Investigating the Effect of Small Molecule Epigenetic Modulators on RNA Editing

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Word Count: 8,397

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Introduction

RNA Editing

RNA editing is an exciting frontier of gene editing that promises to revolutionize the treatment of genetic disorders. Developments in large-scale genome sequencing have elucidated many underlying causes of human disease, leading to the rapid development of DNA and RNA editing therapies [1]. While DNA editing potentially offers permanent cures to genetic mutations, off-target editing can have serious adverse effects, such as wide-scale genomic changes [1]. RNA editing does not permanently affect the genome, making it an adjustable and reversable therapy [2].

RNA editing is a widespread and common post transcriptional modification of the mammalian transcriptome, mainly mediated by ADAR (adenosine deaminase acting on RNAs) enzymes [2]. In 1986, Benne et al. observed an RNA-editing process after noticing frame-restoring nucleotides in the *coxII* gene that were not encoded in the original DNA from trypanosome mitochondria [3]. This observation seemed to defy the central dogma of molecular biology. In 1987, the ADAR protein family was discovered and characterized, and these enzymes were found to endogenously convert adenosine bases to inosine in double stranded RNA (dsRNA) [4]. It was revealed that the *GRIA2* gene requires an ADAR-mediated recoding event for functionality, establishing that RNA editing was not a random cell event, but vital mechanism for post-transcriptional modification in the nervous system [4]. Advances in high-throughput screening, specifically using next-generation sequencing (NGS), have resulted in large-scale identification of RNA editing sites [5].

ADAR enzymes are commonly found in the central nervous system (CNS) and play a crucial role in endogenous RNA editing [4]. ADARs can be broadly grouped into three categories: ADAR1 (with isoforms ADAR1p110 and ADAR1p150), ADAR2, and ADAR3 [2, 4]. Each shares a similar catalytic deaminase domain starting from the C-terminus, followed by dsRNA binding domains[6]. Of these enzymes, ADAR1 and ADAR2 have catalytic activity, and can be used to perform site-specific edits via hydrolytic deamination [2, 7]. Once the dsRNA binds to the respective ADAR enzyme, the catalytic domain converts adenosine to inosine (A->I). Inosine is read as guanine, effectively creating an A->G edit [7].

ADAR Family Proteins

ADAR1 has two different isoforms, p110 and p150 [7, 8]. Unlike ADAR2 and 3, ADAR1 has three dsRNA binding domains, and is widely expressed in various tissues [6]. The p110 is constitutively expressed in the nucleus, whereas the p150 isoform can move between the nucleus and the cytoplasm [9]. ADAR1 primarily acts as a safeguard against autoinflammatory responses to cellular dsRNA, and ADAR1 knockouts have shown mid gestational lethality because of failed embryonic hematopoiesis [8]. Widespread editing from ADAR1 is essential for preventing endogenous dsRNA from being recognized as foreign by the immune system and triggering an immune response [8]. Instead of making precise edits, ADAR1 maintains global cell homeostasis by regulated dsRNA. ADAR1 has also been shown to form a complex with Dicer (an essential endonuclease enzyme) to promote microRNA (miRNA) processing and RNA interference (RNAi) in developing embryos [10].

ADAR2 was identified as the enzyme responsible for a critical recoding event within the GRIA2 transcript [11]. It performs a site-specific A->I alteration at the Q/R sire of the GLUA2 subunit of the AMPA receptors [12]. ADAR2 is highly expressed in neurons and is essential for regulating calcium permeability in neuronal synapses [11]. Because of the ability to perform highly site-specific edits, ADAR2 has strong therapeutic potential, particularly in targeting disease-inducing single nucleotide polymorphisms (SNPs)[11, 12]. ADAR2 binds RNA in both the nucleoplasm and nucleoli, and the editing efficiency is dependent on dsRNA availability [13]. More than half of all adenosines measuring over 100bp (base pair) can be edited by ADAR2, but only a few (like GLUA2) are selectively edited, indicating that the secondary structure of substrates determines selectivity [10].

Unlike ADAR1 and ADAR2, ADAR3 is catalytically inactive, and it is assumed that ADAR3 plays a role in RNA stabilization and gene expression [14]. Despite being catalytically inactive, ADAR3 is vital in neural systems in mammals [15]. In a study exploring mammalian neurons, *Mladenova et al* showed that mice who lack exon 3 of ADAR3 have short- and long-term memory deficiency, as well as increase levels of anxiety [15, 16]. It is thought that ADAR3 plays a negative regulatory role in ADAR editing, as it can bind to dsRNA but remains catalytically inactive [16].

RNA Editing Trans-Regulators

RNA editing is a highly conserved and regulated mechanism in mammalian systems, yet relatively few *cis* or *trans* regulators of ADAR-mediated editing have been discovered [17]. Trans-regulators (or trans-regulatory elements) can be defined as a molecule that regulates gene expression than the one that it binds with[17, 18]. Identifying regulators of ADAR substrates or proteins would greatly enhance the efficacy of existing ADAR-mediated editing technologies for therapeutic use. Inspired by the work of *Freund et al.*, who identified ILF3 as a broadly negative regulator of RNA editing using Biotin Identification (BioID), I decided to perform a small molecule screening, which will be elaborated on later in this thesis [17]. This will serve a dual purpose: on one hand, it will allow us to identify novel *trans*-regulators of editing activity and simultaneously offer a chemical tool to improve the efficacy of existing RNA editing technology.

ADAR2 Editing Technologies

Treatments for genetic mutations, particularly in neural diseases like Amyotrophic Lateral Sclerosis (ALS) and other muscle dystrophies, have led to the development of ADAR2-related technologies[2, 11]. These technologies can broadly be divided into ones that entail delivery or overexpression of ADAR enzymes coupled with guide RNA (gRNA), and ones that rely on chemically modified antisense oligonucleotides (ASOs). While each approach has varying degrees of efficacy, the goal of all the listed technologies is to make a specific edit of a transcript to correct a pathogenic mutation, such as a premature stop codon.

The REPAIR (RNA Editing for Programmable A to I Placement) technology, first developed in 2017, used a catalytically inactive Cas13 (dCas13) to perform site-specific A→I editing, mediated by ADAR2 [19]. Editing rates of REPAIR for the endogenous targets *KRAS* and *PPIB* were 27.1% and 13%, respectively [19]. After specificity-enhancing mutations, it was observed that REPAIR achieved no off target edits [19]. Because of the size of the dCas13, delivery to the cell was a major issue, and to overcome this, *Cox et al* used adeno-associated viral (AAV) vectors for transportation [19]. While REPAIR seems like a promising technology,

the low editing rate and delivery issues make it unsuitable as a wide-spread therapy. There is also a high risk of immunogenicity, as the dCas13 is of bacterial origin [20]. Repeated dosing of this therapy could lead to an immune response, reducing editing efficacy and posing a risk to the patient.

The RESTORE (Recruiting Endogenous ADAR to Specific Transcripts for Oligonucleotide-mediated RNA editing) technology is another RNA editing method that improves upon the REPAIR technology, as it bypasses the need for complex protein delivery [21]. RESTORE consists of an engineered, short ASO (that is chemically modified for enhanced stability and specificity) conjugated to an ADAR recruiting domain, which is capable of endogenous ADAR-mediated RNA editing [21]. The gRNA domain enables high site-specificity, and after several rounds of improvements to the ADAR-recruiting domain, *Merkle et al.* achieved an editing yield of 21% in fibroblasts when targeting STAT1 (signal transducer and activator of transcription 1), which was further increased to 32% with the addition of IFN-α (a type I interferon produced by immune cells in response to infection) [21, 22]. AIRNA, the parent company of the RESTORE technology, has continued to improve upon RESTORE, and in a recent poster presentation titled "Optimized RESTORE+ oligonucleotides for an efficacious and safe RNA base editing treatment for alpha-1 antitrypsin deficiency", AIRNA achieved 90% editing rates in mouse hepatocytes *in vitro*, and 50% editing *in vivo* [23]. This rapid increase in editing efficiency displays the potential therapeutic impact of RNA editing.

