From Assay Development to Novel Inhibitor Discovery: Disrupting Mitochondrial RNA Editing in Trypanosomes

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August 2024

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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2. Abstract

Trypanosomatid diseases, including human African trypanosomiasis, Chagas disease, and leishmaniasis, are debilitating infections caused by kinetoplastids that threaten the lives of millions of people and livestock. Although recent initiatives and programs aimed at controlling, reducing, and eliminating these so-called "neglected tropical diseases", there is still a need for novel drugs with desirable lead target profiles. Mitochondrial transcripts undergo a unique uridine insertion/deletion RNA modification in these parasites to become translatable and functional. This RNA editing pathway is mediated by a multiprotein complex called the "editosome", which has desirable features underpinning its suitability as a validated drug target, such as essentiality for parasite viability, absence in the host organism, multiple enzyme components, and conservation among disease-causing kinetoplastids. Unfortunately, no enzyme-specific inhibitors have been found within the context of the editosome complex, and there is limited information on how the editosome components assemble to perform the editing process. This dissertation aims to identify novel small molecule inhibitors as chemical probes for the editosome and as starting points for antiparasitic drug discovery.

First, I describe the development of three high-throughput amenable fluorescence resonance energy transfer (FRET) reporter-based RNA editing assays for precleaved deletion, insertion, and ligation that bypass the rate-limiting endonucleolytic cleavage step. The multiplex ribozyme-based insertion/deletion editing (RIDE) assay was introduced by modifying the reporter ribozyme sequence to simultaneously monitor U-insertion and deletion editing. These assays exhibit higher editing efficiencies with shorter incubation times and require significantly less purified editosome and 10,000-fold less ATP than previously published full-round *in vitro* RNA editing assays. This enables the identification of ATP-competitive inhibitors, as demonstrated with α, β-methylene ATP (a non-hydrolyzable ATP analog) as proof of principle.

Second, I present the high throughput screen (HTS) conducted in collaboration with Sanford Burnham Prebys to identify RNA editing inhibitors. We successfully miniaturized our FRET-

based full-round RNA editing assay, replicating the complete RNA editing process. We used it to screen 100,000 compounds against purified editosomes derived from *Trypanosoma brucei*, identifying seven primary hits. We sourced and evaluated various analogs to enhance the inhibitory and parasiticidal effects of these primary hits. Mode of action studies, utilizing the assays developed in the previous chapter, revealed that these inhibitors target essential catalytic activities, including the RNA editing ligase and interactions among editosome proteins. Although the primary hits did not exhibit any growth inhibitory effect on parasites, eight analog compounds effectively kill *T. brucei* and/or *Leishmania donovani* parasites at low micromolar concentrations. Our findings introduce novel molecular scaffolds with the potential for broad antitrypanosomal effects.

Third, I present the discovery of the editosome inhibitors by screening a library of widely used human drugs using the RIDE assay. Mode of action (MOA) studies of the identified hits, along with hit expansion efforts, unveiled compounds that interfere with RNA-editosome interactions and novel ligase inhibitors with IC_{50} values in the low micromolar range. *In vitro* and *in silico* analyses of our novel ligase inhibitor, epigallocatechin gallate, demonstrated binding characteristics similar to those of ATP. Overall, these inhibitors demonstrated potent trypanocidal activity, and considering their established safety profiles in humans, they are promising candidates for drug repurposing.

My work provides efficient, sensitive, HTS-amenable fluorescent-based assays for the identification and mechanistic characterization of small-molecule RNA editing inhibitors. In addition, the novel molecular scaffolds discovered in this dissertation, from pilot-scale and HTS screens, can serve as leads for pan-kinetoplastid drug development and as valuable research probes for studying the dynamic assembly and disassembly of the editosome.

3. Résumé

Les maladies à trypanosomatides, y compris la trypanosomiase humaine africaine, la maladie de Chagas et la leishmaniose, sont des infections débilitantes causées par des kinétoplastides qui menacent la vie de millions de personnes et de bétail. Bien que des initiatives et des programmes récents visent à contrôler, réduire et éliminer ces maladies tropicales dites « négligées », il reste encore un besoin de nouveaux médicaments avec des profils de cibles principaux souhaitables. Chez ces parasites, les transcrits mitochondriaux subissent une modification unique d'insertion/suppression de l'uridine pour être traduites et devenir fonctionnels. Cette voie d'édition de l'ARN est médiée par un complexe multiprotéique appelé « éditosome », qui possède des caractéristiques désirables supportant son adéquation en tant que cible thérapeutique validée, telles que son importance pour la viabilité du parasite, l'absence chez l'organisme hôte, plusieurs composants enzymatiques, et la conservation parmi les kinétoplastides pathogènes. Malheureusement, dans le contexte du complexe éditosome, aucun inhibiteur enzymatique spécifique n'a été trouvé, et il y a peu d'informations sur la façon dont les composants de l'éditosome s'assemblent pour effectuer le processus d'édition. Cette thèse vise à identifier de nouveaux inhibiteurs de petites molécules comme sondes chimiques pour l'éditosome et comme points de départ pour la découverte de médicaments antiparasitaires.

Tout d'abord, je décris le développement de trois essais d'édition de l'ARN basés sur des rapports de transfert d'énergie par résonance de fluorescence (FRET) adaptables à haut débit pour la suppression, l'insertion et la ligature préclivées, qui contournent l'étape limitante de clivage endonucléolytique. En modifiant la séquence du ribozyme rapporteur, l'essai d'édition multiplex basé sur le ribozyme d'insertion/suppression (RIDE) a été introduit pour surveiller simultanément l'édition d'insertion et de suppression de l'uridine. Ces essais présentent des efficacités d'édition plus élevées avec des temps d'incubation plus courts et nécessitent beaucoup moins d'éditosome purifié et 10 000 fois moins d'ATP que les essais d'édition de l'ARN *in vitro* complets publiés auparavant. Cela permet l'identification d'inhibiteurs compétitifs de l'ATP, comme démontré avec l'ATP α, β-méthylène (un analogue non hydrolysable de l'ATP) comme preuve de principe.

Ensuite, je présente le criblage à haut débit (CHD) réalisé en collaboration avec Sanford Burnham Prebys pour identifier les inhibiteurs de l'édition de l'ARN. Nous avons réussi à miniaturiser notre essai d'édition de l'ARN complet basé sur le FRET, qui réplique le processus complet d'édition de l'ARN, et l'avons utilisé pour cribler 100 000 composés contre des éditosomes purifiés dérivés de *Trypanosoma brucei*, identifiant sept hits primaires. Nous avons sourcé et évalué divers analogues pour améliorer les effets inhibiteurs et parasiticides de ces hits primaires. Les études sur le mode d'action, utilisant les essais développés dans le chapitre précédent, ont révélé que ces inhibiteurs ciblent des activités catalytiques essentielles, y compris la ligase de l'édition de l'ARN et les interactions entre les protéines de l'éditosome. Bien que les hits primaires n'aient montré aucun effet inhibiteur sur la croissance des parasites, huit analogues tuent efficacement les parasites *T*. brucei et/ou *Leishmania donovani* à de faibles concentrations micromolaires. Nos résultats introduisent de nouvelles structures de basesmoléculaires avec un potentiel d'effets anti-trypanosomiase larges.

Enfin, je présente la découverte d'inhibiteurs de l'éditosome par le criblage d'une bibliothèque de médicaments humains largement utilisés à l'aide de l'essai RIDE. Les études de mode d'action (MOA) des hits identifiés, ainsi que les efforts d'optimisation des hits, ont révélé des composés qui interfèrent avec les interactions ARN-éditosome et des nouveaux inhibiteurs de ligase avec des valeurs d'IC_{50} de l'ordre des faibles micromolaires. Les analyses *in vitro* et *in silico* de notre nouvel inhibiteur de ligase, l'épigallocatéchine gallate, ont démontré des caractéristiques de liaison similaires à celles de l'ATP. Dans l'ensemble, ces inhibiteurs ont montré une activité trypanocide puissante et, compte tenu de leurs profils de sécurité établis chez l'homme, ils sont des candidats prometteurs pour le repositionnement de médicaments.

Mon travail fournit des essais efficaces basés sur la fluorescence, sensibles et adaptables au CHD pour l'identification et la caractérisation mécanistique des inhibiteurs de petites molécules de l'édition de l'ARN. De plus, les nouvelles structures de base moléculaires découvertes dans cette thèse grâce à des criblages à petite échelle et CHD peuvent servir de leads pour le développement de médicaments pan-kinétoplastides, ainsi que de sondes de recherche précieuses pour étudier l'assemblage dynamique et le désassemblage de l'éditosome.

4. Acknowledgments

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr. Salavati. Words cannot fully capture the immense respect and appreciation I hold for you. You have been the best academic father anyone could ask for, providing unwavering support, guidance, and wisdom throughout my doctoral journey. Your patience, non-judgemental attitude, and encouragement have been instrumental in my success. I am incredibly fortunate to have had the privilege of working under your mentorship.

I extend my heartfelt thanks to my research advisory committee members, Dr. Albert Berghuis and Dr. Thavy Long. Your positive attitude and guidance have been crucial in navigating the challenges of my research. Your unwavering faith in my abilities, especially during times when I felt overwhelmed, has been a source of immense strength and motivation.

I am deeply grateful to my past and current lab mates: Akshaya, Nisha, Linhua, Amin, Homa, Pourya, Tamara, and Vanessa. Your hard work, camaraderie, and insightful discussions have been invaluable. Dr. Vaibhav Mehta, a special thank you for training me and being not only a mentor but a great friend. Your guidance and friendship have been immensely valuable to me. I am also deeply thankful to all the students and staff members of the Institute of Parasitology for creating a supportive and collaborative environment.

My gratitude extends to the NIH and CIHR for funding the research that has made this thesis possible.

To my amazing parents, Nadali and Kolsoum, your unwavering support and curiosity about my research have been a source of inspiration. To my wife's parents, Amrollah and Akram, your infinite support has been incredibly encouraging. To my family, Forough and Ali, thank you for always being there and bringing joy into my life.

Above all, I want to thank my beloved wife, Arezou Kamelshahroudi. You have been the lighthouse guiding me through the stormy seas of life and research. Without you, this thesis

would have been impossible. Your unwavering support, love, and belief in me have made this journey worthwhile. Thank you for being my greatest source of strength and happiness.

5. Contribution to knowledge

Mitochondrial RNA editing is a conserved pathway in kinetoplastids that involves the insertion and/or deletion of uridine residues. This process is mediated by a large protein complex called editosome, which has been the subject of research for a long time. However, there is still a gap in knowledge regarding the dynamic assembly and disassembly of this protein complex. Furthermore, while it has been proposed as a suitable target for drug discovery against kinetoplasts, no enzyme-specific inhibitors have yet been identified. To this aim, we developed multiple *in vitro* assays and discovered several RNA editing inhibitors through screening small molecule libraries. In summary, the main contributions of this research to the advancement of knowledge are:

1. Developing fluorescent-based *in vitro* HTS amenable precleaved assays that can monitor one or more RNA editing enzymes. The sensitivity of these assays allows for the identification of competitive inhibitors. Furthermore, the multiplex RIDE assay unifies both editing types in a single reaction tube, and the novel fluorescent gel-based assays provide powerful tools to define the mode of action of editosome inhibitors.
2. Conducting a large-scale high throughput screening and identification of several novel RNA editing inhibitors with various modes of action, including enzyme-specific inhibitors, subcomplex-specific inhibitors, and inhibitors with broad-spectrum effects on editosome. Additionally, several compounds with antiparasitic activity against *T. brucei* and *L. donovani* were discovered that could be further optimized towards new lead compounds.
3. Performing a pilot-scale screening on the NIH clinical collection using the newly developed multiplex assay, leading to the discovery of novel scaffolds demonstrating inhibitory effects on RNA editing, as well as broad spectrum effect on trypanosomes. Among the hits, EGCG showed great potency against both the editosome and KREL1 in the sub-nanomolar to low-micromolar range. Docking tools revealed that EGCG has the same binding poses as ATP in the KREL1 binding pocket. Considering the safety profiles

of the hits, they are promising candidates for repurposing towards anti-kinetoplastid treatments

6. Contribution of Authors

This thesis contains three manuscript-based chapters. Chapters III, IV, and V are reprints of published papers. Chapter III, published in the RNA Journal, is co-authored by me as the first author, Vaibhav Mehta, Rufaida Hassan Khan, Daniel Moses, and Reza Salavati. As the first author of the paper, I contributed to developing and optimizing FRET and gel-based editing assays, data analysis, and manuscript preparation. Dr. Vaibhav Mehta conceptualized multiplexing the assay, performed radiolabeled assays, and mentored me in experimental design and execution. Rufaida Hassan Khan assisted in assay optimization, and Daniel Moses contributed to manuscript preparation.

Chapter IV, published in Biochemical Pharmacology, is co-authored by me, Arezou Kamelshahroudi, Vaibhav Mehta, Fu-Yue Zeng, Ian Pass, Thomas D.Y. Chung, and Reza Salavati. I share co-first authorship with Arezou Kamelshahroudi. In this chapter, I contributed to editosome preparation, performing mode of action assays, data analysis, and manuscript preparation. Arezou Kamelshahroudi contributed to editosome preparation, performing mode of action assays, and data analysis. Dr. Vaibhav Mehta assisted in editosome and substrate RNA preparation. Fu-Yue Zeng and Ian Pass contributed to high-throughput screening miniaturization, execution and dose response full round assay conducted on the initial hits. Dr. Thomas D.Y. Chung contributed to project management, manuscript review, and editing.

Chapter V, published in ACS infectious diseases, is co-authored by me, Arezou Kamelshahroudi, Vanessa Pitsitikas, Kenneth A Jacobson and Reza Salavati. I contributed to executing the pilot-scale screen, dose-response studies using the editosome, data analysis, docking studies, and manuscript preparation. Arezou Kamelshahroudi conducted the mode of action studies. Vanessa Pitsitikas performed dose-response studies on recombinant REL1 and REL2/A2. Dr. Jacobson contributed to the ligase inhibitor expansion.

Dr. Reza Salavati provided intellectual input, supervised the projects, secured funding, and contributed to the experimental design, data analysis, and the preparation, review, and editing of the manuscripts in all of these studies.

