mass spectroscopy to identify potential kinases

x. Confirm that potential kinase(s) identified phosphorylate NT5C2 in vivo
    1. Perform pulldown assay for said kinase according to standard procedure, using wild type T-ALL cells and concurrently pulling down an empty vector construct (negative control)
        a. Perform Western blot of resulting sample according to standard procedure, screening for said potential kinase. If a distinct band is present in the NT5C2 column and not the empty vector, the potential kinase binds specifically to NT5C2
    2. In an identical manner perform pulldown for the potential kinase
        a. Perform standard Western blot of resulting sample, screening for NT5C2. If a distinct band is present in the potential kinase column but not the empty vector column, the potential kinase binds to NT5C2 exclusively

xi. Search for kinase inhibitor(s) in public and commercial databases

xii. Screen the kinase inhibitor(s) by applying to T-ALL cells and observing growth and 6-MP resistance
    1. This stage depends heavily on the nature of kinase(s) identified and may lead to highly varied experimentation to develop T-all therapy via NT5C2 kinase inhibition as observation continues and cell responses arise. As such, this and potential future stages can only be generalized generalized.

2. Perform MTT and malachite green assays with HTP abalogs to identify best inhibitors
3. Determining Effect of NT5C2 inhibition in vivo procedures
    a. Knock out NT5C2 in human T-ALL cells using CRISPR/Cas9
        i. gRNAs for NT5C2 designed using BenchLink. gRNA-GFP complexes obtained from supplier
        ii. Transfect gRNAs into 293T packaging cells
        iii. Infuse T-ALL cells with CRISPR
            1. Infect each gRNA complex onto 10cm plate of T-ALL cells, leaving one negative control of a plate not infected with gRNA
            2. Pipet associated Cas9 protein onto each plate
            3. Incubate 24 hours at 37°
    b. perform T7 Endonuclease Assay from small sample of cells (not pooled, not sorted) to confirm CRISPR activity
        i. Isolate DNA of T-ALL cells using Quiagen DNeasy blood & tissue kit, following standard provided protocol
            1. Protocol constitutes cell lysis and repeated denaturation of non-DNA components, interspaced by centrifugation to remove said products
        ii. Determine resulting DNA concentration in water using NanoDrop and dilute 1:10 for PCR
        iii. Amplify resulting DNA / obtain NT5C2 from whole genome
            1. PCR according to standard protocol
                a. Create PCR master mix of 1.2 uL Taq Pol, 85.8 uL water, 3 uL each of forward and reverse primer, 1.2 uL DMSO, 1.2 uL 50 mM $MgCl_2$, 9.6 uL 5 mM dNTP, 12 uL buffer. This mix is optimized for 3 samples; adjust quantities proportionally if needed
                b. NT5C2 primers: F Exon 13, R Exon 3

2. To separate tubes, add 50 ug of each DNA sample, and 40x master mix by volume
3. Heat cycle samples in accordance with Kapa Master mix guide

iv. PCR purify DNA product & Begin Annealing Reaction
1. Use Quiagen Gel Extraction Kit, following standard protocol provided by manufacturer
a. Protocol constitutes repeated suspension of sample in wash buffers and centrifugation to remove unwanted contents, followed by elution in water

v. Perform T7 Annealing Reaction
1. Count purified DNA weight/volume concentration using NanoDrop
2. Perform twice: add 200ug DNA, 2uL NEBuffer 2, and water up to 19uL for each DNA sample
3. Add 1X T7 Endonuclease to each mixture for one of the above groups (the other a T7 negative control). Incubate all for 15 minutes at 37°C
4. Perform gel electrophoresis for all samples (1 hour 150V). If NT5C2 KO is successful, T7 and gRNA-positive samples should display multiple bands instead of 1 (as in the controls), as T7 Endonuclease cleaves non perfectly matched DNA strands that would result from NHEJ repair after Cas9 activity

vi. Select cells in which NT5C2 was successfully knocked out
1. Use FACS sorting to obtain cells that underwent CRISPR genome alteration by gating for GFP expression above 10^5, as gRNA complex contained GFP
2. Set up single cell suspension of gRNA-positive cells in 96-well plate using FACS sorting, as NHEJ ensures each individual cell will have unique post-CRISPR mutations (some which may not knock out *NT5C2* gene) and as such a variety of variations should be observed
3. After clones grow to sufficient quantity, perform T7 assay on each surviving well of clones to confirm CRISPR activity again. Proceed only with cell clone groups with confirmed CRISPR activity
4. For each clone group, perform Western blot according to standard procedure, screening for NT5C2. Proceed with all groups for which an NT5C2 band is absent, as their mutations resulted in successful NT5C2 knockout

vii. Determine in vitro effects of absence of NT5C2
1. Observe cell growth under normal conditions
2. Treat cells with 6-MP to determine degree of chemoresistance

4. To deal with potentially hazardous chemicals and biological agents agents, personal protective equipment (gloves, lab coat, eye protection protection) is worn at all times. In addition, all potential biohazard materials are immediately disposed of into designated biohazard waste containers. When working with cells, all operations are performed under a tissue culture hood.