To improve upon the issues of efficacy and delivery, *Monian et al* from the pharmaceutical company Wave Life Sciences created short length, modified oligonucleotides (called AIMers) that mediate A→I editing using endogenous ADAR2 [24]. The "stereopure" oligonucleotides have a chemically modified backbone that has enhanced editing efficiency [24]. When targeting the endogenous *ACTB* transcript in non-human primate liver, AIMers achieved a 50% editing rate [24]. Furthermore, AIMers could be modified with GalNAc (N-acetylgalactosamine) conjugates for improved delivery [24]. GalNAc conjugates were originally used to delivery short-interfering RNA (siRNA) to liver hepatocytes, as the interaction between tris-GalNAc and Asialoglycoprotein receptors induces endocytosis [25]. While the AIMer technology overcomes delivery and efficiency concerns, the modified oligonucleotides are difficult to manufacture compared to other RNA editing technologies.

LEAPER

One of the most exciting ADAR-mediated RNA editing technologies, which this project focuses on, is the LEAPER technology. LEAPER, or Leveraging Endogenous ADAR for Programmable Editing of RNA, uses gRNA to recruit endogenous ADAR2 to perform site-specific A→I edits. Out of all the approaches, this technique has the highest editing rate of approximately 80%, and only requires an engineered gRNA to work [26]. However, to successfully recruit ADAR2, the gRNA needs to be over 70 nucleotides long [26]. The gRNA has a deliberate mismatch at the site of the desired edit, and after ADAR2 is recruited, it will perform the A→I edit on the endogenous target strand of RNA. LEAPER offers several advantages compared to other technologies; it is relatively easy to design specific sequences to modify any target, it has high specificity, and it has a high editing efficiency. The goal of this project was to improve the editing rate of LEAPER through the addition of small epigenetic modulators. It is worth noting that in 2023, LEAPER 2.0 was published, which exhibits improved efficiency, delivery, and specificity [27]. LEAPER 2.0 consists of AAV-delivered

circular ADAR-recruiting RNAs (arRNAs) to achieve editing efficiencies of approximately 80% with no discernible toxicity, even at high dosages [27]. While using a humanized mouse model of Hurler syndrome, *Yi et al.* was able to edit the premature stop codon to restore functionality of the affected gene [27]. I focused on LEAPER instead of LEAPER 2.0 for several reasons; LEAPER is easy to manufacture, and at this stage in the project, I was not focusing on *in vivo* delivery.

Limitation of Current Strategies

While each technology employs a unique mechanism of action to induce a site-specific edit, they each share four major limitations: Off-target editing, delivery, editing efficiency, and stability. The first of these issues, off-target editing, poses the biggest challenge, because even if the editing technology makes the correct edit, it could potentially cause more damage to the patient if it makes an edit at an undesired site. Technologies that use shorter gRNAs (around 20-30bp) must overcome the desired threshold of on vs. off target editing events. Because the LEAPER technology uses a 71nt ASO, it is theoretically more specific than technologies that use a shorter gRNA, as there are more base pairs the gRNA must bind to for ADAR-recruitment [26].

Delivery is another limitation of current RNA editing technologies. ASOs are notoriously difficult to deliver to the cell because they are usually large, negatively charged, and must enter the nucleus [28]. RNA can also easily degrade in the wrong environments, creating yet another obstacle for delivery methods to overcome [28]. The three primary solutions for delivery are AAVs, lipid nanoparticles (LNPs), and conjugation [28]. AAVs can efficiently deliver genetic material to the cell but cannot deliver large proteins and may generate an immune response for patients [28]. LNPs, famously used in mRNA vaccine delivery, are a non-viral delivery alternative [28]. However, LNPs tend to have specificity for the liver, limiting the capability to deliver RNA to other tissues [28]. Conjugation, specifically chemical conjugation, links a cell-recognizable ligand to the technology of interest, enabling the RNA complex to pass into the cell [24]. An example of this is the AIMer technology, which uses a GalNAc ligand for delivery into hepatocytes [24]. Conjugation delivery is highly dependent on receptor availability and expression, making it less versatile for widespread delivery [28].

Achieving high editing efficiencies is the final limitation for current RNA editing technologies. The maximum attainable editing rate is inherently capped by several biological factors, including delivery, and in the case of LEAPER technology, the endogenous ADAR2 enzyme pool [2, 7, 26]. RNA is dynamic in nature and has a short half-life, so effective treatments would require continuous deliveries of therapeutics to maintain a state of sustained correction [2, 7, 10].

Structural Genomics Consortium Group Probes

The Structural Genomics Consortium (SGC) is a global public/private partnership across institutes such as Karolinska Institutet, McGill University, and University College London, that has a vast library of potent and well-characterized small epigenetic modulators. All compounds and research are released in the public domain to promote and accelerate protein functionality and drug discovery. Previous studies have used these highly site-specific probes to understand cellular mechanisms [29]. An example of this is JQ1 (a small molecule in the SGC library), which was used to investigate the biological functions of bromodomains in differentiation and

tumor progression [30]. This project was inspired by previous work from the Wood/Rinaldi Lab that used the SGC library in spinal muscular atrophy (SMA) research. Kordala et al. found a molecule within the SGC library (a selective type I PRMT inhibitor named MS023) that promoted exon 7 inclusion in SMN2 pre-mRNA and increased SMN protein levels by 1.6 fold [29]. Encouraged by these results, I followed a similar approach to Kordala et al to hopefully find molecules in the SGC that promote higher rates of ADAR2 editing using LEAPER technology. Molecules from the SGC library that decrease editing rates were also of interest during the screening, as this would be an indication that the editing pathway was disrupted, which would also lead to a better understanding of ADAR2 editing promoters and suppressors. I broadly hypothesized that compounds in this library would have a regulatory effect on ADAR2 regulation, which could increase the editing efficiency of existing ADAR-mediated editing technologies. Furthermore, identification of the mechanism of action of successful candidates would lead to a greater understanding of ADAR2 regulation.

Materials and Methods

Cell Culture

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium-Glutamax (DMEM) (Gibco 10564-0I I) enriched with 10% fetal bovine serum (FBS) (Gibco A5256701) and 1% Antibiotic/Antimycotic solution (Merck A5955). Cells were maintained at 37°C in a 5% CO_2 atmosphere and were passaged every 4 days using TrypLE™ Express (Gibco 12604-013).

Cell Counting

To measure the concentration of cells for cell culture, Plate Reader assay, and FCM assay, an automatic cell counter (Logos Biosystems, LUNA-11, Catalog No. L40002) was used. After harvest using TrypLE™ Express and suspension in standard media (10% FBS, 90% DMEM), 10 µL were transferred to the sample slide. Total cell count was determined, and concentration was adjusted accordingly for each protocol.

Fluorescent Screening of SGC Probes

For transfection, $1x10^6$ cells per milliliter (10% FBS, 90% DMEM) of HEK-reporter cells were seeded to a 6-well plate (CytoOne, Catalog No. CC7682-7506) at a total volume of 1 mL per well and incubated overnight. The next morning, the medium was discarded, and the cells were washed with 1 mL of Phosphate-Buffered Saline (PBS). The transfection mixture was prepared in two separate 1.5mL Eppendorf tubes. Tube A contained 150 µL of OptiMem (Gibco, Catalog No. 5 1985-047) and 9 µL of Lipofectamine 2000 (Invitrogen, Catalog No.11668019). Tube B contained 150 µL of OptiMem and 3 µL of 100 µM 71nt gRNA (Integrated DNA Technologies). These tubes were incubated for 30 minutes at room temperature and combined to create the final transfection mixture. The PBS wash was removed and replaced with the transfection mixture. After 4 to 6 hours of incubation at 37°C, the transfection mixture was removed, and the cells were harvested using 200 µL of TrypLE™ Express and suspended in the standard medium. The cells were then centrifuged at 4000rpm for 3 minutes. The supernatant was discarded, and the cells were resuspended in the standard medium. From this, 100 µL of the resuspended cells were seeded to a 96-well plate (CytoOne, Catalog No. CC7682-7596). 100µL of standard medium (10% FBS 90% DMEM) containing a 5µM concentration of the SGC compound was also added to each well, resulting in a final concentration of 200 µL per well.

Cells were incubated overnight. The next day, the media was removed from each well and washed with 100 µL of PBS. Cells were harvested using 50 µL of TrypLE™ Express and suspended in 500µL of PBS. The contents were transferred to a separate 1.5mL Eppendorf tube and centrifuged at 4000rpm for 2 minutes. The supernatant was discarded, and the cell pellets were resuspended in 200µL of PBS and transferred to a black 96-well plate (Greiner, Ref. No. 655077) for Plate Reader Analysis.