7. Lists of Abbreviations, Figures, and Tables

7.1 List of Abbreviations

Abbreviations: ABMA, α, β-methylene ATP; ATA, aurintricarboxylic acid; ATP, adenosine triphosphate; cryo-EM, cryo-electron microscopy; DMSO, dimethyl sulfoxide - "universal" compound solvent; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; ES, editing site; ExoUase, uridyl exonuclease; FAM, fluorescein maleimide - thiol reactive dye of isomerically pure 6-FAM; FRET, Förster resonance energy transfer; gA6, Guide RNA for pre-edited ATPase 6 mRNA editing; gHHR, Guide RNA for pre-edited hammerhead ribozyme editing; gHHRc, Guide RNA with an extra cytidine in it sequence for pre-edited hammerhead ribozyme editing; gRNA, guide ribonucleic acid; GRBC, Guide RNA-binding complex; HAT, human African trypanosomiasis; HHR, hammerhead ribozyme; HTS, high-throughput screening; IC_{50}, inhibitory concentration at 50% inhibition; IDT, Integrated DNA Technologies - a company; kDNA, kinetoplast DNA; KMRP1/2, kinetoplastid mitochondrial RNA-binding proteins 1 and 2; KREL, kinetoplastid RNA editing ligase; KREN, kinetoplastid RNA editing endonuclease; KREPA, kinetoplastid RNA editing protein; KRET, kinetoplastid RNA editing TUTase; KREX, kinetoplastid RNA editing exonuclease; MEAT1, mitochondrial editing-like complex-associated TUTase 1; MIC, minimum inhibitory concentration; MPsome, mitochondrial 3′ processome; MRB1, Mitochondrial RNA Binding Complex 1; monoclonal antibody, mAb; MOA, mode of action; OB, oligonucleotide binding; PAGE, polyacrylamide gel electrophoreses; PDD, phenotypic drug discovery; PAMC, olyadenylation Mediator Complex; qPCR, quantitative polymerase chain reaction; RECC, RNA-editing catalytic complex; REH2C, RNA Editing Helicase 2 Complex; REMC, RNA Editing Mediator Complex; RESC, RNA Editing Substrate Binding Complex; RGG, arginine-glycine-glycine; RIDE, ribozyme-based insertion/deletion editing; RT, room temperature; SAR, structure activity relationship; S/B, signal-to-background; SC, subcomplex; TAP-tagged, tandem affinity purification; TDD, target-based drug discovery; TUTase, terminal uridyltransferase; Us , uridylates; uridine insertion/deletion, U-indel

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8. CHAPTER I: Introduction

Neglected tropical diseases such as human African trypanosomiasis (HAT), Chagas disease, and leishmaniasis are caused by protozoan parasites belonging to the Kinetoplastida class (Stuart, Brun et al. 2008). These diseases collectively threaten the lives of millions of people and livestock, primarily in tropical and subtropical regions. HAT, caused by *T. brucei* and transmitted by tsetse flies, continues to be a major health issue in sub-Saharan Africa, leading to severe morbidity and mortality (WHO 2023). Chagas disease, caused by *T. cruzi* and spread by reduviid bugs, predominantly affects Central and South America, resulting in chronic heart and digestive system disorders and significant mortality (Gómez-Ochoa, Rojas et al. 2022). Leishmaniasis, which includes cutaneous, mucocutaneous, and visceral forms (also called kala-azar), is caused by more than twenty different *Leishmania* species. It is distributed across tropical and subtropical areas and can be fatal, particularly the visceral form if left untreated (WHO 2016). While these diseases primarily affect millions in tropical and subtropical regions, an increasing number of cases are being reported in developed countries due to factors such as blood transfusions, organ donations from infected individuals, and infections among returning soldiers, immigrants, and travelers. This highlights the urgent need for new therapeutic approaches (Aronson, Sanders et al. 2006, Keynan, Larios et al. 2008, Ready 2010, Manne-Goehler, Umeh et al. 2016, Field, Horn et al. 2018, Amanda Irish 2022).

Despite ongoing efforts to control, reduce, and eliminate these neglected tropical diseases, current treatment options remain inadequate (Field, Horn et al. 2017, De Rycker, Wyllie et al. 2023). The available drugs are often toxic, costly, and require invasive administration routes (Supuran 2023). Additionally, resistance to many existing drugs has emerged, exacerbating the need for new therapeutic agents with improved efficacy and safety profiles. One promising approach in drug discovery targets unique biological processes in kinetoplastids, which are essential for the parasites' survival but absent in mammalian hosts, making them ideal drug targets (Field, Horn et al. 2017). One such pathway is mitochondrial RNA editing, which involves the specific insertion and/or deletion of uridines (Us) in mitochondrial transcripts, converting immature transcripts into translatable functional mRNAs (Benne, Van den Burg et al.

1986, Fidalgo and Gille 2011, Salavati, Moshiri et al. 2012, Field, Horn et al. 2017). This post-transcriptional editing process is crucial for properly translating essential components of the mitochondrial oxidative phosphorylation system. A set of short non-coding RNA sequences called guide RNA (gRNA) carry the information on where the editing should occur (Blum, Bakalara et al. 1990, Blum and Simpson 1990), and the entire process is mediated by a large multiprotein complex known as the editosome. Composed of multiple enzymes, such as endonucleases, terminal uridylyl transferases, exonucleases, and ligases (Goringer 2012, Aphasizheva, Zhang et al. 2014, Read, Lukes et al. 2016, Aphasizheva, Alfonzo et al. 2020), the editosome's essentiality for parasite survival (Schnaufer, Panigrahi et al. 2001, Drozdz, Palazzo et al. 2002, Huang, O'Hearn et al. 2002, Aphasizhev, Aphasizheva et al. 2003, Wang, Ernst et al. 2003, Carnes, Trotter et al. 2005, Salavati, Ernst et al. 2006, Guo, Ernst et al. 2008, Ernst, Panicucci et al. 2009, Carnes, Gendrin et al. 2023, Davidge, McDermott et al. 2023), along with its absence in mammalian hosts and its large drug binding landscape, makes it an attractive target for antiparasitic drug development (Salavati, Moshiri et al. 2012).

Despite suggestions that the basic mechanism of RNA editing involves the stepwise coordinated activity of the enzymes, the detailed mechanisms of its dynamic assembly/disassembly and how the RNA editing holoenzyme interacts with RNA substrates remain poorly understood. Selective small molecule inhibitors are powerful tools for studying the conformational dynamics of multi-subunit protein complexes like the ribosome or spliceosome (Ermolenko, Spiegel et al. 2007, Jurica 2008, O'Brien, Matlin et al. 2008, Effenberger, Urabe et al. 2017). Thus, discovering potent small molecules can be useful for the functional analysis of the editosome and also serve as starting points in drug discovery programs.

This dissertation addresses these gaps by identifying novel small-molecule inhibitors of the editosome and developing assays to facilitate high-throughput screening (HTS) for potential drugs. Hence, the specific objectives of this research are to: (a) develop HTS-compatible fluorescence resonance energy transfer (FRET)-based RNA editing assays, (b) discover novel scaffolds with an inhibitory effect on *in vitro* RNA editing through HTS using the full-round RNA editing assay previously developed in our lab, and identify the hits' mode of action against

the editosome, (c) conduct a pilot-scale screen of a library of established human drugs using the RIDE assay, pursuing a drug repurposing strategy to discover drugs that inhibit RNA editing.

T. brucei was used as a "model" system throughout this research since this early-branching eukaryote has been studied extensively over the past decades and has developed into a valuable model organism. Therefore, the editosome complex and its components are derived from *T. brucei*.

The research presented in this dissertation provides significant contributions to the field of neglected tropical disease drug discovery. By developing efficient and sensitive assays for identifying RNA editing inhibitors, this work lays the foundation for discovering new antiparasitic agents. The novel molecular scaffolds identified through HTS, and pilot-scale screens have the potential to serve as leads for broad-spectrum kinetoplastid drug development. Moreover, these inhibitors offer valuable research tools for dissecting the dynamic assembly and function of the editosome, advancing our understanding of this critical biological process.

9. CHAPTER II: Literature review

9.1 Trypanosomatid pathogens and their burden

The three related trypanosomatid pathogens, "the Tritryps", namely *Trypanosoma brucei*, *Trypanosoma cruzi*, and various *Leishmania* species, represent a significant threat to global public health. Despite their shared genomic organization and cellular structures, these organisms cause distinct diseases in humans, each transmitted by its respective vector (Aronson, Sanders et al. 2006, Keynan, Larios et al. 2008, Stuart, Brun et al. 2008, Ready 2010, Manne-Goehler, Umeh et al. 2016, Field, Horn et al. 2018, Amanda Irish 2022).

T. brucei is an extracellular parasitic protist, and two of its subspecies are human pathogens and cause HAT (*T. b. gambiense,* responsible for 92% of the cases and *T. b. rhodesiense,* causing the remainder) (WHO 2023). The third subspecies (*T. b. brucei*), causes nagana in livestock and is not pathogenic to humans, making it a safe choice for study. Infection begins with a bite from the tsetse fly (*Glossina* spp.) bite which is the insect vector of the unicellular parasite. HAT, or sleeping sickness, progresses in two stages. Stage I, the haemolymphatic stage, involves the parasite proliferating in the blood and lymph. The second stage, neurological dysfunction, occurs when the parasite crosses the blood-brain barrier and invades the central nervous system, and is fatal if left untreated.

In the 20th century, Africa faced two major outbreaks of HAT caused by *T. b.* gambiense. Initial efforts nearly eradicated the disease by the 1960s, but neglect and conflict led to a resurgence in the 1990s, peaking at approximately 40,000 reported cases (Barrett 2006, Brun, Blum et al. 2010, Franco, Simarro et al. 2014). Recent efforts by the WHO have reduced the incident of HAT, with 992 and 663 cases reported in 2019 and 2020, respectively. Despite this progress, interruptions due to the COVID-19 pandemic pose a risk of resurgence, as evidenced by the number of cases rising to 802 in 2021 and 837 in 2022. Climate change may further influence HAT distribution and incidence (Lord, Hargrove et al. 2018, Aliee, Castaño et al. 2021, Franco, Cecchi et al. 2022,

WHO Accessed on May 2024). It is also worth noting that the total costs of either the control or elimination program for HAT has been estimated to be nearly US$1 billion over the next decade (Sutherland and Tediosi 2019).

T. cruzi is an intracellular parasite that inhabits different cell types in humans, including heart and muscle cells, fibroblasts, gut, and central nervous system (Rassi, Rassi et al. 2010, Rassi, Rassi et al. 2012, Guhl and Ramírez 2021, De Fuentes-Vicente, Santos-Hernández et al. 2023). It causes Chagas disease, also called American trypanosomiasis, which can eventually progress and lead to serious cardiac and digestive problems, underscoring its clinical complexity and long-term health implications. An estimated 6–7 million individuals globally are believed to harbor *T. cruzi* infections, resulting in roughly 12,000 fatalities annually (WHO 2024). Chagas disease spreads to humans and numerous animal species through large reduviid bugs of the subfamily Triatominae. Chagas disease transmits through the feces of infected blood-sucking triatomine bugs, primarily through insect-to-human contact. Infection arises when the bug's feces enter an open wound or mucous membrane, such as the nose or eyes (Rassi, Rassi et al. 2010, Rassi, Rassi et al. 2012). This disease progresses through two stages: acute and chronic. The latter phase, often linked to heart muscle complications (referred to as Chronic Chagas Cardiomyopathy, or CCM), takes decades to manifest (Nunes, Beaton et al. 2018).

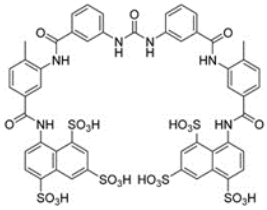
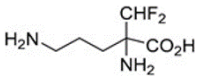
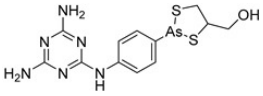
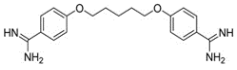
Over twenty different *Leishmania* species are responsible for the three main forms of leishmaniasis: visceral (also called kala-azar, the most dangerous form), cutaneous (the most common form), and mucocutaneous. Leishmaniasis is one of the most widespread neglected tropical diseases, affecting 98 countries globally, with an estimated 700,000 to 1 million new cases annually. The clinical manifestations of leishmaniasis are influenced by the interplay between parasite traits, vector biology, and host factors, with immune responses being the most pivotal among the host factors (Colmenares, Kar et al. 2002, Tripathi, Singh et al. 2007, Burza, Croft et al. 2018). These intracellular parasites are transmitted by the bite of infected female phlebotomine sandflies and infect macrophages (WHO 2016). *Leishmania* species, known primarily for causing cutaneous disease, may trigger disseminated illness in individuals with

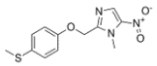
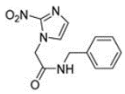
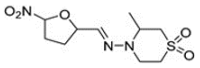
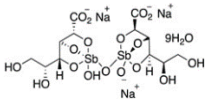
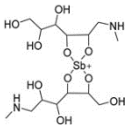
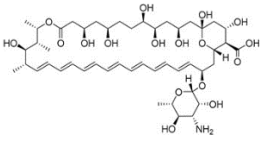
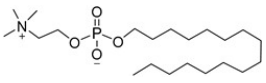
compromised cellular immunity, such as those living with human immunodeficiency virus (Handler, Patel et al. 2015, Kantzanou, Karalexi et al. 2023).

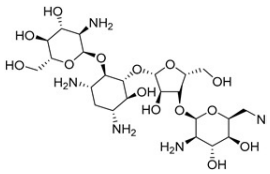
9.2 Anti-trypanosomatid therapeutics

The current regimen against the Tritryps is listed in Table 9.1. There are many drawbacks associated with the current treatments of the Tritryps diseases, including inefficacy, toxicity, an invasive route of administration, limited distribution to rural areas, and emerging resistance to the drugs (Castro, de Mecca et al. 2006, Kedzierski, Sakthianandeswaren et al. 2009, Field, Horn et al. 2017) Specifically, most HAT drugs are very old and are quite toxic to any cell that they enter (Koning 2020). A study in 2021 argued that the recently approved Fexinidazole for HAT is worse than the conventional nifurtimox-eflornithine combination therapy. They concluded that treatment with Fexinidazole resulted in more deaths and relapse occurrences (Lutje, Probyn et al. 2021). Hence, there is an urgent need for developing new drugs to address these unmet medical needs. Also, target product profiles for new drugs against trypanosomatid diseases have been suggested (Katsuno, Burrows et al. 2015, Rao, Barrett et al. 2019, DNDi 2024). For example, in the case of Chagas disease, the medications should prevent progression to chronic disease and have a shelf life of over two years in tropical conditions. For leishmaniasis, the new drugs should be effective against all forms of visceral and cutaneous leishmaniasis, offering a short-course treatment that ensures a relapse-free cure. For HAT, the ideal drug must be effective against both *T. b. gambiense* and *T. b. rhodesiense* in both stages of the disease.

