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Research Plan

The Research Plan/Project Summary is a succinct detailing of the rationale, research question(s), methodology, and risk assessment of your research project and should be completed before the start of your experimentation. Any changes you make to your study should to be added to the final document. Leave any bold headings in your final research plan and answer all bullets in required sections. The layout should appear as it is presented here. `

The research plan for ALL projects should include the following:

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.

Mutant NT5C2-containing ALL cells have demonstrated increased sensitivity to treatment with mizoribine, an IMPDH inhibitor, likely due to depletion of intracellular purine supply due to inhibition of synthesis (IMPDH) and activation of removal (NT5C2). Preliminary evidence suggests that knockout and knockdown of NT5C2 has little effect on cell homeostasis and growth. In addition, NT5C2 splicing variants have been associated with hereditary spastic paraplegia. These indicate that there may be unknown compensatory mechanisms and/or endogenous roles of NT5C2. Therefore, to identify other pathways that are affected by NT5C2, a whole-genome CRISPR screen will be performed with mizoribine to identify which genes confer additional sensitivity or resistance to that drug. Also, RNA sequencing of NT5C2 CRISPR KO cells, and CRISPR rescue cells, will be performed to identify any compensatory mechanisms for NT5C2 inhibition and comparisons of KO vs. WT NT5C2 in vivo will be performed to confirm the non-essential nature of NT5C2 and identify what if any effects can be seen. Finally, a whole-genome CRISPR screen will be performed with HTP-2, a potential NT5C2 inhibitor identified through previous research, to identify and pathways affected by NT5C2 pharmaceutical inhibition.

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

Research questions:

1. Identify effect of NT5C2 knockout on cell, including compensatory mechanisms
2. Identify pathways affected by NT5C2 pharmaceutical inhibition

C. RESEARCH METHODS

1. 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. Bacterial minipreps performed with Zymo Miniprep Kit, according to standard procedure.
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 MgCl2, 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
3. Perform MTT assays once daily for 7 days, comparing growth of equal quantities of plated NT5C2 KO and WT cells

c. Perform rescue of NT5C2 KO cells
1. Rescue gRNAs for NT5C2 designed using BenchLink. gRNA-GFP complexes obtained from supplier
2. Select for bacteria with plasmids with puromycin, and extract. Bacterial minipreps performed with Zymo Miniprep Kit, according to standard procedure.
3. Transfect gRNAs into 293T packaging cells
4. Infuse T-ALL cells with CRISPR
a. Infect each gRNA complex onto 10cm plate of T-ALL cells, leaving one negative control of a plate not infected with gRNA
b. Pipet associated Cas9 protein onto each plate
c. Incubate 24 hours at 37 degrees
5. Confirm successful rescue using western blotting; perform growth curve assays to identify any effect

2. Perform whole-genome CRISPR screening to identify genes conferring resistance or sensitivity to mizoribine
a. Whole-genome GeCKO gRNA pooled library transfected into lentiviral vectors
i. Add 3 ug DNA of interest, 2.7 ug GagPol combined lentiviral packaging plasmid, .3ug VSV-G lentiviral envelope plasmid, 18uL jetPEI transfection reagent, and 250 uL NaCl to wells. Suspend jetPEI in an additional 250 uL NaCl prior to adding it to the DNA solution.
ii. Vortex 30 seconds and incubate at 37° for 30 minutes
iii. Drip each of the solutions (one for every vector) onto individual plate of >1,000,000 293T cells
iv. Incubate for 72 hours at 37°
b. Infect human T-ALL cells with produced lentiviral vector
i. Prepare 96 well plates for all genes, adding 1 x 10^6 target cells per well
ii. Centrifuge contents of packaging cell plates (500 x g for 5 minutes).
iii. Extract supernatant containing virus, and filter to remove any remaining cells
iv. Add 1ug/mL polybrene infection reagent to viral supernatant, for final concentration of 8ug/mL
v. Add 500 viral supernatant to each well for final 1:1 cell:virus ratio by volume

vi. Centrifuge cells with viral vector for 90 minutes at 30°C and 1000 x g
vii. Incubate cells 4-6 hours
viii. Add 1 mL RPMI
ix. Incubate 4-6 hours, then change media (removing viral vector remnants)
x. Add 1ug/mL puromycin to select for infected cells

c. Pool experimental and control (no CRISPR) cells into flask and take representative samples from each (day 0)
d. Add 50 uM mizoribine to experimental group, and take representative samples at Day 7 and Day 14 (final day)
e. Extract RNA from all time points using QuiaGen RNA Extraction Kit according to standard procedure, and send samples for MaGeCK analysis
f. Perform validation experiments with any genes of interest using shRNA knockdown of said genes in cells, via MTT assays with mizoribine and HTP
g. If successful, repeat whole-genome screening with HTP-2 in place of mizoribine

3. Identifying compensatory mechanisms
    a. Extract RNA of generated NT5C2 KO, WT, and mutated ALL cells using QuiaGen RNA extraction kit and standard protocol
    b. Perform RNA sequencing on samples to compare gene expression of each variant
    c. Perform validation experiments with any genes of interest using shRNA knockdown of said genes in cells, via MTT and malachite green assays

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:

- CUTLL1 cell line
    - Obtain from T-ALL patient sample
    - All work will be performed under hood, in gloves and lab coat
    - Disposed of in designated biohazard waste containers after being bleached
- REH cell line
    - Purchase from supplier; originally sourced from T-ALL patient sample
    - Will be transfected into lentiviral All work performed under hood, in gloves and lab coat
    - Dispose of in designated biohazard waste containers after being bleached
- 293T cell line
    - Purchase from supplier; original source human fetal kidney
    - Biosafety Level Assessment: Level 2
    - All work will be performed under hood, in gloves and lab coat
    - Dispose of in designated biohazard waste containers after being bleached
- Jurkat cell line
    - Purchase from supplier; original source human peripheral blood
    - Biosafety Level Assessment: Level 1
    - All work will be performed under hood, in gloves and lab coat
    - Dispose of in designated biohazard waste containers after being bleached
- Lentiviral vectors

- Will be generated via transfection
- Biosafety Level Assessment: Level 2
- All direct-contact work will be 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 contacts will be 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 will be used in buffer composition. Where applicable, all concentrations of a substance to be used are listed. Unless otherwise indicated, all chemicals/drugs will be:

- 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
- pL.CRISPR.efs.gfp (AND OTHER) plasmids; other materials involved in gecko gRNA cloning
- QuiaGen RNA kit
- Zymo Miniprep Kit
- Polybrene
- Jetpei infection reagent
- Agilent Quick Site mutagenesis kit
- QuiaGen RNA extraction
- Mizoribine (50 uM)
- SGC-CBP30

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.

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5. Belver, L., & Ferrando, A. (2016). The genetics and mechanisms of T cell acute lymphoblastic leukaemia. *Nature Reviews Cancer*, *16*(8), 494.
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THERE ARE NO ADDENDUMS TO THIS RESEARCH PLAN