Flow Cytometer Screening and Analysis

The Flow cytometer analysis was performed using a similar protocol from the initial transfection method. Following transfection of the gRNA as described in the transfection protocol, cells were harvested using 200 µL of TrypLE™ Express and resuspended in the standard medium. Next, cells were seeded to a 24-well plate (CytoOne, CC7682-7524) format at $1x10^6$ cells per milliliter at a volume of 500 µL. Each well was treated with the respective compound from the SGC library. Cells were then incubated overnight at 37°C in a 5% CO_2 atmosphere. After overnight incubation, the media was discarded. The cells were harvested by 200 µL of TrypLE™ Express and suspended in standard media. The cells were centrifuged at 4000 rpm for 2 minutes and the supernatant was discarded. The cells were then washed in 500 µL of PBS and were re-centrifuged at 4000 rpm for 2 minutes. The supernatant was discarded, and cells were resuspended in PBS to yield a final concentration of $1x10^6$ cells per milliliter at a minimum volume of 250 µL. The samples were then acquired on the Sony ID17000™ flow cytometer (Sony Biotechnology), and 10,000 events were acquired per sample. Data analysis was conducted using Floreada.io, and gating strategies selected for singlet cells. The final gating was determined by the negative controls (non-transfected cells), which differentiated populations that expressed both mCherry and eGFP. The editing rate was quantified by dividing the number of cells expressing both mCherry and eGFP by cells only expressing mCherry.

RNA Extraction

500 µL of $1x10^6$/ml HEK-reporter cells in standard media of were seeded to a 6-well plate. To this, 500 µL of standard media with 5µM of SGC compound was added and incubated at 37°C in 5% CO_2 atmosphere overnight. Cells were then washed one time with 1 mL of PBS. From this point, the protocol of the Maxwell® RSC simplyRNA Cells Kit was followed, using the Maxwell® RSC.

cDNA Synthesis

Complimentary DNA (cDNA) was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Catalog No. 4368814). Each sample contained 1000 ng of RNA totaling a volume of 10 µL. The mixture was incubated according to the Reverse Transcription Kit protocol. Following synthesis, cDNA was diluted 1:5 in RNase-free water and stored at -20°C before the use in real-time PCR (qRT-PCR).

qRT-PCR Protocol

RT-qPCR reactions were prepared with the SYBR Green™ Gene Expression Master Mix (Applied Biosystems #4369016) and then transferred to the StepOnePlus™ real-time PCR system, which ran and analyzed the samples. *GAPDH* (housekeeping), *ADAR1,* and *ADAR2*

primers were used for amplification. Each sample was completed as duplicate. Fold changes were calculated using the ΔΔCt method.

Protein Extraction

500 µL of HEK293T-PHFTAG-reporter cells maintained in standard media were seeded to a 6-well plate at a concentration of $1x10^6$ cells/mL. To this, 500 µL of standard media treated with SGC compounds (resulting in a final concentration of 5µM) was added and incubated overnight at 37°C in 5% CO_2 atmosphere. Cells were then washed one time with 1 mL of PBS, and frozen. Once all samples were collected, cells were thawed on ice, harvested, and pelleted. 100 µL of radioimmunoprecipitation assay (RIPA buffer) was added and incubated for 30 minutes on ice. Following incubation, cells were centrifuged at 12,500 rpm for 10 minutes at 4°C. The soluble fraction was collected, transferred to clean microcentrifuge tube, and stored at -20°C until further use.

BCA Protein Assay and Western Blot

Total protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Catalog No. 23225). All samples were triplicated to ensure reliability. Bovine Serum Albumin (BSA) was used to generate a standard curve to calculate the protein concentration of each sample. Protein samples were combined with LDS sample buffer 4x (Invitrogen, Ref. NP0007) and Sample Reducing Agent 10x (Invitrogen, NuPAGE, Ref. NP0004). Samples were boiled for 10 minutes at 70°C, then put on ice for 3 minutes, then briefly centrifuged for further collection. From this, 20 µL of each sample was added to a 4-12% Bis-Tris gradient gel (Invitrogen, Catalog No. XPO4125BOX). The proteins were separated by size using MOPS running buffer at a constant 150V for 30-60 minutes. After running, the sorted proteins were transferred to a membrane for Western Blot analysis.

Immunoblotting

Following completed electrophoresis, the proteins were transferred to a membrane using Bio-Rad Trans-Blot® Turbo™. After membrane transfer, the membrane was put in a blocking solution (Intercept blocking buffer 927-70001) for 1 hour. The membrane was then washed with Tris-Buffered Saline with Tween-20 (TBST) to remove the blocking buffer. The membrane was then incubated with the primary antibody (GeneTex, ADAR2ab GTX54916) overnight in the cold room. The next day, after four 15-minute washes in TBST, the secondary antibody (rat-IgG-HRP, GeneTex, Catalog No. GTX628478-01) was added to the membrane. After a one-hour incubation, the membrane was washed 4 more times for 15 minutes. Clarity Western ECL Substrate (Bio-Rad, cat. 1705061) was added to the membrane, and chemiluminescence detection was completed by the Bio-Rad ChemiDoc™ Imaging System. To quantify band intensity, ImageJ analysis was completed. Anti-GAPDH (GeneTex, Catalog No. GTX627408) was also used to ensure equal protein loading, and the band intensity of each sample was compared to GAPDH intensity.

Statistical Analysis

Statistical significance was determined with a minimum of n = 3 biological replicates using GraphPad Prism 10 software and one-way ANOVA tests with Dunnett's correction for

figures 4 and 5. P values less than 0.05 were statistically significant. Statistical tests and error bars are detailed in figure legends.

Results

Generation of a Cell Reporter System for RNA editing

The initial step of this project was focused on establishing a robust and specific cell reporter system. This work was completed by Taichi Fukunaga prior to my arrival at the lab (**Figure 1A**). The reporter system emulated the approach that was used in the LEAPER paper, which used a Doxycycline (DOX)-inducible plasmid construct (**Figure 1A**). This work validates the reporter system described in the LEAPER paper [26]. A HEK-293 cell line was transfected with a sequence that contained an mCherry sequence, followed by a UAG stop codon, immediately preceded by an eGFP sequence (**Figure 1A**). When treated with DOX, the cells would express mCherry, which was detectable under the light microscope the next day. After mCherry expression was confirmed, I could then transfect the cells with the 71nt gRNA. Upon transfection, the gRNA can enter the nucleus, bind to the reporter sequence, and recruit ADAR2 to make a specific edit at the site of the stop codon. ADAR2 would perform an A→I edit, changing the UAG stop codon to UI(G)G codon, allowing the translation of the eGFP sequence (**Figure 1A**). The next day, the treated cells would express both mCherry and eGFP. Both fluorescent proteins allowed us to visualize the editing activity of ADAR2 within the cell. I also tried the 71nt gRNA at different concentrations to determine which dose would be most efficacious during our screen (**Figure 1B**). I found that while 40nM of gRNA caused editing events, the signal was too weak to measure changes in editing (**Figure 1B**). I decided to use 200nM of 71nt gRNA, which gave us stronger signals in cells, as well as more cells expressing eGFP. The top row of images in **Figure 1B** confirms mCherry expression upon DOX induction. There is no eGFP expression in the vehicle, which has no gRNA treatment. Moderate eGFP expression is visible when treated with 40nM of 71nt gRNA (**Figure 1B**). When treated with 200 nM of 71nt gRNA, higher levels of eGFP are expressed. Finally, when treated with 200 nM of 19nt gRNA, no eGFP is expressed, validating efficacy of the 71nt gRNA ASO (**Figure 1B**).

Screening of Small Molecules with CLARIOstar

While I was able to detect editing activity under light microscopy, I needed a way to quantify the data. I decided to use the CLARIOstar plate reader analysis, which measures emitted light from the fluorescent mCherry and eGFP proteins [26, 27]. I concluded the best way to measure editing activity would be to divide the eGFP values by the mCherry values to give produce an editing ratio. If more eGFP was expressed (due to the editing activity of the 71nt gRNA), I would have a larger ratio, enabling the detection of small changes in editing rates once I started to screen molecules from the SGC library. I planned to screen all 54 molecules in the SGC library, with biological triplicates of each compound for redundancy. Due to the format of our assay, I decided to screen 10 compounds at a time, for a total of 6 screens. Once the screening was completed, I would select the most impactful compounds for further analysis, in the hope that some compounds would share the same targets.