Table 9.1 Current drug treatments for tritryps diseases *((Berman, Waddell et al. 1985, Saha, Mukherjee et al. 1986, McCarthy, Wortmann et al. 2015, Field, Horn et al. 2017, Moreira Vanessa, de Jesus Luís Cláudio et al. 2017, Fairlamb and Horn 2018, Koning 2020, Matos, Viçosa et al. 2020, Kourbeli, Chontzopoulou et al. 2021, Altamura, Rajesh et al. 2022, Albisetti, Halg et al. 2023, De Rycker, Wyllie et al. 2023))*

Disease	Drug	Structure	Comment	Route of administration	Proposed mechanism of action
Human African trypanosomiasis	Suramin		• Only suitable for first-stage infection with *Trypanosoma brucei rhodesiense* • Associated with toxicity	Intravenous injections	Polypharamacology (inhibiting DNA helicase, glycolytic enzymes, glycerophosphate oxidase, serine oligopeptidase and RNA editing ligase)
	Eflornithine (monotherapy or in combination with nifurtimox: NECT)		• Suitable for second-stage disease • High cost • Not efficacious against *T.b. rhodesiense*	Intravenous injections	Inhibits trypanosome ornithine decarboxylase
	Melarsoprol		• Suitable for second-stage disease • Highly toxic and causes substantial levels of drug-related mortality due to reactive encephalopathy • High levels of treatment failure reported in some regions	Intravenous injections	Not clear exactly but inhibits trypanothione reductase, also results in inhibition of energy metabolism
	Pentamidine		• Only suitable for first-stage infection with *T. b. gambiense* • Associated with toxicity	Intramuscular injections	Not clear exactly but accumulates strongly in the trypanosome's single mitochondrion and binds to the kinetoplast DNA (kDNA). Also, inhibits mitochondrial topoisomerase II activity.

Disease	Drug	Structure	Comment	Route of administration	Proposed mechanism of action
	Fexinidazole		•Suitable for first-stage and non-severe second-stage infection caused by *T. b. gambiense*	Oral	The precise mechanism is not known but it is suggested that fexinidazole is metabolized in the body into two active metabolites (M1/M2) that damage DNA and proteins.
Chagas disease	Benznidazole		• Tolerability, toxicity, incompliance	Oral	Inhibits the synthesis of DNA, RNA, and proteins
	Nifurtimox		• Tolerability, toxicity, incompliance	Oral	Creates oxygen radicals, reacts with nucleic acids of the parasite and cause significant DNA breakdowns
Leishmaniasis	Pentavalent Antimonials (Sodium stibogluconate)		• First-line treatment • High resistance in some regions of India • Toxicity	Intramuscular or intravenous injection	Inhibits macromolecular biosynthesis in amastigotes and can inhibit glycolysis step of metabolism and fatty acid oxidation of the parasite
	Meglumine antimonate		• First-line treatment • High resistance in some regions of India • Toxicity	Intramuscular or intravenous injection	Promote oxidative stress-derived DNA damage and and can inhibit glycolysis step of metabolism and fatty acid oxidation of the parasite
	Amphotericin B		• Very effective in regions with resistance to pentavalent antimonials • High toxicity	Intravenous injections	Targets membrane sterols that result in a loss of the permeability barrier to small metabolites
	AmBisome®	liposomial formula of amphotericin B	• Well tolerated High cost	Intravenous injections	
	Miltefosine		• Very high efficacy for VL • Teratogenic • Increasing treatment failures	Oral	Disrupting parasite's intracellular Ca^{2+} homeostasis

Disease	Drug	Structure	Comment	Route of administration	Proposed mechanism of action
	Paromomycin		• Ototoxicity	Intramuscular injection	Not completely understood. However, inhibition of translocation and recycling of ribosome subunits and modification in mitochondrial membrane potential have been proposed.

9.3 Drug discovery strategies

Two distinct paradigms exist for novel drug discovery: phenotypic drug discovery (PDD) and target-based drug discovery (TDD). Within the framework of PDD, chemical entities capable of eliciting alterations in cellular phenotypes are identified and subsequently subjected to comprehensive mode of action studies and iterative optimization processes. Crucially, PDD does not necessitate a priori knowledge of specific molecular targets and has been empirically validated as a powerful tool for the identification of novel trypanocidal agents (Sykes, Baell et al. 2012, Jones and Avery 2013, Buckner, Buchynskyy et al. 2020). However, this methodology is not without its inherent challenges. For instance, it necessitates exhaustive follow-up assays to discern and exclude false positives, demands meticulous molecular profiling to ensure the consistency of the mechanism of action, and requires the elucidation of underlying biological mechanisms (Moffat, Vincent et al. 2017).

Conversely, TDD involves establishing *in vitro* assays designed to identify compounds capable of inhibiting specific molecular targets, such as enzymes. These identified compounds are subsequently subjected to iterative optimization processes to enhance their potency (Patrick 2018). This strategy has been successfully employed to discover novel therapeutic agents for the Tritryps diseases (Sharlow, Lyda et al. 2010, Moshiri, Mehta et al. 2015, Zimmermann, Hall et al. 2016, Salas-Sarduy, Landaburu et al. 2017, Martinez de Iturrate, Sebastian-Perez et al. 2020, Saldivia, Fang et al. 2020, McNae, Kinkead et al. 2021, Perez, Bouvier et al. 2021). However, a

significant challenge inherent to TDD is the potential disconnect between *in vitro* and *in vivo* potency, suggesting that a compound's efficacy in a controlled environment may not translate to effectiveness within a living organism. This discrepancy often arises from inadequate cellular uptake of the drug (McNae, Kinkead et al. 2021).

Ultimately, both PDD and TDD have their respective advantages and limitations, and neither can be deemed categorically superior to the other, despite a larger number of approved drugs historically favoring the phenotypic approach (Sadri 2023). A detailed comparative analysis of these methodologies is provided in (Moffat, Vincent et al. 2017). For this research, our focus will be on target-based screening.

The initial phase of TDD necessitates the identification of an appropriate molecular target. Trypanosomatids, which diverged early in evolutionary history, exhibit a plethora of unique biochemical features that render them attractive targets for drug discovery (Field, Horn et al. 2017). These features include a compartmentalized glycolysis, divergent kinetochore and origin recognition complexes, a single motile flagellum with a paraflagellar rod, a highly intricate mitochondrial DNA structure known as kinetoplast DNA (kDNA)—the hallmark of the Kinetoplastid order of parasites—and extensive editing of kinetoplast-encoded transcripts (Damasceno, Marques et al. 2021). Several drugs have been found to directly impact the mitochondrial physiology and ultrastructure of these parasitic protists (Rodrigues and de Souza 2008, Sen and Majumder 2008, de Souza, Attias et al. 2009). Our laboratory has dedicated several years to investigating the unique and essential mitochondrial RNA editing process. Building on this foundation, our efforts concentrate on identifying inhibitors of this specific biochemical pathway in *T. brucei,* the model organism.

9.4 Energy regulation, mitochondrion, and kinetoplast of T. brucei

T. brucei contains a single large, ramified mitochondrion that spans the length of the parasite and undergoes extensive remodeling and remarkable metabolic rewiring during its digenetic life cycle, indicating substantial alterations in the *T. brucei*'s energy metabolism (Souza, Carvalho et

al. 2010, Zíková 2022). These parasites can swiftly adjust their metabolism in response to environmental changes and differentiation cues (Zíková 2022). In the glucose-rich bloodstream of their mammalian host, the bloodstream form of *T. brucei* generates most of its cellular ATP through aerobic glycolysis. Conversely, in the glucose-poor and amino acid-rich tissues of the tsetse fly, the procyclic form of *T. brucei* switches to an amino acid catabolism and relies on mitochondrial oxidative phosphorylation for ATP production. Nevertheless, the molecular mechanisms underlying the functional remodeling of mitochondria are still not well understood (Lamour, Rivière et al. 2005, Stuart, Brun et al. 2008, Smith, Bringaud et al. 2017, Zíková 2022).

The electron transport chain in *T. brucei* comprises five enzymes that interact with the ubiquinone pool within the inner mitochondrial membrane. Complex I (NADH dehydrogenase), the largest electron transport chain complex, consists of at least 46 subunits from both nuclear and mitochondrial genomes. Despite extensive research, the biological significance of complex I in *T. brucei* remains unclear, as it appears to play a minimal role in electron transfer and proton translocation in procyclic and bloodstream forms (Surve, Heestand et al. 2012, Duarte and Tomás 2014). *T. brucei* has an alternative type II NADH dehydrogenase, NDH2, which transfers electrons from NADH to ubiquinone without proton pumping (Fang and Beattie 2002). NDH2 is vital for procyclic cell growth, supporting the proton motive force via the ubiquinone/ubiquinol pool linked to complexes III and IV. Moreover, the inner mitochondrial membrane enzyme houses an FAD-dependent glycerol-3-phosphate dehydrogenase, crucial for bloodstream cells that rely entirely on glucose oxidation; it sustains the NAD+/NADH redox balance within the glycosome and facilitates a high rate of glycolysis(Albert, Haanstra et al. 2005, Škodová, Verner et al. 2013). The canonical complex II (succinate dehydrogenase), directly involved in the TCA cycle by oxidizing succinate to fumarate and supplying electrons to the ubiquinone pool, is vital for the insect forms of *T. brucei* and aids in the oxidative metabolism of proline (Coustou, Biran et al. 2008). Its activity increases significantly during the metacyclic stage (Christiano, Kolev et al. 2017), although it is not essential for bloodstream form parasites (Alkhaldi, Martinek et al. 2016, Zíková 2022).

In procyclic *T. brucei* parasites, complexes III (ubiquinol:cytochrome c oxidoreductase) and IV (cytochrome c oxidase) are primary contributors to the mitochondrial membrane potential (Horváth, Horáková et al. 2005), but their abundance decreases during metacyclic development (Doleželová, Kunzová et al. 2020, Naguleswaran, Fernandes et al. 2021), leading to reduced membrane potential and ATP production by oxidative phosphorylation. This reduction activates AMP-activated kinase, which downregulates ATP-consuming pathways during differentiation (Herzig and Shaw 2018). Complexes III and IV are completely absent in both long slender and short stumpy bloodstream forms (Naguleswaran, Fernandes et al. 2021). Mitochondrial F_oF_1 - ATP synthases (complex V) primarily generate ATP from proton movement across the mitochondrial membrane, though they can also hydrolyze ATP when mitochondrial physiology changes (Schnaufer, Clark-Walker et al. 2005, Zíková, Schnaufer et al. 2009, Walker 2013). In *T. brucei*, this enzyme is crucial for procyclic forms and is required for development in the fly vector, but its role and regulation during differentiation remain unclear. Additionally, in the bloodstream forms of *T. brucei*, the enzyme's hydrolytic activity is upregulated to maintain the vital mitochondrial membrane potential, highlighting its importance in parasite survival and potential as a therapeutic target (Acestor, Zíková et al. 2011, Panicucci, Gahura et al. 2017). A recent study showed that ATP/ADP carrier is the exclusive transporter capable of importing ATP into the mitochondrial matrix, fueling the hydrolytic function of the F_oF_1-ATP synthase. However, the removal of ATP/ADP carrier does not impact parasite growth, virulence, or the levels of mitochondrial membrane potential ($\Delta\Psi m$) since the long slender bloodstream form mitochondrion can generate ATP via substrate-level phosphorylation pathways (Taleva, Husová et al. 2023).

Another unique feature of the double membrane-bound mitochondrion is the peculiar, dense disc-shaped mitochondrial nucleoid, or kinetoplast, which measures approximately 450 nm in diameter and 150 nm in height (Jakob, Hoffmann et al. 2016)). This structure consists of fibrous structured kDNA and associated proteins (Englund, Guilbride et al. 1996, Jakob, Hoffmann et al. 2016). The term 'kinetonucleus' was introduced by Robertson in 1913 to describe this entity (Robertson 1913), and later, in 1924, Bresslau and Scremin identified DNA within this structure, subsequently termed kinetoplast (Steinert, Firket et al. 1958, Burton and Dusanic 1968,

Amodeo, Bregy et al. 2023). kDNA, the mitochondrial DNA network, is composed of interlocked DNA rings (Figure 9.1), including about 50 large circular DNAs (20–25 kb, known as maxicircles) and 5000-10000 small heterogenous circular DNAs (1 kb, known as minicircles) (Jensen and Englund 2012, Cooper, Wadsworth et al. 2019). kDNA accounts for up to 20% of the total cellular DNA (Riou and Delain 1969, Mensa-Wilmot, Hoffman et al. 2019) and is located adjacent to the basal body of the flagellum, connected to it by the tripartite attachment complex, which may organize kDNA replication and division (Lukes, Guilbride et al. 2002, Ogbadoyi, Robinson et al. 2003, Povelones 2014, Schneider and Ochsenreiter 2018). Maxicircles in trypanosomes, functionally equivalent to the other eukaryotic mitochondrial DNAs, encode 18 genes, including components of the mitochondrial respiratory chain and ribosomal RNAs (9S and 12S). These maxicircle genes are often incomplete and may lack a start codon (ATG) (Borst, Fase-Fowler et al. 1980), resulting in “cryptogenes” that require post-transcriptional maturation to become translationally competent and functional, leading to the discovery of RNA editing, discussed further in section *7.2.5* (Benne, Van den Burg et al. 1986). Minicircles constitute approximately 95% of the kinetoplast mass and encode non-translatable trans-acting RNA sequences called gRNAs, which carry the information for RNA editing. Moreover, the minicircle repertoire changes based on species, strains, and life stages (Kirby, Sun et al. 2016, Rusman, Floridia-Yapur et al. 2021).