3. POTENTIALLY HAZARDOUS BIOLOGICAL AGENTS RESEARCH:
(ANSWER THE BELOW ITEMS FOR EACH AGENT USED)

- CUTLL1 cell line
    - Obtained from T-ALL patient sample
    - All work performed under hood, in gloves and lab coat
    - Disposed of in designated biohazard waste containers after being bleached
- 293T cell line
    - Purchased from supplier; original source human fetal kidney
    - Biosafety Level Assessment: Level 2
    - All work performed under hood, in gloves and lab coat
    - Disposed of in designated biohazard waste containers after being bleached
- Jurkat cell line
    - Purchased from supplier; original source human peripheral blood
    - Biosafety Level Assessment: Level 1
    - All work performed under hood, in gloves and lab coat
    - Disposed of in designated biohazard waste containers after being bleached
- Lentiviral vectors
    - Generated via transfection
    - Biosafety Level Assessment: Level 2
    - All direct-contact work performed by qualified scientist under designated viral hood while wearing 2 pairs of gloves, lab coat, and protective body covering
    - Virus and all instruments it contacted immediately bleached and disposed of in designated biohazard waste containers

4. HAZARDOUS CHEMICALS, ACTIVITIES & DEVICES:
(ANSWER THE BELOW ITEMS FOR EACH THING USED)

Most chemicals below used in buffer composition. Where applicable, all concentrations of a substance used are listed. Unless otherwise indicated, all chemicals/drugs are:

- Disposed of in designated hazard waste containers
- Non-flammable
- Non-radioactive
- Handled only while wearing personal protective equipment (lab coat, gloves, eye protection)
- Evaluated for safety by supplier/manufacturer, resulting safety information/protocol relayed through published material safety data sheet (MSDS) and followed diligently

- 10% (v/v) adult bovine serum
- 1%, 0.2% (w/v) Triton X-100
- 1× PBS
- 1x PBST
- Biotin, 1 mM
- 1% bovine serum albumin, fraction V
- 250 mM luminol
- 90 mM coumaric acid
- Tris·Cl, 30%
    - 50 mM, 1 M, 10 mM; pH 8.5, 7.4, 6.8
- 500 mM, 50 mM NaCl
- 2%, 0.2% SDS (w/v)

- AEBSF 1mM
- Aprotinin 800nM
- Bestatin 50µM
- E64 15µM
- Leupeptin 20µM
- Pepstatin A 10µM
- 1 mM, 20 mM DDT
- 3% (v/v) sodium azide
- 4.5% (v/v) hydrogen peroxide
- 12% sucrose
- 0.004% bromophenol blue
- 0.1% (w/v) deoxycholic acid
- 1 mM, 250 mM EDTA
- 50 mM HEPES, pH 7.5
- 0.5% (w/v) deoxycholic acid
- 0.5% (w/v) NP-40
- 250 mM LiCl
- T7 Endonuclease I 5 ug/mL
- Puromycin 1 ug/mL
- BSA 100ug/mL
- DMEM

D. BIBLIOGRAPHY

List at least five (5) major references (e.g. science journal articles, books, internet sites) from your literature review. If you plan to use vertebrate animals, one of these references must be an animal care reference.

1. Dieck, C. L., Tzoneva, G., Forouhar, F., Carpenter, Z., Ambesi-Impiombato, A., Sánchez-Martín, M., ... & Ferrando, A. A. (2018). Structure and Mechanisms of NT5C2 Mutations Driving Thiopurine Resistance in Relapsed Lymphoblastic Leukemia. *Cancer cell*, *34*(1), 136-147.
2. Tzoneva, G., Dieck, C. L., Oshima, K., Ambesi-Impiombato, A., Sánchez-Martín, M., Madubata, C. J., ... & Sulis, M. L. (2018). Clonal evolution mechanisms in NT5C2 mutant-relapsed acute lymphoblastic leukaemia. *Nature*, *553*(7689), 511.
3. Hnízda, A., Fábry, M., Moriyama, T., Pachl, P., Kugler, M., Brinsa, V., ... & Trka, J. (2018). Relapsed acute lymphoblastic leukemia-specific mutations in NT5C2 cluster into hotspots driving intersubunit stimulation. *Leukemia*, 1.
4. Weng, A. P., Ferrando, A. A., Lee, W., Morris, J. P., Silverman, L. B., Sanchez-Irizarry, C., ... & Aster, J. C. (2004). Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*, *306*(5694), 269-271.
5. Belver, L., & Ferrando, A. (2016). The genetics and mechanisms of T cell acute lymphoblastic leukaemia. *Nature Reviews Cancer*, *16*(8), 494.
6. Roux, K. J., Kim, D. I., Burke, B., & May, D. G. (2018). BioID: a screen for protein-protein interactions. *Current protocols in protein science*, *91*(1), 19-23.