During the first screen, I found a molecule called Bromosporine (BSP) that appeared to increase editing rates by 46%, which was very promising data (**Figure 2B**). As the name

suggests, BSP is part of a class of inhibitors that target bromodomains, and was found to reactivate HIV-1 replication as part of an Acquired Immunodeficiency Syndrome (AIDS) study [31]. The efficacy of BSP was my first indication that bromodomains may influence ADAR-mediated RNA editing.

An unforeseen inconsistency was observed during our second screen. The editing rates of the control group were not consistent between screens, necessitating a change in transfection protocol. I hypothesized that the variation was due to inconsistent cell seeding during the first two days, and while the 71nt gRNA always induced GFP translation, the transfection stage could not be closely controlled. To account for this, I decided to normalize each screen to the two control groups from the respective screen. This way, I could compare editing rates between different screens in a consistent manner, as each day 2 seeding would have the same relative amount of gRNA from the morning. After switching to this new protocol, I observed consistent transfection for the rest of the project.

After all 54 compounds had been screened, I compiled all the normalized data and generated a heat map, which allowed us to clearly visualize the different effects the compounds had (**Figure 2A**). To account for errors, I decided that compounds that had a relative editing rate increase of 20% or lower would be excluded from the next step. This left us with 11 statistically significant compounds that increased editing efficiency of the LEAPER technology by more than 20% (**Figure 2B**). Surprisingly, I did not see any compounds that decreased editing by more than 20%. Two molecules, OF-1 and NI-57, decreased editing rates by 6.8% and 7.1%, respectively (**Figure 2A**). Interestingly, they both targeted bromodomains, specifically Bromodomain and Plant Homeodomain Finger-Containing proteins (BRPF). This was our second clue that targeting bromodomains has a significant and regulatory effect on the ADAR2 editing system. The 11 compounds that significantly increased rates of editing were as follows: BSP (46%), IOX1 (39%), BAY-678 (38%), IOX2 (36%), UNC1999 (36%), JQ1 (29%), MS049 (25%), PFI-5 (22%), NVS-PAK1-1 (22%), OICR-9424 (22%), and GSK343 (22%) (**Figure 2B**). BSP and JQ1 target bromodomains; IOX1 and IOX2 target 2-oxoglutarate oxygenases; BAY-678 targets serine proteases; UNC1999, MS049, PFI-5, and GSK343 target methyltransferases; NVS-PAK1-1 targets serine and threonine-protein kinases; and OICR-9424 targets WD40. To determine which compounds would be selected for further analysis, I eliminated compounds that had a high deviation between individual wells. For instance, one of the wells containing IOX1 had a 91% increase in editing rate, while the other wells only had 15% and 11% respectively (**Figure 2A**). For this reason, IOX1, IOX2, MS049, PFI-5, NVS-PAK1-1, and OICR-9424 were excluded from the next screen. I chose to include two additional compounds, I-BRD9 and PFI-1, for further analysis. While I-BRD9 only increased editing efficiency by 17% and PFI-1 only increased rates by 5%, both are pan-bromodomain inhibitors like JQ1 and BSP (**Figure 2A**). I reasoned that it was important to characterize all inhibitors that have the same targets as our top-performing compounds.

Dose Dependency Effect of Hit Compounds

From the initial screen, 7 compounds were selected for further analysis. These included BSP, JQ1, I-BRD9, and PFI-1, which are pan-bromodomain inhibitors; as well as UNC1999 and GSK343, which targets EZH2, a gene that functions as a histone methyltransferase within the Polycomb Repressive Complex 2 (PRC2); and BAY-678, which specifically targets neutrophil elastases[32]. I conducted a dose dependency analysis to determine the efficacy and potency of

each compound, as well as validate the results from the initial screening. I determined that a 5x reduction in concentration was sufficient, starting at 5μM and ending at 0.2μM. Each well was triplicated for consistency. After a one-way ANOVA test of the dose dependency assay, I found no statistical significance. There was small evidence of a dose-dependent response; for instance, the 5μM JQ1 treatment improved the editing rate by 53%, but the 1μM concentration only improved the editing rate by about 7.4% (**Figure 3**). I saw single outliers that made it appear that I-BRD9 and BAY-678 had a stronger effect than I had seen in the first screen. For this reason, as well as concerns about general specificity and reliability, I decided to switch the assay format to the flow cytometer instead of the plate reader. The flow cytometer measures the fluorescent signal of each individual cell and would give us a clear marker if the cell was expressing mCherry and eGFP. Given the results of the dose dependency screen, I decided to focus on only the most potent molecules for the flow cytometer analysis. These were JQ1 and BSP. After normalization, 5μM of JQ1 increased editing rates by 45%, and 5μM of BSP increased editing rates by 60% (**Figure 4A**). I also saw a clear dose dependent response. For each reduction in compound concentration of JQ1, the editing efficiency dropped from 45%, to 37%, to 28%, respectively (**Figure 4A**). The same was true for BSP, as the editing rates dropped from 60%, to 32%, to 11%, respectively (**Figure 4A**). An ordinary one-way ANOVA test with multiple comparisons showed that 5 μM and 1μM concentration, BSP and JQ1 significantly improved editing efficiency of the LEAPER technology ($P = <0.0001$) (**Figure 4A**). After considering the results from the multiple dose dependency screens and the plate reader assays, I concluded that only JQ1 and BSP had significant and consistent effects on the editing efficiency of the LEAPER technology.

Confirmation of the Primary Screening by Flow Cytometry

I decided to change to flow cytometer analysis after the first round of dose dependency for more accurate measurement and higher consistency. In the flow cytometry analysis, editing ratio was determined by dividing the cells that expressed both mCherry and eGFP by the cells that only expressed mCherry. The cells were divided into quadrants based on the negative control, which is generally grouped in Q3. This enabled me to exclude cells that did not express mCherry from the editing efficiency calculation, giving us a more exact measurement of editing efficiency (**Figure 4B-D**).

Assessment of Minimal gRNA Length by Flow Cytometry

Now that JQ1 and BSP were established as potent molecules that increase rates of editing of the LEAPER technology, I tested several shorter length ASO for editing activity. I designed three new ASOs that targeted the same sequence as the 71nt ASO but were all shorter in length. The LEAPER paper established that the minimum length of an ASO to recruit ADAR2 had to be larger than 70nt but given that our compounds improved editing rates as much as 70%, I wanted to test this theory [26]. **Figure 4B** shows the editing activity of the 71nt, 37nt, 31nt, 25nt, and 19nt ASOs. There is no activity in the negative control, which does not contain any ASOs. I confirmed the effect of my selected compounds, JQ1, and BSP, which increased the editing rate by about 30% and 70%, respectively. There was no editing activity of the 19nt, 25nt, 31nt, and 37nt (**Figure 4B**). This confirmed the LEAPER paper's claim that there is a minimum nucleotide length required and suggested that the mechanism of action of our two compounds was not a

direct interaction between the guide sequence and ADAR2. This analysis also once again validated the increase in editing efficiency of the LEAPER technology after JQ1 and BSP treatment.

Investigation of Mechanism of Action

Next, I needed to determine the mechanism of action of JQ1 and BSP. The results from the assay with the smaller length ASOs suggested that these compounds do not directly interact with ADAR2 and the exogenous gRNA. I decided to test mRNA levels of ADAR1 and ADAR2 after a 24-hour treatment of BSP and JQ1. **Figure 5D** shows the relative ADAR1 mRNA levels, where JQ1 and BSP are normalized to the non-treatment group. After treatment, both JQ1 and BSP decreased mRNA levels of ADAR1, with a 25% and 40% reduction, respectively. GAPDH was used as a housekeeping protein, and ADAR1 levels were compared to the levels of GAPDH to determine the difference in expression. This was evidence that JQ1 and BSP both play a regulatory role in ADAR1 expression. **Figure 5C** shows the relative quantities of ADAR2 mRNA compared to the non-treatment group. Again, GAPDH was used as a housekeeping protein. There was an increase in ADAR2 mRNA expression as JQ1 increased expression by 79% and BSP increased expression by 35% (**Figure 5C**). Both compounds had a profound effect on ADAR1 and ADAR2 levels, suggesting that they play a regulatory role in ADAR expression levels. After a one-way ANOVA test, only JQ1 was found to significantly increase ADAR2 mRNA levels (**Figure 5C**, $P = 0.05$). The low sample size ($n = 3$) likely causes BSP to have no statistical effect. To determine the speed of how quickly JQ1 and BSP affect mRNA ADAR2 expression, a 2-hour and 6-hour qRT-PCR was completed. **Figure 5A** suggests that after 2 hours, BSP slightly increases ADAR2 mRNA expression by 41%. After 6 hours, both JQ1 and BSP increased ADAR2 mRNA expression by 78% and 61%, respectively. However, neither result is statistically significant, as the *P*-value was 0.11 for JQ1 and 0.2 for BSP.