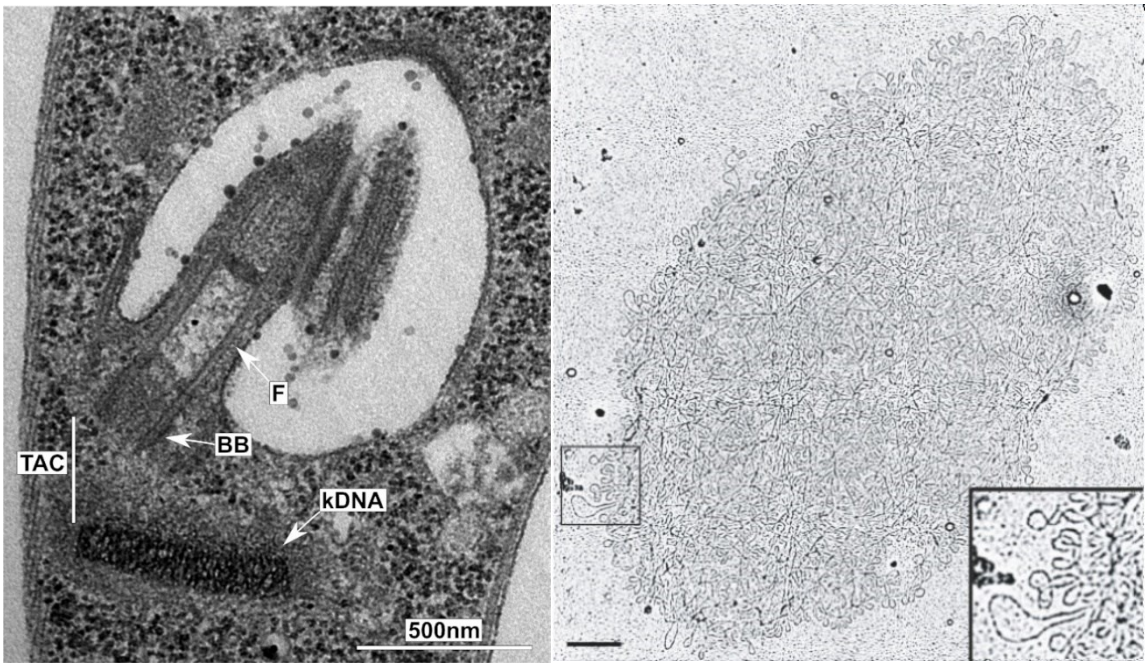


Figure 9.1 Mitochondrion and kDNA network of a kinetoplsatid *(A) A Transmission electron microscopy image of a Trypanosoma brucei mitochondrion showing kinetoplast, basal body, flagellum, and tripartite attachment complex (TAC) (Amodeo, Bregy et al. 2023) (B) The intercalated network of minicircles and maxicircles (Roy Chowdhury, Bakshi et al. 2010) (Available via license: CC BY 4.0)*

9.5 Mitochondrial RNA editing

In 1986, Benne *et al* found an inconsistency between a mitochondrial transcript (cytochrome-c oxidase subunit II (COII)) and its corresponding DNA sequence on the maxicircle, leading to the discovery of the first type of RNA editing in *T. brucei* and *Crithidia fasciculata* (Benne, Van den Burg et al. 1986). Subsequently, other types of RNA editing were discovered in different organisms (Randau, Stanley et al. 2009, Li and Mason 2014). These modifications include either insertion/deletion or base substitution of nucleotides. These modifications affect various RNA substrates, including mRNA (Powell, Wallis et al. 1987), tRNA (Alfonzo, Blanc et al. 1999, Lavrov, Brown et al. 2000), rRNA (Decatur and Fournier 2003), and miRNA (Choudhury, Tay et al. 2012, García-López, Hourcade et al. 2013).

Mitochondrial RNA editing in trypanosomes is an essential post-transcriptional modification of maxicircle genes that entails inserting and/or deleting uridylates at specific sites, as dictated by

gRNAs (Goringer 2012). RNA editing corrects frameshifts and generates initiation and termination codons (minimal editing) or entire open reading frames (extensive or pan editing) in otherwise noncoding sequences (Lukes, Hashimi et al. 2005). Twelve maxicircle protein-coding genes undergo various degrees of editing, with three genes edited minimally to moderately and nine extensively) (Table 9.2) (Goringer 2012). These transcripts mostly serve as precursors of the oxidative phosphorylation system, detailed in section *7.2.4,* which is used by the insect stage procyclic form of *T. brucei* as the main energy generation source. Although the mammalian bloodstream form of *T. brucei* relies exclusively on glycolysis for ATP production, it requires an active mitochondrion for other essential metabolic pathways, such as fatty acid metabolism, calcium homeostasis, and apoptosis (Helfert, Estevez et al. 2001, Stephens, Lee et al. 2007, Roldán, Comini et al. 2011).

The site specificity of RNA editing is governed by 30-60 nucleotide noncoding gRNAs that mostly act in trans, although there is one instance of cis-editing (Golden and Hajduk 2005)) (Blum and Simpson 1990, Pollard, Rohrer et al. 1990, Sturm and Simpson 1991). These gRNAs have three important functional domains: I) a 5′ anchor region, which is complementary to the pre-mRNA downstream of the first editing site (ES); II) a central region or guiding section that specifies editing of the mRNA sequence; III) 3′ poly U-tail that presumably stabilizes gRNA/Pre-mRNA hybrid (Blum, Bakalara et al. 1990, McManus, Adler et al. 2000, Simpson, Sbicego et al. 2003, Aphasizheva, Alfonzo et al. 2020).

Mitochondrial transcript	Respiratory complex/ function	No. of U insertions/ U deletions	Length of edited mRNA (nt)	stage	reference
ND1	Complex I	Not edited	-	Unknown	
ND3		210/13	452	BF/PF	(Read, Wilson et al. 1994)
ND4		Not edited	-	BF	
ND5		Not edited	-	BF	
ND7		553/89	1,238	BF/PF	(Koslowsky, Bhat et al. 1990)
ND8		259/46	574	BF	(Souza, Myler et al. 1992)

ND9		345/20	649	BF	(Souza, Shu et al. 1993)
Cyb	Complex III	34/none	1,151	PF	(Feagin, Jasmer et al. 1987)
COI	Complex IV	Not edited	-	PF	
COII		4/none	663	PF	(Benne, Van den Burg et al. 1986)
COIII		547/41	969	BF/PF	(Feagin, Abraham et al. 1988)
A6	Complex V	447/28	811	BF/PF	(Bhat, Koslowsky et al. 1990)
S12	Ribosomal protein S12	132/28	325	BF/PF	(Marchal, Ismaili et al. 1993) (Maslov, Sturm et al. 1992)
S3 (former MURF5)	Ribosomal protein S3	Not edited	-	Unknown	(Ramrath, Niemann et al. 2018)
MURF1	Unknown function	Not edited	-	Unknown	
MURF2		26/4	1,111	BF	(Feagin and Stuart 1988)
CR3		148/13	299	BF	(Stuart, Allen et al. 1997)
CR4		325/40	567	BF	(Corell, Myler et al. 1994)

Table 9.2 Maxicircle genes undergoing post-transcriptional modification *Abbreviations: ND, NADH: ubiquinone oxidoreductase subunits 1, 3, 4, 5, 7, 8, and 9); CYb, apocytochrome b; CO, cytochrome oxidase (subunits I-III); A6, ATP synthase subunit 6 (complex V); S12, ribosomal protein S12; S3, ribosomal protein S3; MURF, maxicircle unidentified reading frame; CR, G- versus C-strand-biased gene subunits 3 and 4; BF, edited in Bloodstream form, PF, edited in procyclic form (modified from reference (Stuart, Allen et al. 1997, Hajduk and Ochsenreiter 2010, Goringer 2012)*

9.6 RNA editing mechanism

Early experimental evidence suggested that RNA editing is catalyzed in a stepwise manner by a high molecular mass multiprotein complex called the editosome, named in analogy to ribosomes and the spliceosome (Pollard, Harris et al. 1992, Göringer, Koslowsky et al. 1994, Köller, Nörskau et al. 1994, Corell, Read et al. 1996, Kable, Seiwert et al. 1996, Seiwert, Heidmann et al. 1996, Peris, Simpson et al. 1997, Rusché, Cruz-Reyes et al. 1997, Aphasizhev, Aphasizheva et al. 2003). Protein components of this complex have been identified using various biochemical methods, including mass spectrometry of the editosome complex purified through various methods such as serial ion exchange and gel permeation chromatography, immunoaffinity chromatography of editosome proteins, and tandem affinity purification (TAP-tag) of tagged

components of editosome in *T. brucei* (Panigrahi, Schnaufer et al. 2003). Editosome proteins have been found to be conserved in three related trypanosomatid pathogens: *T. brucei*, *T. cruzi*, and *L. major* (Worthey, Schnaufer et al. 2003). Preliminary data from our lab indicate that monoclonal antibodies (mAbs) specific to the *T. brucei* editosome show cross-reactivity with *T. cruzi* and *Leishmania major* extracts. Using these mAbs, we were able to immunoprecipitate functional editosomes in all three species (unpublished data). This suggests the potential for new inhibitors to serve as broad-spectrum trypanocidal compounds.

The coordinated activities of editosome components begin with the hybridization of the gRNA and its cognate pre-edited mRNA through Watson-Crick-type base-pairing and noncanonical G:U wobble base pairing, forming a three-helix junction RNA hybrid (Corell, Read et al. 1996, Goringer 2012, Aphasizheva and Aphasizhev 2016, McDermott, Luo et al. 2016, Read, Lukes et al. 2016). An endonuclease recognizes the mismatch in the duplex and cleaves the pre-mRNA downstream of the editing site at the first unpaired nucleotide. Following endonucleolytic activity, uridylates (Us) are added by a 3'-terminal uridylyl transferase (TUTase) or removed by 3' exouridylylase (ExoUase) at the 3' end of the 5' fragment, based on the information dictated by the gRNA. The two fragments are then ligated by RNA editing ligase (Figure 9.3 obtained from (Aphasizheva, Alfonzo et al. 2020)). Subsequently, the edited mRNA can serve as a substrate for another gRNA in pan-editing scenarios, with the editing progressing from the 3’ to the 5’ end of the transcript. Upon completion, a helicase releases the mRNA for further processing (Li, Herrera et al. 2011). While only a small subset of mRNAs are fully edited, the majority are either unedited or partially edited, with some evidence suggesting alternative editing that lead to dead-end products (Sturm and Simpson 1990, Koslowsky, Jayarama Bhat et al. 1991, David, Flegontov et al. 2015, Simpson, Bruno et al. 2016, Zimmer, Simpson et al. 2018). All catalytic steps of the RNA editing process (endonuclease, exoUase, TUTase, and ligase) and most RNA editing factors have been shown to be essential for the viability of *T. brucei* (Schnaufer, Panigrahi et al. 2001, Drozdz, Palazzo et al. 2002, Huang, O'Hearn et al. 2002, Kang, Rogers et al. 2005, Kang, Gao et al. 2006, McDermott, Carnes et al. 2019), making them viable targets for drug discovery programs. Despite the well-established basic mechanism involving the stepwise coordinated activity of the enzymes, the dynamic assembly/disassembly of editosome remains

underexplored due to the lack of potent, selective small molecule inhibitors. These inhibitors have proven to be had proven to be powerful tools for studying the conformational dynamics of multisubunit protein complexes like ribosome or spliceosome (Ermolenko, Spiegel et al. 2007, Jurica 2008, O'Brien, Matlin et al. 2008). Small molecule probes can disrupt the dynamic interactions of protein complexes, providing snapshots of the complex's lifetime (Disney 2008). Given that the editosome consists of sub-complexes (Schnaufer, Ernst et al. 2003) and functions in a stepwise manner, an enzyme-specific inhibitor could halt RNA editing, allowing the observation of different stages of editosome assembly. Presumably, sub-complexes are recruited sequentially to perform their functions and advance the editing process. Thus, inhibition of a specific step could help identify a partial editosome (a sub-complex) interacting with the RNA substrate, highlighting the need for novel, mechanism-specific inhibitors to dissect the RNA editing process.

9.7 RNA editing holoenzyme

All the crucial proteins required for RNA editing are part of the RNA editing holoenzyme, which is an RNA-mediated assembly of three main complexes: the RNA Editing Catalytic Complex (RECC), the RNA Editing Substrate Binding Complex (RESC), and the RNA Editing Helicase 2 Complex (REH2C) (Aphasizheva, Alfonzo et al. 2020). In the following sections, proteins and complexes are named according to the new consensus nomenclature (Aphasizheva, Alfonzo et al. 2020). The complete, updated list of proteins involved in mitochondrial RNA editing and their annotations can be found in (Aphasizheva, Alfonzo et al. 2020)Table1.

9.8 RNA Editing Catalytic Complex (RECC)

All enzymes involved in the RNA editing reaction cycle—including endonuclease, TUTase, ExoUase and ligase activities—are found within the RNA Editing Catalytic Complex (RECC), formerly known as the ~20S editosome or RNA editing core complex (Rusché, Cruz-Reyes et al. 1997, McManus, Shimamura et al. 2001, Rusché, Huang et al. 2001, Madison-Antenucci, Grams

et al. 2002, Ernst, Panicucci et al. 2003, Panigrahi, Allen et al. 2003, Panigrahi, Schnaufer et al. 2003, Simpson, Aphasizhev et al. 2004, Stuart, Schnaufer et al. 2005, Carnes, Trotter et al. 2008). The components of the RECC are encoded by nuclear genes and then transported into the mitochondrion (Böhm, Katari et al. 2012). This protein-only complex has been characterized as a high-molecular-mass assembly with an apparent Svedberg sedimentation coefficient (S-value) of 20S on glycerol gradients (Pollard, Harris et al. 1992, Corell, Read et al. 1996) and consists of 21 proteins, including enzymes, structural and RNA-binding proteins (Aphasizheva, Alfonzo et al. 2020). Using transmission electron microscopy and cryo-electron microscopy (cryo-EM), the structure of TAP-tagged editosome preparations has been characterized as having a bipartite appearance with two prominent globular subdomains (Golas, Böhm et al. 2009, Li, Ge et al. 2009, Goringer 2012). The editosome reaction center is proposed to be situated at the interface of the insertion and deletion subcomplexes, creating a catalytic core with bifunctional properties. Unfortunately, the lack of high-resolution structures impedes the understanding of the mechanistic details of their reaction cycle. Additionally, only the crystal structure of five editosome proteins (KREPA6, KREPA3, KREL1, KRET2, and KREPA1) have been resolved (Czerwoniec, Kasprzak et al. 2015). However, with the advent of new versions of AlphaFold and AlphaFold Multimer, the structures of individual proteins and entire complexes can now be predicted with acceptable accuracy, enabling further analysis (Liu, Guo et al. 2023, Abramson, Adler et al. 2024).