While the previous experiment proved that JQ1 and BSP affect mRNA levels, I wanted to see if protein levels of ADAR2 were also affected. I conducted a western blot experiment to detect the difference in protein expression after a 24-hour treatment of JQ1 and BSP (**Figure 5E**). **Figure 5E** is a western blot that displays the protein levels of the control group, JQ1, and BSP after 24 hours. I used biological triplicates for this experiment. There is an increase in both band thickness and darkness in the JQ1 and BSP bands compared to the non-treatment group, suggesting that ADAR2 protein levels are higher after treatment. After completing a one-way ANOVA test, neither result is statistically significant ($P = 0.0856$, $P = 0.1653$) because of the low sample size ($n = 3$). A t-test shows that the result is statistically significant. **Figure 5F** is a graph of the ImageJ analysis, and BSP and JQ1 have elevated protein levels with an approximate 100% increase in relative expression.

Discussion

RNA editing offers several key advantages to conventional DNA editing, which makes it an attractive system for therapeutic applications Unlike DNA editing, RNA editing is non-permanent, so off-target occurrences are relatively less damaging [2]. Additionally, RNA editing can utilize the cell's endogenous editing mechanisms which avoid issues of delivering complex exogenous molecules [2]. Technologies like LEAPER and REPAIR are extremely site-specific and reduces the rate of off-target editing events. Furthermore, DNA editing technologies that rely

on double-stranded DNA breaks (DSBs) often have high occurrences of large deletions, chromosomal translocations, and sometimes entire chromosome loss [33].

RNA editing is a particularly exciting and revolutionary technology to treat major neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Amyotrophic Lateral Sclerosis [2]. Because neurons are considered to be post-mitotic cells, DNA editing technologies that rely on cell proliferation are often not as effective [34]. ADAR2 is highly expressed in neuronal brain populations, as well as other skeletal tissues, like the heart and muscle [35]. ADAR2-related RNA editing technologies, therefore, have high potential to treat the aforementioned neurogenerative diseases, as their effectiveness is not limited to dividing cells and they are enriched in the tissues most affected by these disorders.

Low rates of efficiency are the biggest hurdle that RNA editing technologies must overcome to become effective therapies, and this project sought to identify and characterize small molecules that could increase editing efficiency of existing RNA editing technologies. I focused on LEAPER technology because of the simplicity and specificity of the system. While I originally tested the LEAPER technology in a HEK-Reporter-hADAR2 (ADAR2 overexpression) cell line, I quickly moved to a basic HEK-Reporter cell line, as I found that the overexpression was not necessary to determine editing rates. LEAPER offered a distinct advantage for research because it only used a single molecule, and no exogenous ADAR2 was required. Unlike AIMERs, there are no post-transcriptional modifications required for RNA editing. Designing and creating the 71nt gRNA, as well as the 19nt, 25nt, 31nt, and 37nt was simple and fast, as I outsourced this work to Integrated DNA Technologies (IDT).

To increase the editing rate of the LEAPER technology, I decided to screen through the SGC library, as these probes are potent epigenetic modulators that have many downstream effects. When I started this project, I foresaw two different possibilities. The first, and most probable, was that I would find several compounds that increased the rate of editing through epigenetic changes in the cell, likely changing the expression levels of ADAR2. The second and more unlikely possibility was that the several compounds in the SGC library would directly interact with either ADAR2 or the 71nt ASO to increase editing efficiency. Early on in our screen, I identified two EZH2 inhibitors, UNC1999 and GSK343, that increased the rate of editing in the reporter cell line. After a short investigation, I found that researchers at Northwestern had discovered a direct link between EZH2 and ADAR2 [36]. In their proposed project, they want to "identify precisely how EZH2 and ADAR interact and edit the substrates in prostate cancer"[36]. I decided to eliminate both GSK343 and UNC1999 after the dose dependency analysis because they had significantly lower editing rates than JQ1 and BSP, but I want to note that my work confirms that EZH2 and ADAR2 may be linked, as Cao et. al suggests [36]. In the future, if the work at Northwestern continues and proves that there is a structural interaction between EZH2 inhibitors and ADAR2, it would be worthwhile investigating this further with regards to the LEAPER technology.

During the primary screening of the SGC library, I encountered several problems that led us to switch from the plate reader analysis to a flow cytometer-based assay. The first problem was consistency in the editing rates of our controls between screens. To enable comparison across different screening days, each well was normalized to the positive control of its respective experiment. The second problem was because our editing ratio was determined by the amount of eGFP expressed by the amount of mCherry expressed, I could potentially see false editing

improvements if mCherry levels were decreased. Compounds in the SGC library are potent and potentially cytotoxic, and if mCherry levels were decreased because of low cell counts or other unforeseen effects, I would falsely identify compounds that increased the editing rate. Knowing this, each well was observed using light microscopy to double-check that mCherry levels remained consistent. Switching to the flow cytometer allowed us to control the exact number of cells counted per well (which was set at 10,000 cells per well) and determine if each cell was expressing mCherry and/or eGFP. The reason I avoided using the flow cytometer during the initial screen was due to material costs of the ASO. During a practice experiment, in which I tested the reporter system in the Flow Cytometer, I established that the 24-well plate was the minimal size for successful transfection, but compared to the 96-well plate, more ASO and SGC compounds were required. I rationalized that any compound that could effectively increase the editing rate during the first screen was sufficient, despite the relative inaccuracies I observed using the plate reader. In short, our first screen gave us a general idea of the editing efficiencies of each compound, and the dose dependency (using the flow cytometer) validated the results from the initial screen.

Compound concentration was an important consideration for this project. If the concentration during the screens was too high, there was a high risk of apoptosis, and if the concentration was too low, I could not observe the effects of the small molecules on LEAPER editing efficiency. One of my advisors, Taichi Fukunaga, had previous experience with drug discovery and determined that 5 μM was the maximum concentration I could work with for consistent results. After a brief search of previous research that used JQ1, I confirmed that 5 μM was a sufficient concentration for screening, despite the broad range of IC_{50} values of the SGC library [37]. During dose dependency, I hoped to see the same editing efficiency in the 0.2μM concentration, but this was not the case. A lower concentration would decrease cytotoxicity and costs if this were to become an add-on therapy. It is worth noting that cell irregularities were observed if treated with BSP and JQ1 for over 36 hours. This effect was noticeable in the qT-PCR experiments, and I hypothesize that some of the variations in the data were a result of cell death.

I was successful in finding two promising compounds that increased the editing efficiency of the 71nt treatment, as proven from both the initial screen and the dose dependency analysis. Discovering the mechanism of action was difficult, as both JQ1 and BSP are epigenetic modulators with many different downstream effects. While I did not find a direct structural interaction between JQ1/BSP and ADAR2, I did find that bromodomain inhibitors play a role in ADAR regulation, as seen from the qt-PCR and western blot results. The overexpression after BSP and JQ1 treatment partially explains the increase in editing activity. If protein levels of ADAR2 were higher in the cell, there would be an increased likelihood of ADAR2 recruitment by the 71nt gRNA. I found it interesting that JQ1 and BSP drove a decrease in ADAR1 mRNA, which led me to wonder if decreased ADAR1 levels could affect editing efficiency. ADAR1, specifically the p150 isoform, plays a critical role in preventing aberrant immune responses to endogenous dsRNAs [38]. There is no evidence that ADAR1 could interact with our reporter system and drive mCherry or eGFP expression. I conclude that pan-bromodomain inhibitors have a regulatory role in ADAR mRNA and protein levels, driving an increase in ADAR2 expression and a decrease in ADAR1 expression.