The common core of the RECC encompasses enzymes such as U-specific Kinetoplastid RNA editing exoribonuclease 2 (KREX2), 3′ Kinetoplastid RNA editing Terminal uridylyl transferase 2 (KRET2), and Kinetoplastid RNA editing ligases (KREL1 and KREL2, specific for U-deletion and U-insertion editing, respectively). Additionally, this core contains oligonucleotide binding (OB)-fold containing Kinetoplastid RNA editing proteins A1-6 (KREPA1-6) and two proteins, each with a U1-like zinc finger and degenerate RNase III motifs (KREPB4 and KREPB5) (McManus, Shimamura et al. 2001, Panigrahi, Gygi et al. 2001, Rusché, Huang et al. 2001, Schnaufer, Panigrahi et al. 2001, Aphasizhev, Aphasizheva et al. 2003, Ernst, Panicucci et al. 2003, Worthey, Schnaufer et al. 2003). Various methods have been used to define the RECC interactome including yeast two-hybrid/coimmunoprecipitation (Carnes, Trotter et al. 2005, Stuart, Schnaufer et al. 2005, Trotter, Ernst et al. 2005, Carnes, Trotter et al. 2008), subcomplex

reconstitution with recombinant proteins (Trotter, Ernst et al. 2005, Lerch, Carnes et al. 2012), tandem affinity purification (Carnes, Trotter et al. 2005), and chemical cross-linking/mass spectrometry (McDermott, Luo et al. 2016). Figure 9.2 shows the cross-linking map within the RECCs purified from TAP-tagged KREN1 or KREPB5 procyclic *T. brucei* cells (McDermott, Luo et al. 2016). The common core consists of two separate stable heterotrimeric insertion and deletion subcomplexes (Figure 9.3) (Schnaufer, Ernst et al. 2003). The insertion module comprises KRET2, KREPA1, and KREL2, while the deletion module comprises KREX2, KREPA2, and KREL1. KREPA1 and KREPA2 play crucial roles in enhancing the activity of their interacting partners (Ernst, Panicucci et al. 2009, Gao, Rogers et al. 2010, Park, Budiarto et al. 2012, Moses, Mehta et al. 2023) and also interact with KREPA3 and KREPA6, respectively, to connect the deletion and insertion subcomplexes. KREL1 is essential for the parasite's viability and *in vivo* editing and can compensate for KREL2, which is not crucial for the parasite (Huang, Cruz-Reyes et al. 2001, Rusché, Huang et al. 2001, Schnaufer, Panigrahi et al. 2001, Cruz-Reyes, Zhelonkina et al. 2002, Drozdz, Palazzo et al. 2002, Schnaufer, Ernst et al. 2003, Panigrahi, Ernst et al. 2006). Additionally, besides TUTase 2, which is part of the RECC, TUTase 1 resides within the mitochondrial 3′ processome (MPsome) and plays a role in gRNA maturation by adding a U-tail (Aphasizhev, Sbicego et al. 2002, Aphasizhev, Aphasizheva et al. 2003, Ernst, Panicucci et al. 2003). Although KREX2 is part of the deletion subcomplex and is not essential for parasite survival, KREX1, which is part of the KREN1 endonuclease module and is vital for the parasite, is associated with exoUase activity (Kang, Rogers et al. 2005, Ernst, Panicucci et al. 2009, Carnes, Lewis Ernst et al. 2012).

Three distinct isoforms of the RECC with highly dynamic components have been characterized, each sharing twelve core proteins and containing a mutually exclusive RNase III endonuclease (KREN1, KREN2 and KREN3) along with an interacting partner (KREPB8, KREPB7 and KREPB6, respectively) (Trotter, Ernst et al. 2005, Panigrahi, Ernst et al. 2006, Carnes, Trotter et al. 2008). Each isoform is specialized: the variant with KREN1/KREPB8, which also includes the U-specific exoribonuclease KREX1, is specific for U-deletion; the one with KREN2/KREPB7 facilitates U-insertion; and the one with KREN3/KREPB6 is responsible for a specific cis-editing U-insertion of COII mRNA (Golden and Hajduk 2005, Carnes, Trotter et al.

2008, Carnes, Soares et al. 2011). However, KREN2 and KREN3 have been shown to having overlapping specificities for insertion editing sites, though they occur at distinctly different relative frequencies (Carnes, McDermott et al. 2017). Furthermore, OB-fold-containing proteins (KREPA1-6) and RNase III domain-containing proteins (KREPB4 and KREPB5) play a crucial role in maintaining the structural integrity of the complex (Guo, Carnes et al. 2012, Davidge, McDermott et al. 2023).

A recent study showed that editing of consecutive insertion and deletion sites is non-processive (Carnes, McDermott et al. 2023), explaining the presence of numerous partially edited mRNAs found *in vivo*. This indicates that the RECCs repeatedly bind and release the RNA substrate for each catalytic cycle. However, many aspects remain poorly understood, including the mechanisms regulating differential editing of mRNA transcripts and the varying functions of RECC proteins during different life cycle stages (McDermott, Carnes et al. 2015, McDermott, Guo et al. 2015, McDermott and Stuart 2017, McDermott, Carnes et al. 2019, Smith, Doleželová et al. 2020, Carnes, McDermott et al. 2022).

For the purposes of this dissertation, we shall equate the terms "editosome" and "RECC" (RNA Editing Core Complex). Throughout this work, the term "editosome" will refer to the RECC. This definition is consistent with the primary focus of our assays and experiments, which concentrate on the central catalytic activities of the RECC.

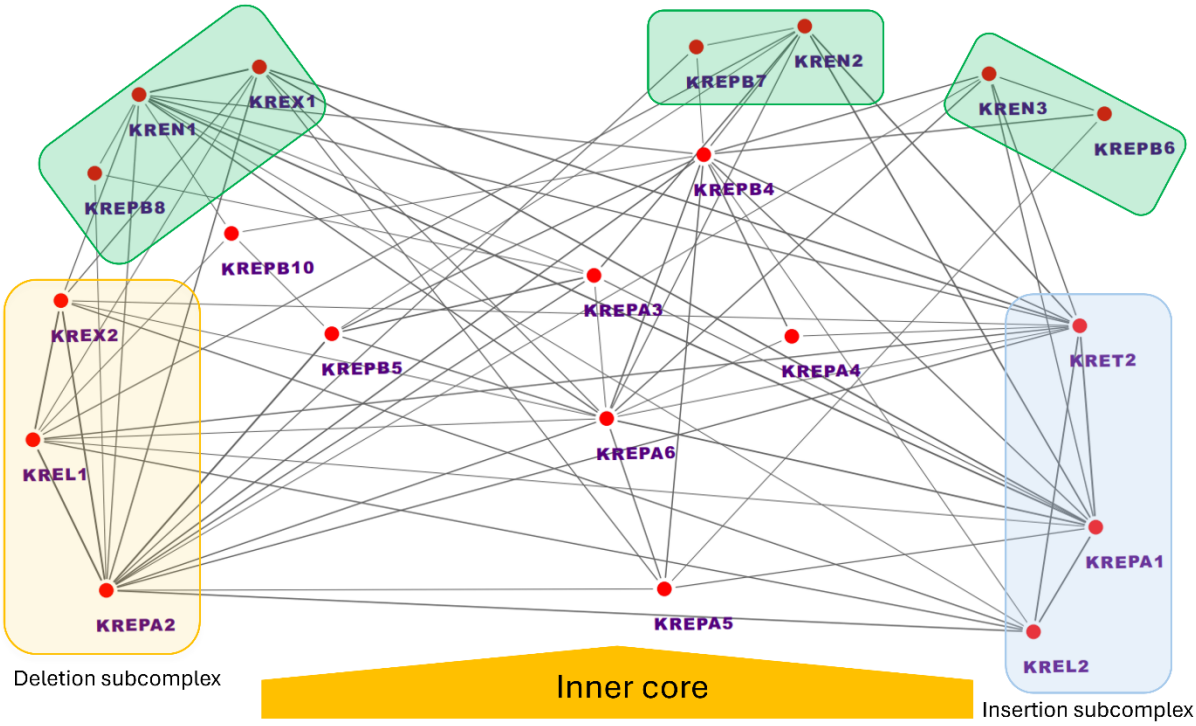


Figure 9.2 Complex network of the interprotein cross-links within RECC based on results from a CXMS experiment *(McDermott, Luo et al. 2016). Observed cross-links between pairs of proteins are shown as gray lines. Each node represents a protein within the RECC. The insertion subcomplex (blue rectangle), deletion subcomplex (pale orange rectangle), and the inner core proteins (orange pentagon arrow) are defined in the map. Each of these isoforms of the RECC contains one of the three green rectangles, each containing an endonuclease and its interacting partner/s.*

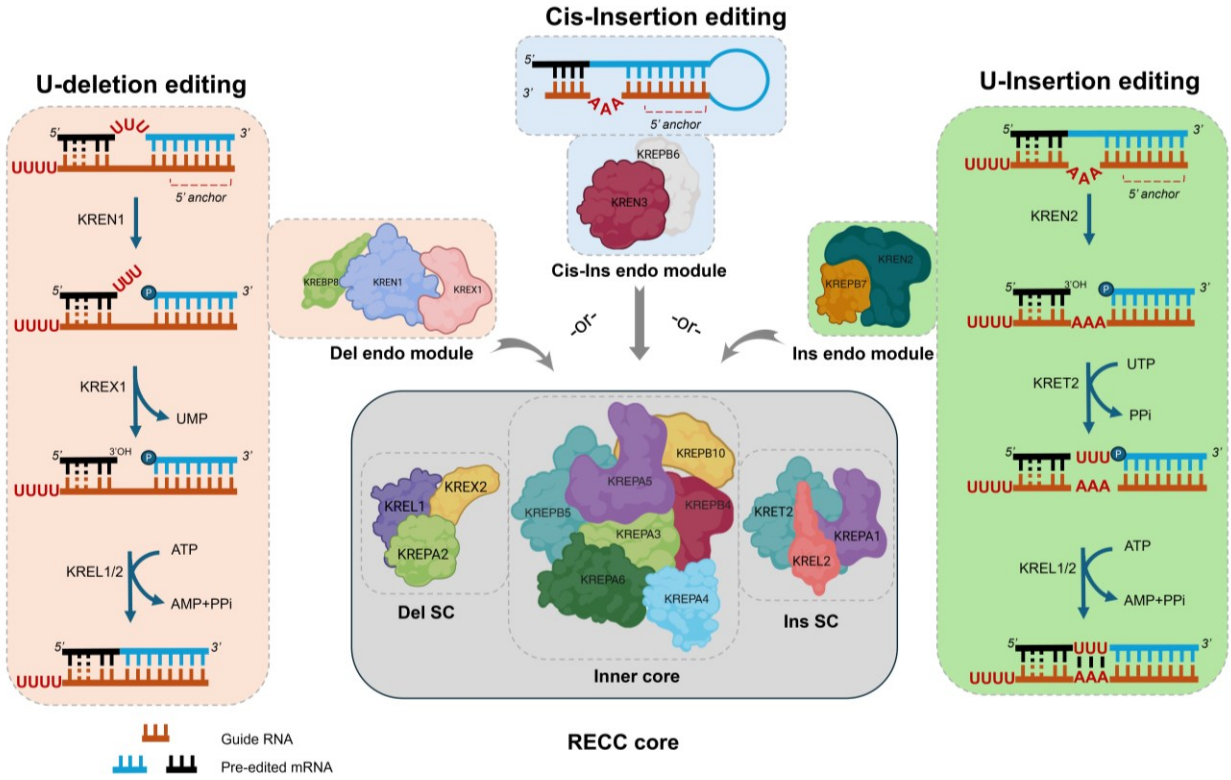


Figure 9.3 Schematic representation of the enzymatic steps of uridine insertion/deletion editing mediated by the three isoforms of RECC. *Uridine insertion/deletion (U-indel) RNA editing mechanism and the associated endonuclease modules for each type of editing are shown. RECC components and their organization within the complex are depicted. The 5' anchor refers to the 5' sequence of the gRNA that anneals with the pre-edited mRNA; SC, subcomplex. Adapted from (Aphasizheva, Alfonzo et al. 2020).*

9.9 RNA Editing Substrate Binding Complex (RESC), RNA Editing Helicase 2 Complex (REH2C), and Accessory Factors

Following the discovery and characterization of RECC components, many multiprotein complexes involved in RNA editing were identified. One such complex, the Mitochondrial RNA Binding Complex 1 (MRB1), was discovered by various groups using distinct methods, including mAbs and tandem affinity purification-tag (TAP-tag) (Hashimi, Zikova et al. 2008, Panigrahi, Zikova et al. 2008, Weng, Aphasizheva et al. 2008). MRB1 is associated with kDNA

transcripts and initiates RNA editing (Acestor, Panigrahi et al. 2009, Ammerman, Hashimi et al. 2011, Ammerman, Tomasello et al. 2013, Read, Lukes et al. 2016). Its two major subunits, known as RESC1 and RESC2 (formerly GRBC1 and GRBC2, respectively), form a stable α2β2 heterotetramer that binds gRNA both *in vitro* and *in vivo* (Aphasizhev, Aphasizheva et al. 2003, Aphasizheva, Zhang et al. 2014, Aphasizheva, Alfonzo et al. 2020). These proteins, unique to kinetoplastids, are essential and their knockdown results in the elimination of gRNAs (Weng, Aphasizheva et al. 2008, Hashimi, Cicova et al. 2009). A recent study reported the cryo-EM structure of RESC1–RESC2, which forms an obligatory domain-swapped dimer. It was proposed that two RESC1–RESC2 heterodimers binding to complementary RNA strands, each containing a 5′-triphosphate, account for the observed heterotetramer formation (Dolce, Nesterenko et al. 2023). Besides their role in the MRB1 core, RESC1 and RESC 2 have been suggested to have additional functions (Ammerman, Downey et al. 2012, McAdams, Simpson et al. 2018).

RESC, a 21-component subset of the RNA editing holoenzyme, binds RNA editing substrates, intermediates, and products. It coordinates pre- and post-editing processing events through RNA-mediated contacts with 5′ and 3′ modification complexes and auxiliary factors, and it provides a scaffold for the transient association of RECC. (Aphasizheva, Alfonzo et al. 2020) (Carnes, Gendrin et al. 2023). RESC is composed of the Guide RNA Binding Complex (GRBC, including RESC1-6), RNA Editing Mediator Complex (REMC, including RESC7, RESC9, RESC11A, RESC12/12A, and RESC13), RESC organizers (RESC8, RESC10, and RESC14) and in some cases the Polyadenylation Mediator Complex (PAMC, RESC 15-18) (Fisk, Ammerman et al. 2008, Ammerman, Hashimi et al. 2011, Ammerman, Downey et al. 2012, Kafková, Ammerman et al. 2012, Ammerman, Tomasello et al. 2013, Aphasizheva, Zhang et al. 2014, McAdams, Harrison et al. 2019, Aphasizheva, Alfonzo et al. 2020). Mitochondrial RNAs maintain the association between RESC components (GRBC, REMC, and PAMC) and play an essential role in attracting RECC and the polyadenylation complex (Aphasizheva, Zhang et al. 2014). Furthermore, REMC and PAMC connect GRBC to RECC and the polyadenylation complex, respectively.

A recent study reported cryo-EM structures of three states of the RESC complex (RESC-A, RESC-B, and RESC-C) and characterized the RNA-binding specificity for each subunit involved (Liu, Wang et al. 2023). It showed that RESC5 and RESC6 are shared between RESC-A (gRNA-stabilizing particle) and RESC-B (binding gRNA-mRNA), while other components are replaced ([RESC10 and RESC14 replace RESC2 and RESC1], [RESC7 and RESC8 replace RESC3 and RESC4]). The smaller RESC-C complex, composed of RESC5-8, RESC10, and RESC14, contains both gRNA and mRNA. It is unclear whether RESC-C serves as an intermediate in the progression from RESC-A to RESC-B or represents a reconfiguration of RESC-B during editing (Liu, Wang et al. 2023). They proposed a mechanism for the assembly of a ribonucleoprotein substrate that the catalytic RECC utilizes.

Most RESC subunits are crucial for cell viability and lack recognizable motifs, except for RESC8, RESC13, RESC14, and RESC19 (Aphasizheva and Aphasizhev 2016, Read, Lukes et al. 2016, Cruz-Reyes, Mooers et al. 2018, Aphasizheva, Alfonzo et al. 2020). Within the GRBC, RESC3 is essential for maintaining the integrity of the GRBC core. It interacts with all other GRBC core proteins (RESC1, RESC2, RESC4, RESC5, and RESC6), as well as subunits of the REMC subcomplex (RESC11A, RESC12, RESC12A, and RESC13), and RESC8 and RESC10 (Huang, Faktorová et al. 2015). However, RESC3 is not critical for the fitness of procyclic *T. brucei* when cultured in a glucose-containing SDM-79 medium. RNAi targeting of RESC4 did not significantly change the levels of pre-edited and partially edited RNA forms but caused a gradual loss of fully edited mRNAs, both short- and long-tailed (Aphasizheva, Zhang et al. 2014). RESC5 is essential for both stages of the parasite's life cycle and is crucial for GRBC's structure and function (Ammerman, Tomasello et al. 2013). It establishes a robust interaction with RESC6 within the core of GRBC (Ammerman, Hashimi et al. 2011, Ammerman, Downey et al. 2012, Ammerman, Tomasello et al. 2013) and together with RESC7 facilitates the RNA-mediated interaction between GRBC and REMC (Ammerman, Tomasello et al. 2013, Aphasizheva, Zhang et al. 2014). Moreover, the recently discovered RESC19 (RBP7910) preferentially interacts with poly(U) and poly(AU)-rich RNA sequences (Nikpour and Salavati 2019) and shows significant affinity for $(CG)_n$ Z-DNA in both single-stranded and double-stranded forms (Ehlert, Poorinmohammad et al. 2023), suggesting its potential role in regulating

RNA editing of mitochondrial transcripts. It was also proposed to have a regulatory role in *T. brucei* differentiation due to its upregulation at various time points during the differentiation from bloodstream form to procyclic form, but not in the procyclic stage (Zamani, Poorinmohammad et al. 2024). This underscores the importance of studying regulatory mechanisms during the differentiation either through editosome proteins (Dejung, Subota et al. 2016) or by analyzing mitochondrial mRNA abundances (Smith, Tylec et al. 2023) rather than focusing solely on the endpoint in each life stage.

REMC subcomplex, formerly known as the TbRGG2 subcomplex, is another component of the RESC. It appears to be heterogeneous and mainly contains RESC7, RESC9, RESC11A, RESC12/12A, and RESC13. This subcomplex interacts with the GRBC core via protein-protein interactions, and this association is enhanced in the presence of RNA (Ammerman, Downey et al. 2012, Kafková, Ammerman et al. 2012, Aphasizheva, Zhang et al. 2014, Simpson, Bruno et al. 2017, McAdams, Simpson et al. 2018). RESC13 or TbRGG2, for which REMC is typified, is an RNA binding protein with various functions such as annealing RNAs and unwinding gRNA/mRNA duplex (Fisk, Ammerman et al. 2008, Ammerman, Presnyak et al. 2010, Foda, Downey et al. 2012, McAdams, Simpson et al. 2018) and was suggested to have a role in 3' to 5' progression of editing (Sortino, Tylec et al. 2022).

The three-membered REH2C, consisting of KREH2, KH2F1, and KH2F2, is an mRNA-associated ribonucleoprotein subcomplex. It exhibits ATP-dependent 3′–5′ dsRNA unwinding activity and controls editing fidelity (Aphasizheva, Alfonzo et al. 2020). Immunoprecipitation studies showed that the RESC containing REH2C includes a variant of gRNA-bound GRBC that lacks RESC6 (Kumar, Madina et al. 2016). Furthermore, KREH2, a DEAH/RHA RNA helicase, was recently discovered to control non-canonical editing in A6 mRNA differentially by leveraging regulatory gRNAs and repressive RNA structures (Meehan, McDermott et al. 2023). Additionally, it has been suggested to serve as a potential dual-purpose regulatory element considering its upregulation during differentiation from bloodstream to procyclic form and in the procyclic stage (Zamani, Poorinmohammad et al. 2024).

In addition to these complexes mentioned above, auxiliary factors are involved in RNA editing. These include but are not limited to, kinetoplastid mitochondrial RNA-binding proteins 1 and 2 (KMRP1/2, which play a role in matchmaking mRNA with its cognate gRNA and form a heterotetrameric complex (Blom, Burg et al. 2001, Aphasizhev, Aphasizheva et al. 2003, Schumacher, Karamooz et al. 2006, Zikova, Kopecna et al. 2008)), and KRGG1 (which contains an arginine-glycine-glycine (RGG) tripeptide and differentially affects the editing of various mRNA, and is homologous to RESC6 (Vanhamme, Perez-Morga et al. 1998, Carnes, Gendrin et al. 2023)). Also, TAP-tag purification of KRGG1 has co-isolated it with GRBC (Hashimi, Zikova et al. 2008), although it was not categorized as a GRBC component at that time (Aphasizheva, Alfonzo et al. 2020)). Another factor, mitochondrial editing-like complex-associated TUTase 1 (MEAT1), a U-specific TUTase, interacts with RECCs lacking an insertion subcomplex (Aphasizheva, Ringpis et al. 2009, Stagno, Aphasizheva et al. 2010, Aphasizheva, Alfonzo et al. 2020)). Furthermore, KRBP16, which exhibits RNA binding and annealing activities, has been shown to have overlapping functions with KMRP1/2 and plays a role as a mitochondrial gene regulating factor (Pelletier and Read 2003, Fisk, Presnyak et al. 2009)).

9.10 In vitro RNA editing HTS assays

Traditionally, U-indel RNA editing has been monitored using radiolabeled RNA substrates on gels, despite the low detection limit (Kable, Seiwert et al. 1996, Seiwert, Heidmann et al. 1996, Rusché, Cruz-Reyes et al. 1997, Igo, Palazzo et al. 2000, Igo, Weston et al. 2002, Carnes, Trotter et al. 2005). However, only a few high-throughput amenable assays capable of monitoring RNA editing *in vitro* have been developed thus far. Liang *et al.* developed a full-round insertion assay, where "full round" refers to an editing reaction that necessitates all three enzymatic steps of RNA editing. This assay is based on an electrochemiluminescent aptamer-switch, where a signal is produced due to a conformational change within the RNA reporter triggered by the insertion of three uridine residues (Liang and Connell 2009). Zimmerman et al. developed a fluorescent-based assay that allows for monitoring RNA editing Ligase 1 activity and can be adapted for use with other nucleic acid ligases (Zimmermann, Hall et al. 2016). In this assay, the ligation of two fluorophore-labeled RNA substrates, directed by gRNA, brings the fluorophores into proximity,

resulting in Förster resonance energy transfer (FRET). Our lab developed a full-round deletion hammerhead ribozyme (HHR)-driven reporter assay integrated with a FRET detection method (Moshiri and Salavati 2010), where the deletion of three uridine residues from the pre-edited inactive HHR, as specified by gRNA, activates the ribozyme. This activation allows the edited HHR to cleave a FRET substrate bound to it, releasing a fluorescent signal that was otherwise quenched. Del campo *et al.* established an assay that monitors both insertion and deletion editing in one pot (Del Campo, Leeder et al. 2020), using fluorophore-labeled RNAs analyzed by capillary electrophoresis in combination with a laser-induced fluorescence readout. For a comparison of the assays, please see (chapter III, Table 10.1 (Rostamighadi, Mehta et al. 2023)). There are disadvantages associated with each assay, namely high concentration of ATP (limiting sensitivity for finding ATP competitive inhibitors), monitoring only one step of RNA editing, using mitochondrial extract which has more impurities than TAP-tagged purified protein, a lack of quantitative analysis of intermediate products, and complicated instrumentation for performing HTS. These assays have proven valuable for pilot-scale screening efforts aimed at identifying potential RNA editing inhibitors. Each has contributed to advancements in our understanding of RNA editing mechanisms and has yielded promising candidates for further investigation.

9.11 Screening small molecule libraries to find RNA editing inhibitors

The availability of 3D protein structures enables scientists to perform virtual screens to discover novel inhibitors for their protein of interest. Thus far, crystal structures of only five editosome proteins, namely KREL1, KRET2, KREPA1, KREPA3, and KREPA6, have been deposited in the PDB database (Deng, Schnaufer et al. 2004, Deng, Ernst et al. 2005, Wu, Park et al. 2011, Park, Budiarto et al. 2012, Park, Pardon et al. 2012). Among these, the two enzymes, KREL1 and KRET2, have been targeted in virtual screens (Amaro, Schnaufer et al. 2008, Durrant, Hall et al. 2010, Moshiri, Acoca et al. 2011, Demir, Labaied et al. 2014) and some of the hits have shown trypanocidal efficacy. Unfortunately, no experiments have been performed to validate their target engagement within cells. Moreover, KREL1 inhibitors identified through virtual screens were found to inhibit not only the recombinant ligase but also other targets, specifically

MRP1/2, indicating they affect RNA-protein interactions when exposed to the whole editosome complex (Moshiri, Acoca et al. 2011, Mehta, Moshiri et al. 2020). Recently, Acquah and Mooers targeted the U-helix (a structural domain of the RNA editing substrate) using a virtual screen followed by molecular dynamics studies, identifying inhibitors with binding affinities from low-micromolar to nanomolar for the target (Acquah and Mooers 2023).

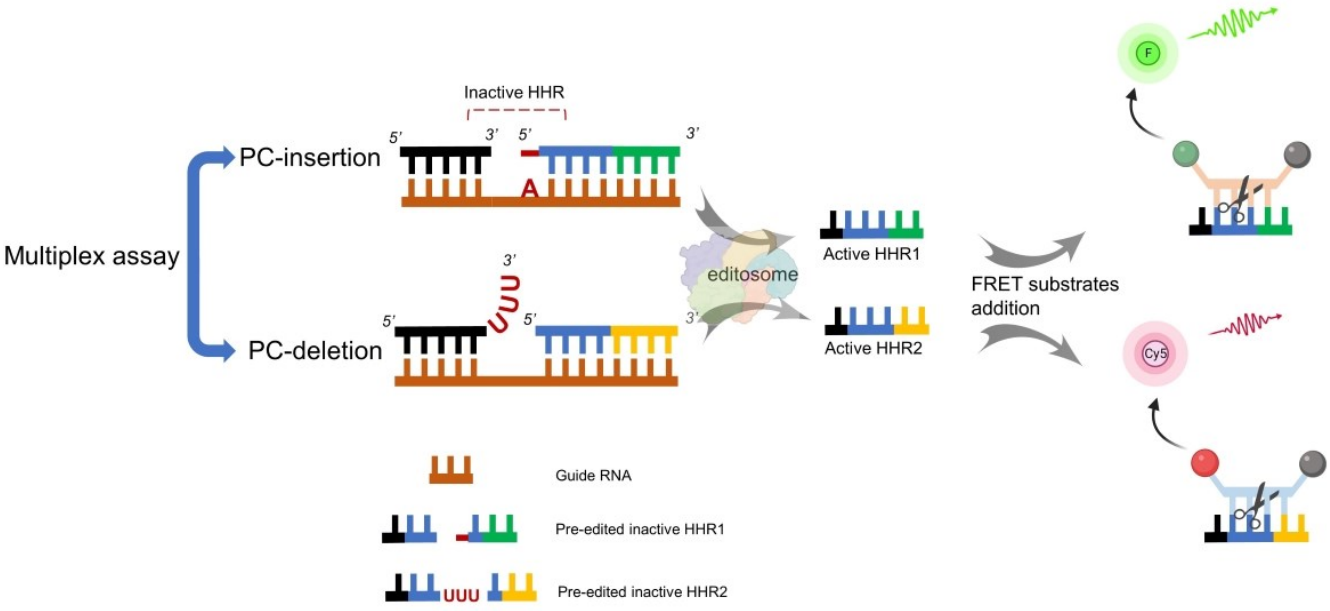
Pilot-scale *in vitro* screens were conducted using the first three assays listed in section *7.2.10* (Liang and Connell 2010, Moshiri, Mehta et al. 2015, Zimmermann, Hall et al. 2016). These assays tested a library of pharmacologically active compounds (LOPAC1280; Sigma, St. Louis, MO) to identify new inhibitors for RNA editing. Although the assays employed different approaches, one might expect overlap among the identified hits due to the common ligation step. However, no common inhibitors were found among the three screens, and the overlapping hits were reported to act on different targets.

For instance, NF023 inhibited editing by only ~35% in the full-round deletion assay in Moshiri's study and was not considered a hit (Moshiri, Mehta et al. 2015). However, in the other two screens, different modes of action were suggested for it. GW5074 and protoporphyrin IX were identified only in Liang's assay, where they inhibited endonuclease or preceding steps of editing (Liang and Connell 2010). Aurintricarboxylic acid (ATA), PPNDS, and NF449 were indicated to inhibit RNA-protein interactions, demonstrating low micromolar IC_{50} values against the editosome only in the HHR assay (Moshiri, Mehta et al. 2015). Ruthenium red was recognized as a broadly active inhibitor in both Moshiri's and Liang's screens. Suramin was found in Liang's screen but was not further characterized due to its interference with the ECL assay; however, it was strongly indicated to inhibit RNA editing ligase in Zimmerman's study (Liang and Connell 2010, Zimmermann, Hall et al. 2016). Mitoxantrone was shown to halt editing non-specifically by promoting aggregation in the HHR editing assay; however, in contrast, it was found to be a genuine detergent-resistant inhibitor (inhibiting via other modes of action rather than forming aggregation) in Liang's assay.