My work shows that pan-BET inhibitors affect ADAR expression, but I wanted to try to determine if there was a specific bromodomain that JQ1 and BSP target. The bromodomains of

the BET protein family include BRD2, BRD3, BRD4, and BRDT[39]. BRD4 is the most researched and well-characterized protein and is a "reader" of lysine acetylation [39]. JQ1 has been shown to inhibit BRD4, resulting in decreased histone acetylation [40]. It regulates many different transcription factors that bind to acetylated histone tails and acts upon different promoter regions [39]. For this reason, I included PFI-1 in the dose dependency screen, because it has the same specific targets as JQ1 and BSP. Notably, PFI-1 had no effect on the rate of editing, which led us to believe that either JQ1 and BSP have a greater binding affinity to the specific bromodomain regulating ADAR expression, or PFI-1 has a stronger affinity to a bromodomain that has no downstream effects on ADAR expression. In the first screen, I-BRD9 increased editing efficiency by 17%, which was below our 20% threshold, but I decided to include it because it had a similar target to JQ1/BSP **(Figure 1A)**. However, the dose dependency assay showed that it did not have a statistically significant impact, and none of the other compounds that target BRD9 (such as BI-9564, LP-99, and TP-472) had any effect. It is worth noting that those last three molecules have affinity for BRD7, so perhaps BRD9 inhibition has a small effect on ADAR expression and BRD7 has no effect. From existing literature, there is little evidence that bromodomains, specifically BRD4, regulate ADAR2 expression. However, one of the conclusions of this project is that BRD4 inhibition using BSP or JQ1 increases mRNA and protein levels of ADAR2.

The focus of this project evolved as I conducted each experiment. I was originally searching for a small molecule compound that would increase the editing efficiency of the LEAPER technology, and this shifted to discovering an underlying mechanism of ADAR regulation. This project lays the groundwork for a single molecule add-on therapy to existing RNA editing technologies, as it was shown that small molecules can increase editing efficiency. Benefits of small molecule therapies include lower costs, easier storage and transportation, and improved delivery to the cell [41]. This past year, I also worked to develop an antibody-related technology to improve ASO delivery. Once this technology is fully developed, JQ1 and BSP could be used in conjunction with existing technologies to improve editing efficiency. For this small molecule technology to become a viable add-on therapy, it would be useful to further analyze the downstream effects of bromodomain inhibition, specifically BRD4. Small epigenetic modulators affect many genes, and if there was a specific enhancer or transcription factor that only controlled ADAR levels, I could find a small molecule that targeted this region. ADAR2 is limited to A → I edits, and therefore cannot alter T, G, or C [2]. ADAR2 can edit premature start and stop codons, and can create start and stop codons, but it cannot yet perform deletions [11]. A common hallmark of neurodegenerative disease is protein misfolding and aggregation, and ADAR2 editing technologies must have the ability to correct these mistakes to effectively treat diseases [42].

Bromodomain inhibitors are currently being used in other unique ways in ASO research. Recently, *Kashyap et. al* conjugated JQ1 to a splice-switching oligonucleotide (SSO) to try to improve ASO delivery [43]. It had been established that JQ1 has a strong affinity for bromodomains, especially BRD4, and has been used in the transportation of cytoplasmic proteins into the nucleus [43]. Gibson et al. pioneered this technique by using a JQ1 "warhead" to induce nuclear transport of cytoplasmic proteins [43, 44]. The JQ1 domain creates a ligand-to-receptor reaction that can be exploited to shuttle other compounds into the nucleus, such as an ASO. There was a statistically significant increase in ASO efficacy after JQ1 conjugation, which promises to enhance other RNA-editing technologies if adapted [43]. There is a possibility that

the augmented editing efficiency of the LEAPER technology upon BSP and JQ1 treatment is due to increased nuclear import, but this argument would not explain the increase of ADAR2 levels. Improved ASO transport into the nucleus would lead to an increase in endogenous ADAR2 editing [43, 44].

Future plans for this research would include testing LEAPER technology with the addition of JQ1 and BSP in a different reporter cell system and targeting a different endogenous target. The original LEAPER paper targeted the *TP53* tumor suppressor gene, and with the addition of either JQ1 or BSP, I could possibly increase the editing efficiency [26]. For further confirmation that JQ1 and BSP can regulate ADAR2 expression, I plan to use different cell lines or types. ADAR proteins are most highly expressed in neurons, human cortical neuronal cells or a neuroblastoma cell line, like SK-N-MC, so I would use one of these cell lines [35]. While the addition of JQ1 or BSP has direct synergy with LEAPER technology, other RNA editing technologies like AIMers or REPAIR rely on pre-engineered proteins. Increasing the abundancy of ADAR2 would likely have no effect on REPAIR editing efficiency, because REPAIR consists of an ADAR2 catalytic domain fused to a dCas13-gRNA complex [19]. While the SGC library is full of epigenetic modulators, it may be worthwhile to screen other libraries of small compounds capable of structural interactions that increase recruitment of ADAR-mediated technologies.

Conclusion

This project demonstrates that small epigenetic modulators can be leveraged to increase the editing efficiency of current ADAR2-mediated RNA editing technologies. Using a validated HEK-293 reporter cell line, I screened through the SGC library to find epigenetic modulators that significantly affected the editing efficiency of the LEAPER technology. After multiple rounds of screening, I identified two pan-BET inhibitors, JQ1 and BSP, as enhancers of editing efficiency. To understand their mechanism of action, I conducted qRT-PCR and Western blot experiments, and I found that both compounds increase mRNA and protein levels of ADAR2. I also found that both compounds decrease ADAR1 mRNA expression. This finding suggests that JQ1 and BSP indirectly regulate ADAR2 expression, and this increase in ADAR2 protein increases the editing efficiency of the LEAPER technology. This discovery provides a clear path for characterizing the exact transcription factors or enhancers responsible for ADAR regulation.

The goal of this project was to discover epigenetic modulators that could be used in combination with ADAR-mediated editing technologies. Using the LEAPER technology as inspiration, I designed four smaller length gRNAs to see if, when used in combination with JQ1 and BSP, I could achieve editing with a smaller gRNA. This was unsuccessful and suggests that pan-BET inhibitors do not play a structural role in improving ADAR2 editing efficiency. It also validates the claim that a minimum length of 70nt is required for the LEAPER method [26]. The work I conducted laid essential groundwork for improving editing efficiency, which is one of the main limitations of ADAR-mediated RNA editing. Future optimization of this combination will help the development of highly effective RNA therapeutics to treat neurodegenerative diseases.

Graphs and Figures

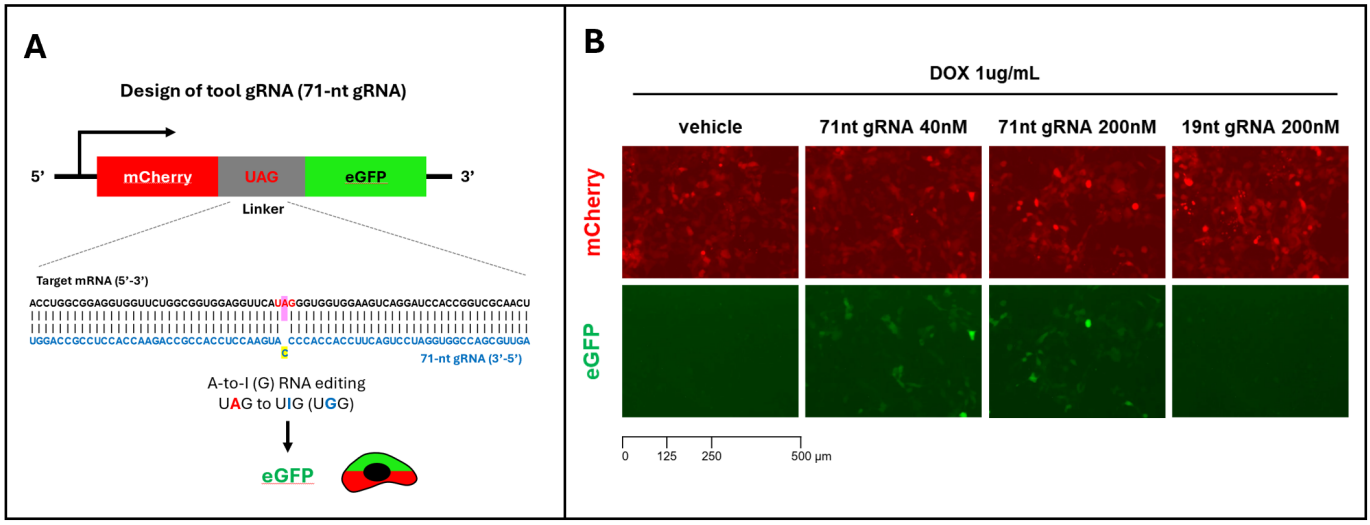

Figure 1: **Creation and validation of HEK-293 cell reporter system.** (A) Schematic of the design of tool gRNA. A linker sequence (with a mismatch stop codon) was inserted between the mCherry and eGFP coding sequence. Upon 71nt gRNA recognition and correction, eGFP is expressed. (B) Light microscopy validation of the fluorescent HEK-reporter cell line. Cells were induced with 1µg/mL of DOX for activation of tool gRNA. Red cells in the top row indicate mCherry expression, and green cells in bottom row indicate eGFP expression. Treatment is indicated by each column. Scale bar is located in the bottom left, with HEK293T cells averaging 10-15µm in size.