The contradictory results, such as non-overlapping hits and different modes of action attributed to a hit, may stem from differences in the stringency of screen cut-offs, assay sensitivity, reaction components (including protein sources and ATP concentrations), incomplete mode of action (MOA) studies, and limiting the RNA editing to a specific step. Hits detected by these assays were either non-specific inhibitors, or their specific targets were not identified in the context of the editosome.

Del Campo et al. also employed their novel fluorescence-based insertion/deletion editing assay to screen a number of UTP analogs and discovered some novel inhibitors (Del Campo, Leeder et al. 2020). However, the use of 0.5 mM ATP and 0.1 mM UTP in this assay decreases the likelihood of identifying competitive inhibitors. Therefore, novel sensitive assays are required for high-throughput screening (HTS) campaigns and mode of action studies.

10. CHAPTER III: Hammerhead ribozyme-based U-insertion and deletion RNA editing assays for multiplexing in HTS applications



This chapter is a reprint of:

Rostamighadi M, Mehta V, Hassan Khan R, Moses D, Salavati R. Hammerhead ribozyme-based U-insertion and deletion RNA editing assays for multiplexing in HTS applications. **RNA**. 2023 Feb;29(2):252-261. doi: 10.1261/rna.079454.122. Epub 2022 Dec 1. PMID: 36456183; PMCID: PMC9891259.

10.1 Abstract

Untranslatable mitochondrial transcripts in kinetoplastids are decrypted post-transcriptionally through an RNA editing process that entails uridine insertion/deletion. This unique stepwise process is mediated by the editosome, a multiprotein complex that is a validated drug target of considerable interest in addressing the unmet medical needs for kinetoplastid diseases. With that objective, several in vitro RNA editing assays have been developed, albeit with limited success in discovering potent inhibitors. This manuscript describes the development of three hammerhead ribozyme (HHR) FRET reporter-based RNA editing assays for precleaved deletion, insertion, and ligation assays that bypass the rate-limiting endonucleolytic cleavage step, providing information on U-deletion, U-insertion, and ligation activities. These assays exhibit higher editing efficiencies in shorter incubation times while requiring significantly less purified editosome and 10,000-fold less ATP than the previously published full round of in vitro RNA editing assay. Moreover, modifications in the reporter ribozyme sequence enable the feasibility of multiplexing a ribozyme-based insertion/deletion editing (RIDE) assay that simultaneously surveils U-insertion and deletion editing suitable for HTS. These assays can be used to find novel chemical compounds with chemotherapeutic applications or as probes for studying the editosome machinery.

10.2 Introduction

Trypanosoma brucei subsp., *Trypanosoma cruzi* and the *Leishmania* spp. are parasitic trypanosomes that cause devastating endemic diseases such as human African trypanosomiasis (HAT), Chagas disease and leishmaniases, respectively (Stuart, Brun et al. 2008, WHO 2015). Treatments available against these diseases are not ideal due to toxicity, inefficacy, and the emergence of resistant parasite strains, while vaccine development remains a challenge (Denise and Barrett 2001, Fairlamb 2003, Delespaux and de Koning 2007, Field, Horn et al. 2017, Pramanik, Alam et al. 2019); hence, there is a pressing need for developing novel trypanocidal therapeutics. Among several unusual biochemical features of the trypanosomes, mitochondrial RNA editing is of considerable interest for drug discovery and development (Fidalgo and Gille 2011, Salavati, Moshiri et al. 2012, Field, Horn et al. 2017). Most mitochondrial transcripts in these organisms are encrypted and require extensive post-transcriptional modifications through specific insertions and/or deletions of uridylate (U) residues in a guide RNA-dependent manner for the maturation (Stuart, Schnaufer et al. 2005, Salavati, Moshiri et al. 2012), ultimately coding for multiple protein components in the oxidative phosphorylation system (Hajduk and Ochsenreiter 2010, Goringer 2012).

An ~800 kDa multi-protein RNA-editing catalytic complex (RECC) catalyzes the trypanosomatid U-indel RNA editing as dictated by short complementary guide RNA (gRNA) (Rusché, Cruz-Reyes et al. 1997, Aphasizhev, Aphasizheva et al. 2003, Panigrahi, Schnaufer et al. 2003, Golas, Böhm et al. 2009, Li, Ge et al. 2009, Aphasizhev and Aphasizheva 2011, Goringer 2012, Voigt, Dobrychlop et al. 2018, Aphasizheva, Alfonzo et al. 2020). Editing catalysis is coordinated in an enzymatic cascade commencing upon hybridization of the 5' anchor region of a guide RNA to its cognate premature mRNA just downstream of the first editing site. Catalysis initiates with endonucleolytic cleavage at the editing site by an RNA editing endonuclease, followed by U-insertion with a terminal uridylyl transferase (TUTase) or U-deletion with a U-specific exoribonuclease (ExoUase), and terminates with ligation of the edited site with an RNA editing ligase (KREL) (Blum, Bakalara et al. 1990, Pollard, Harris et al. 1992, Piller, Decker et al. 1995, Corell, Read et al. 1996, Voigt, Dobrychlop et al. 2018)(for a recent

review see (Aphasizheva, Alfonzo et al. 2020)). The edited site then serves as the anchor region for the following gRNA; numerous accessory proteins and protein complexes such as the RNA editing substrate complex (RESC) govern and ensure RNA chaperoning, editing initiation, progression, efficiency and fidelity (Read, Lukes et al. 2016). The RECC, RESC, and REH2C complexes constitute the functional editing holoenzyme (Aphasizheva, Zhang et al. 2014, Aphasizheva, Alfonzo et al. 2020). The mechanisms through which these protein complexes assemble, interact, and process RNA substrates, are yet to be elucidated. Discovering novel RNA editing inhibitors will serve as excellent tools for unravelling this intricate biochemical process and provide potential chemical scaffolds for therapeutic needs (Mehta, Moshiri et al. 2020).

Based on the traditional editing assays that use radiolabeled RNA substrates (Kable, Seiwert et al. 1996, Seiwert, Heidmann et al. 1996, Rusché, Cruz-Reyes et al. 1997, Igo, Palazzo et al. 2000, Igo, Weston et al. 2002, Carnes, Trotter et al. 2005), several suitable high-throughput screening (HTS) *in vitro* assays have been developed over the past decade. An electrochemiluminescent aptamer-based "full-round" insertion editing assay developed by Liang and Connell (2009), generates a signal upon binding of conformationally changed aptamer, upon successful editing, to streptavidin-coated microtiter wells (Liang and Connell 2009). A HHR reporter-based "full-round" deletion editing assay designed by Moshiri and Salavati (2010), generates an active ribozyme from successful editing, which cleaves a FRET substrate for an indirect but amplified quantification of the RNA editing (Moshiri and Salavati 2010). Zimmerman *et al.* devised a FRET-based RNA ligation assay that employs recombinant RNA editing ligase 1 (KREL1) with a truncated version of recombinant kinetoplastid RNA editing protein A2 (KREPA2) (Zimmermann, Hall et al. 2016). When the above three assays were screened against the library of pharmacologically active compounds (LOPAC®^{1280}; Sigma), a different set of potential hits was identified (Liang and Connell 2010, Moshiri, Mehta et al. 2015, Zimmermann, Hall et al. 2016). While common inhibitors of RNA editing were anticipated with similar mechanisms of action, at least against the RNA ligation activity, no such overlap in the hits and their mechanisms were corroborated upon further investigation. Contradicting results may stem from differences in assay sensitivity, variance in source protein composition, chemical

substrate concentrations (such as ATP), and incomprehensive mechanism of action (MOA) studies (Table 10.1).

Recently Del Campo *et al.* introduced a novel fluorescence-based insertion/deletion editing assay that utilizes capillary electrophoresis to provide quantitative information on editing products and intermediates (Del Campo, Leeder et al. 2020). While the appealing nature of this assay is its ability to monitor not just the final edited product but also several editing intermediates in a high-throughput manner, high chemical substrate concentrations (0.5 mM ATP and 0.1 mM UTP) could potentially thwart the chances of finding competitive inhibitors of the catalytic enzymes.

This manuscript demonstrates the suitability of HHR-based "pre-cleaved" (PC) RNA editing assays for use in HTS applications or as secondary assays that can aid in determining the MOA of known RNA editing inhibitors. PC insertion and deletion assays are significantly more efficient than a "full-round" editing assay, bypassing the rate-limiting endonucleolytic cleavage step. Furthermore, multiplexed monitoring of U- insertion and deletion, simultaneously, is feasible with the PC ribozyme-based insertion/deletion editing (RIDE) assay, paving the way for more efficient inhibitors of the editosome.

	Assay	**Source protein**	**ATP conc.**	**Assay type**	**Signal amplified**	**Multi-plexing**
(Liang and Connell 2009)	Full-round insertion	*L. tarentolae* editing extract	1 mM	Electroluminescent aptamer	No	No
(Zimmermann, Hall et al. 2016)	Ligation	Recombinant RNA editing Ligase	10-20 μM	FRET-based	No	No
(Moshiri and Salavati 2010)	Full-round deletion	*T. brucei* Mitochondrial extract	1 mM	Ribozyme-based	Yes	No
(Del Campo, Leeder et al. 2020)	Pre-cleaved insertion and/or deletion	Tap-tagged purified editosome and *T. brucei*	0.5 mM	Capillary electrophoresis & laser-induced fluorescence	No	Yes

		mitochondrial extract				
(Rostamighadi, Mehta et al. 2023)	Pre-cleaved insertion and/or deletion	TAP-tagged purified editosome (feasible with recombinant proteins)	100 nM	Ribozyme-based	Yes	Yes

Table 10.1. Summary of HTS amenable to RNA editing assays.

10.3 Materials and methods

10.3.1 Preparation of RNA substrates

The PC RNA substrates used in this study (as summarized in Table 10.2) were designed based on the ribozyme reporter previously described (Moshiri and Salavati 2010). In this sequence, additional U residues in the HHR active site served as substrates for the deletion assay, while the removal of a U residue from the HHR active site served as substrates for the insertion assay. For the ligation assay, no modifications to the HHR active site were required (Figure 10.1). These substrates were synthesized and HPLC purified by Integrated DNA Technologies (IDT, Coralville, IA). To minimize any aberrant modifications to the RNA substrates, the 5' ends of the 5' HHR fragments and gRNA were hydroxylated, and the 3' ends of the 3' HHR fragments and gRNA were phosphorylated (Figure 10.1 inset).

For use in radiolabeled editing assays, as performed conventionally on urea denaturing polyacrylamide gel electrophoreses (PAGE), the 5'Del and 5'Ins fragments were 5' end labelled with [γ-^{32}P] ATP using T4 PNK (New England Biolabs) and subsequently purified using 8% urea PAGE as described before (Wang, Salavati et al. 2002).

PC-deletion	
5′Del	5'-/OH/GGAAAGUUGUGACUGAUUU/OH/-3'
3′Del	5'-/Phos/UGAGUCCGUGAGGACGAAACAAUAGAUCAAAUGU/Phos/-3'
gHHRc	5'-/OH/GUUUUGUUCUUAUGGACUCACUCAGUCAUAAUUUUCCUUUUUUUUUU/Phos/-3'
gHHRc competitor	5'-AAAAAAAAAAGGAAAATTATGACTGAGTGAGTCCATAAGAACAAAAC-3'
PC-insertion	
5′Ins	5'-/OH/GGAAAGUUGUGACUGA/OH/-3'
3′Ins	5'-/Phos/GAGUCCGUGAGGACGAAACAAUAGAUCAAAUGU/Phos/-3'
gHHR	5'-/OH/GUUUUGUUCUUAUGGACUCAUCAGUCAUAAUUUUCCUUUUUUUUUU/Phos/-3'
gHHR competitor	5'-AAAAAAAAAAGGAAAATTATGACTGATGAGTCCATAAGAACAAAAC-3'
HHR FRET substrate (used in the above two assays)	
FRET1	5'-/FAM/GAUCUAUUGUCUCACA/IABkFQ/-3'
Additional RNA substrates for use in the RIDE assay	
3′Del2	5'/Phos/UGAGUCCGUGAGGACGAAAUCCGCUGGAAAAUGU/Phos/-3'
FRET2	5'-/Cy5/UCCAGCGGAUCUCACA/IAbRQSp/-3'

Table 10.2. RNA substrates used in the pre-cleaved assays. *Note that the PC-ligation assay uses the 5′ Ins, 3′ Del and gHHRc (and the respective competitor) RNA substrates. FAM: 6-carboxyfluorescein, IABkFQ: Iowa Black FQ quencher, IAbRQSp: Iowa Black RQ quencher.*

10.3.2 Purification of Trypanosoma brucei editosome

Genetically modified procyclic *T. brucei* expressing TAP-tagged kinetoplastid RNA editing ligase 1 (KREL1) was used for functional editosome purifications as described before (Stuart, Panigrahi et al. 2004). Antibodies against four RECC proteins (KREL1, KREPA2, KREPA3, and KREPA4) were used for probing the purified elutions by western blotting (Supplemental Figure S10.1).

10.3.3 Pre-cleaved assays: Development and optimization

Both versions of editing assays, FRET-based and radiolabeled, contained 2 pmol of the 5' and 3' HHR fragments, and 4 pmol of gRNA for the respective type of editing (refer to Figure 10.1 and Table 10.2). Before starting the assays, the RNA mixtures were hybridized by denaturation at 70 °C for 5 min in a water bath and allowed to slow-cool to ambient room temperature, typically lasting 40-60 min. Following hybridization, the RNA mixture was added to a master mix to obtain a final reaction composition of 25 mM HEPES [pH 7.9], 10 mM $Mg(OAc)_2$, 50 mM KCl, 1 mM EDTA, 0.1 µM adenosine triphosphate (ATP), 5 mM $CaCl_2$ and 0.1% Triton X-100. The solution also contained 1 µL of the purified editosome (calmodulin eluate from KREL1-TAP tag purification, at approximately 40 ng/mL) and 100 µM UTP for insertion editing or RIDE assays. Separate master mixes were prepared for the controls, typically missing one or more reaction components or containing known RNA editing inhibitors (Figure 10.2). The plates were sealed and incubated at 28 °C overnight (approximately 16-20 h).