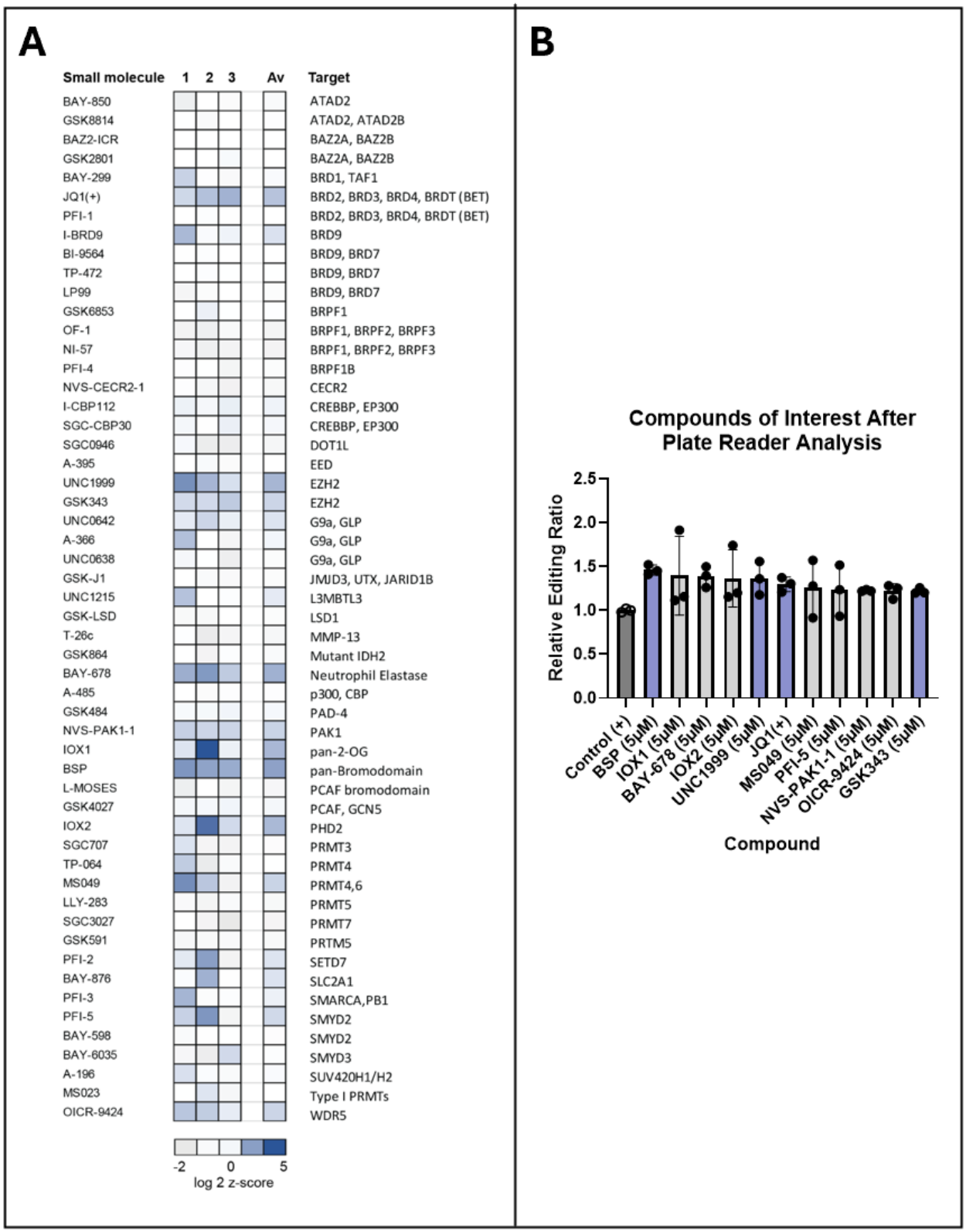

Figure 2: **Primary Screening of the SGC library using plate reader analysis:** (A) Heatmap of the primary screen of the SGC library. Each molecule and their targets are listed, screened in triplicate (in columns 1, 2, and 3). Color intensity reflects the $\log_2$ transformed z-score of the fold

change relative to the control. Darker blue indicates a deviation in editing rates from the average control. (B) Compounds of interest after primary plate reader analysis. Compounds were sorted by average normalized relative editing ratio. Compounds that target bromodomains are highlighted in blue.

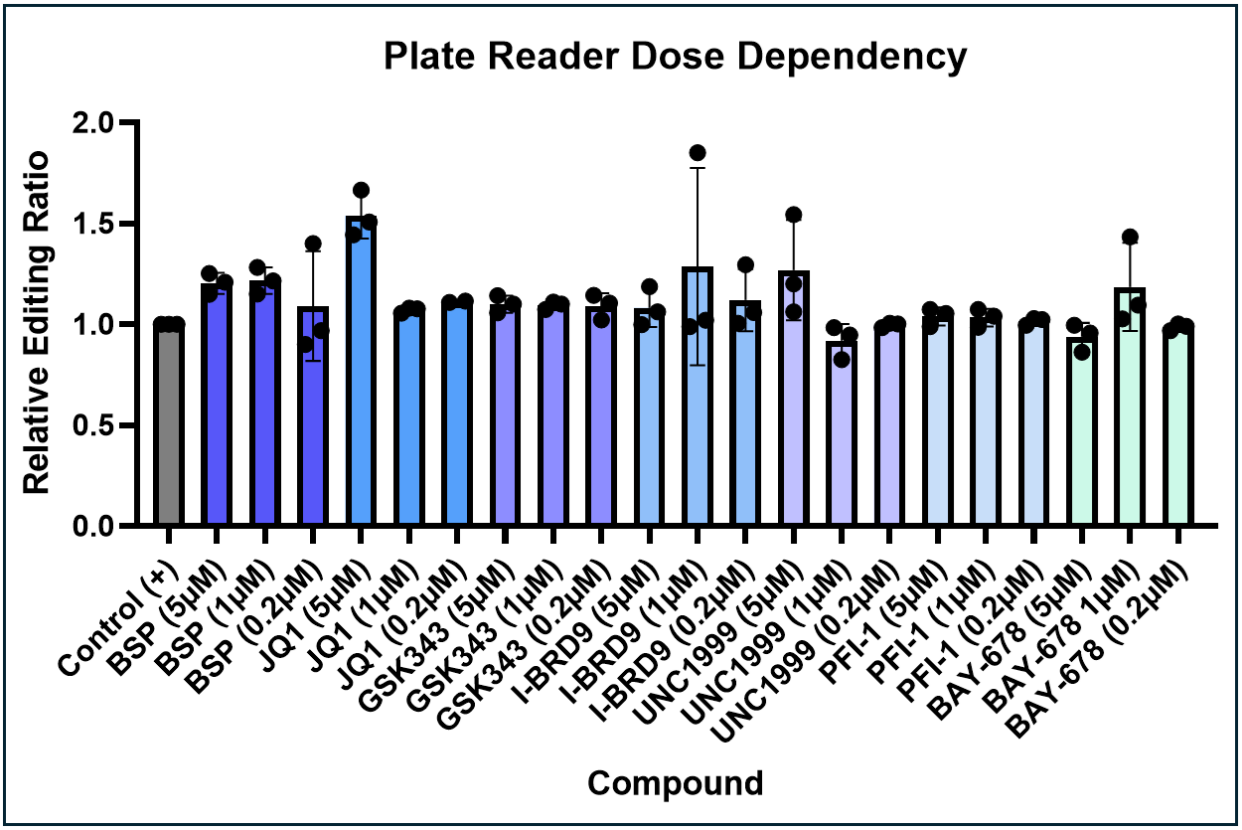

Figure 3: Plate Reader Dose Dependency Analysis. Using the plate reader assay format, a dose-dependency analysis was completed. Biological triplicates were used for each compound. SGC compounds were normalized to the positive control and separated into different colors on the graph. The compound concentrations were 5 μM, 1 μM, and 0.2μM. No statistical significance was found.

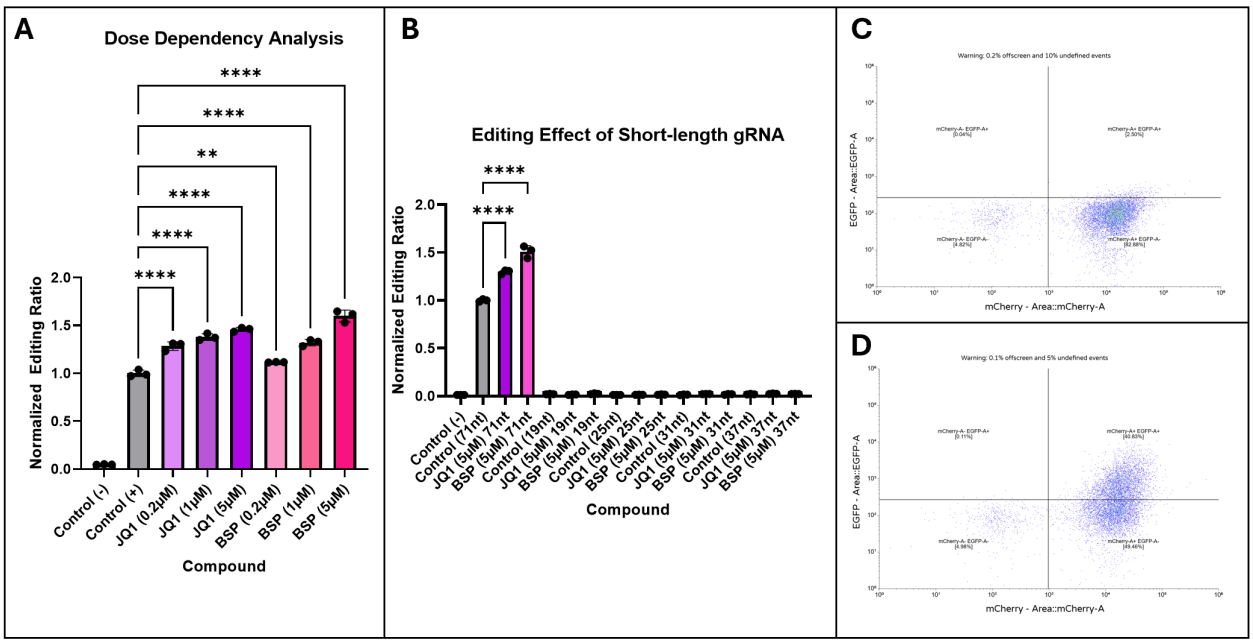

Figure 4: Flow cytometry analysis. (A) Dose dependency analysis using flow cytometry after 24-hour compound treatment. Concentrations of each compound were 0.2 μM, 1 μM, and 5μM. (B) Editing efficiency of short-length gRNAs. Concentration of each compound was 5μM. (C) Floreada.io analysis of negative control (no gRNA) from flow cytometer. Cells expressing only mCherry in Q4. (D) Floread.io analysis of 71nt gRNA after BSP treatment. Cells expressing mCherry and eGFP in Q1. Significance: ** P-value = 0.0086, **** P-value < 0.0001.

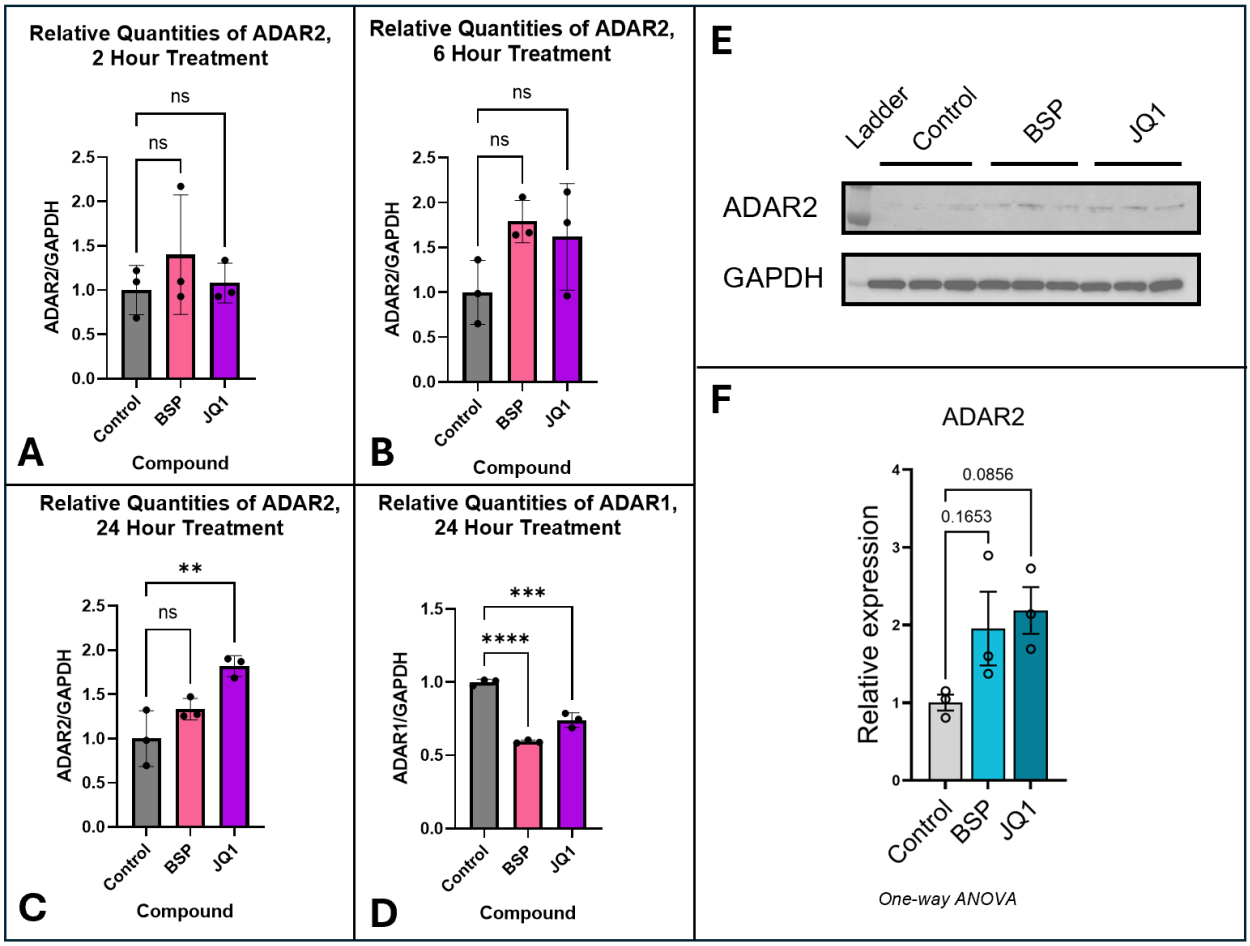

Figure 5: Investigation of the Mechanism of Action. (A) Relative levels of ADAR2 mRNA after 2-hour compound treatment. (B) Relative levels of ADAR2 mRNA after 6-hour compound treatment. (C) Relative levels of ADAR2 mRNA after 24-hour compound treatment. (D) Relative levels of ADAR1 mRNA after 24-hour compound treatment. (E) Western blot confirmation of ADAR2 protein levels after 24-hour compound treatment. (F) ImageJ quantification of ADAR2 protein levels after 24-hour compound, normalized to control. One-way ANOVA P-values = 0.1653 and 0.056, for BSP and JQ1 respectively. Significance: ** P-value = 0.0050, *** P-value 0.0001, and **** P-value < 0.0001.

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