To stop the editing reaction in the radiolabeled assays, 40 pmol of an appropriate DNA guide competitor was added to each reaction, along with 0.1 volumes of 3 M sodium acetate. The RNA was then extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol before reconstitution with a urea loading dye and loading onto a 15% or 6% urea PAGE (30-40 cm gel with S2 sequencing gel electrophoresis apparatus). After running the PAGE for approximately 1.5 – 2 h at 45-50 watts, the gel was scanned on a PhosphorImager (Bio-Rad).

To stop the editing reaction in the FRET-based assays, 40 pmol of an appropriate DNA guide competitor was added to each well and incubated at 85 °C for 10 min. After cooling at RT

for 5 min, 20 pmol of the HHR FRET substrate were added to each well and read kinetically for fluorescein every min at 37 °C in an RT-qPCR machine. The slope calculated from the kinetically measured fluorescent signal outputs represents editing activity. Alternatively, the final measurement could be taken as an endpoint reading after 30-60 min incubation at 37 °C. Assay optimization was facilitated by measuring various ATP, UTP, and purified editosome concentrations while testing for different incubation durations.

10.3.4 Ribozyme insertion/deletion editing (RIDE) assay

Multiplexing in the RIDE assay was performed with minor modifications to the HHR sequence for PC deletion, requiring a different 3' fragment and HHR FRET substrate (Figure 10.1, Figure 10.4, and Table 10.2). The editing reaction was performed as above, with two tri-molecular hybrids ([5'Del, 3'Del2, gHHRc] and [5'Ins, 3'Ins, gHHRc]) prepared independently before assay initiation. For signal measurement, 20 pmol of both HHR substrates, FRET1 and FRET2, were added to the reaction simultaneously to read for fluorescence from the FAM and Cy5 fluorophores.

10.3.5 Z' factor determination

The Z' was calculated for the assays using the following formula where σ is the standard deviation and μ is the mean of the values (Zhang, Chung et al. 1999). n and p stand for negative and positive controls. The number of replicates for each control was 10 and suramin was used as the inhibitor of RNA editing reaction.

$$z - factor = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

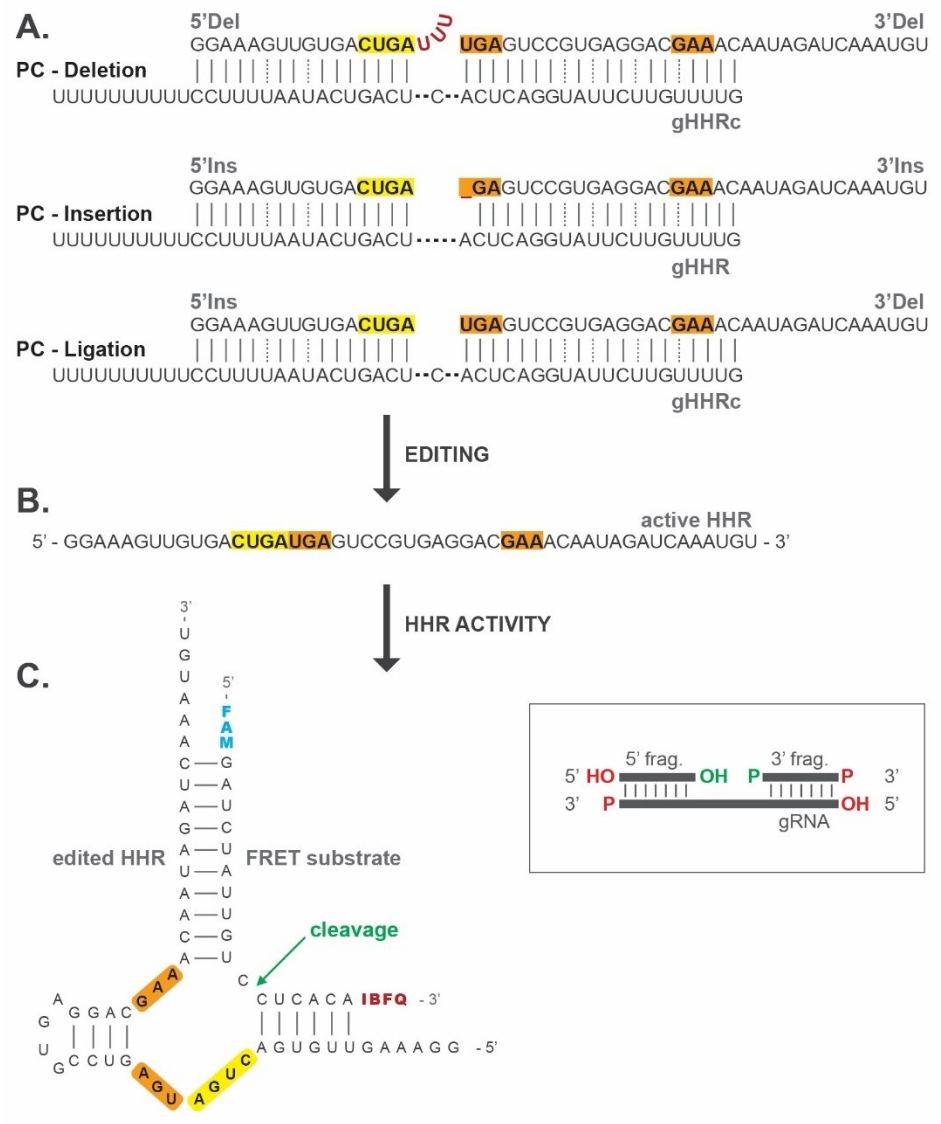


Figure 10.1. Schematic representation of the pre-cleaved RNA editing assays. *(A) Trimolecular hybrids are involved in the PC-deletion, insertion, and ligation assays. Three U residues were designed for removal from the 5'Del fragment in PC-deletion; one missing U residue was designed for addition to the 3'Ins fragment in PC-Insertion; no requirement for U-deletion or insertion in PC-ligation. The Watson-Crick and G·U wobble base-pairs are depicted by solid and dashed lines. The critical nucleotides of the catalytic site of the HHR are indicated in bold and highlighted in yellow and orange (B) RNA editing results in the formation of an active HHR. (C) Enzymatic cleavage activity of the active HHR on the FRET substrate (containing a fluorophore and a quencher). An arrow indicates the cleavage site. The inset depicts the usage of non-conventional termini in the RNA substrates to reduce/eliminate aberrant editing products. Guide RNA gHHRc (gHHR with cytidine in the editing site) was used for efficient RNA editing (Supplemental Figure S10.2).*

10.4 Results

10.4.1 Pre-cleaved RNA editing assay development

To monitor the guide RNA-dependent enzymatic activity of the editosome proteins, we developed three PC HHR-based *in vitro* assays (namely PC-ligation, PC-deletion, and PC-insertion) coupled with a FRET reporter system (Figure 10.1). We trimmed and modified the original version of pre-edited hammerhead A6Rbz (Wang, Salavati et al. 2002, Moshiri and Salavati 2010, Moshiri, Mehta et al. 2015) to bypass the rate-limiting endonucleolytic step by using two fragments of the pre-edited RNA to mimic the editing site post-cleavage, hence termed "pre-cleaved". We used gHHR as the guide RNA in PC-insertion assay and its modified version gHHRc (gHHR with an additional cytidine) for PC-ligation and PC-deletion assays. Inclusion of cytidine in the guide RNA was shown to improve *in vitro* editing efficiency (Supplemental Figure S10.2) and was previously described (Cruz-Reyes, Zhelonkina et al. 2001). As depicted in Fig 8.1, PC-deletion and PC-insertion assays comprise two consecutive enzymatic steps: uridine deletion or insertion dictated by the mismatches against the template gRNA sequence, catalyzed respectively by an ExoUase or a TUTase before ligation. The ligation assay only requires the final ligation step mediated by a KREL to obtain the final active HHR (Supplemental Figure S10.3). The edited product is then detached from the gRNA via adding a gRNA competitor in molar excess. Subsequently, the edited active HHR enzymatically cleaves a FRET-labeled substrate RNA, measured by the signal detected that corresponds to the amount of edited product. Upon testing with intermediate HHR sequences that could potentially arise from these assays, only the fully edited HHR was observed to cleave its FRET substrate (Supplemental Figure S10.4).

The PC-deletion and insertion *in vitro* RNA editing assays were initially monitored using a radiolabeled version of the 5' HHR fragment (Figure 10.2A and 8.2B upper panels). In these experiments, we included three control conditions. In the absence of editosome proteins, there was no change to the radiolabeled 5' HHR fragment (condition 1). Furthermore, in the absence of gRNA, there was non-specific removal of nucleotides in PC deletion and, intriguingly, correct addition of a uridine residue in PC insertion (condition 2). The absence of the 3'HHR fragment

resulted in correct removal of uridine residues likely dictated by the gRNA (condition 3). Other control conditions containing known editing inhibitors (Amaro, Schnaufer et al. 2008, Durrant, Hall et al. 2010, Liang and Connell 2010, Moshiri, Acoca et al. 2011, Salavati, Moshiri et al. 2012, Zimmermann, Hall et al. 2016, Mehta, Moshiri et al. 2020), such as the sulfonated compounds mordant black 25 (MrB) (condition 4) and suramin (condition 5), completely inhibited RNA editing activity. The conditions assayed were replicated in the FRET-based versions for comparison, which resulted in a quantifiable signal obtained only from the fully edited HHR (Figure 10.2A and 10.2B lower panels). Minuscule editing activity was observed without added ATP (condition 6), likely due to the purified editosome containing pre-adenylated KREL proteins. While robust editing was detected in the presence of added ATP in the radiolabeled assay on urea PAGE (condition 7), it surprisingly did not translate to a correlated amount of HHR activity. Upon closer investigation of the final product on a 6% urea PAGE, the final edited product did not correspond to the same length as the positive control active HHR (Supplemental Figure S10.5), indicating that ligation of the 2 HHR fragments occurs rather prematurely. This was circumvented by the delayed addition of ATP 1-hour post incubation initiation (condition 8 in Figure 10.2 and Supplemental Figure S10.5).

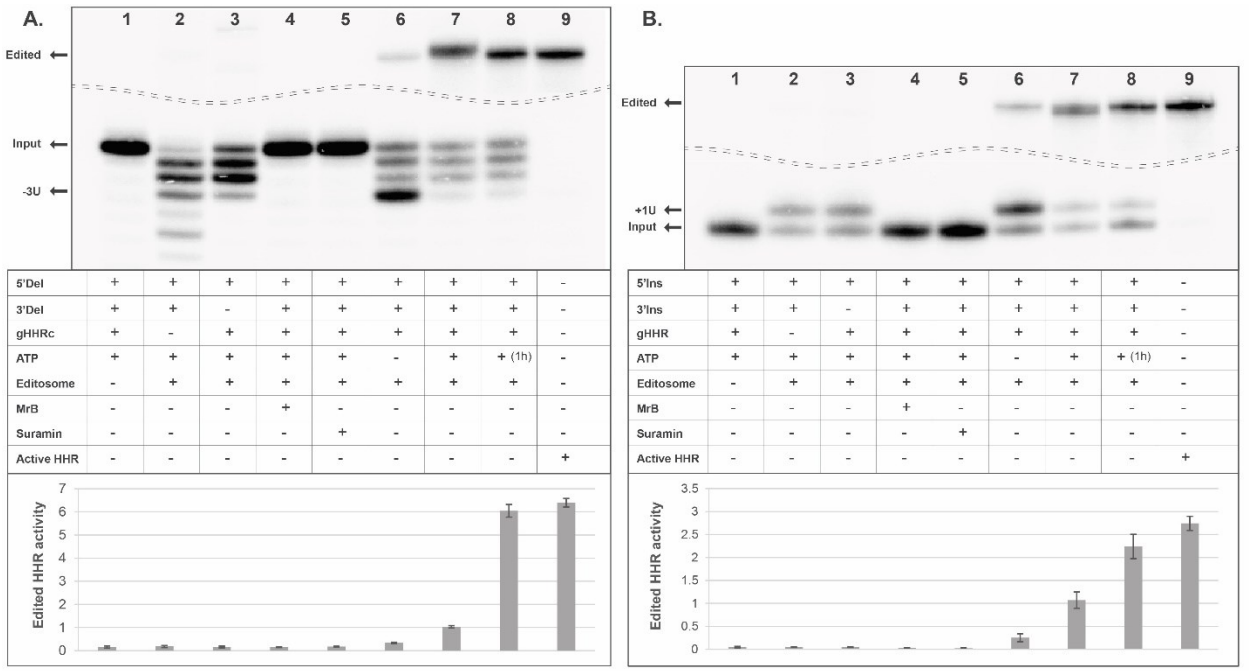


Figure 10.2. Development of pre-cleaved deletion and insertion assays. *Assays are monitored using radiolabeled HHR RNA substrates (top panel) and FRET-based HHR substrates (bottom panel) with several controls (as described in the middle panel). (A) PC-deletion assay and (B) PC-insertion assay with all components except functional editosome (lane 1), guide RNA (lane 2) and 3' fragment (lane 3). Both assays in the presence of RNA editing inhibitors (MrB and suramin, lanes 4 and 5). A faint product/signal was detected without ATP (lane 6). In the presence of all reaction components with discernible product/signal (lane 7). Improved HHR activity is observed when ATP addition is delayed by one-hour post reaction initiation (lane 8). Active HHR (0.5 and 1.5 pmol) was used as a size and activity control in PC-insertion and deletion assay, respectively, to magnify the enhancement in activity (lane 9). Means and standard deviations in the bottom plot were obtained from four replicates. Lanes 6,7 and 8 in both panels were significantly different from no protein controls (lane 1) ($p<0.05$) by one-way ANOVA (GraphPad Prism)*

10.4.2 Optimizing the pre-cleaved assay conditions

The FRET-based PC insertion and deletion assays were screened to establish the optimized concentrations of ATP, UTP (for PC insertion and the RIDE assay), and purified editosome (KREL1-TAP tag calmodulin eluate) and also for optimized incubation durations (Figure 10.3). The optimized ATP concentration was established approximately at 100 nM in the PC deletion assay (Figure 10.3A) and PC insertion assay (data not shown), without compromising significantly on efficiency. Likewise, the optimized UTP concentration for the PC insertion assay was observed approximately at 10 µM (Figure 10.3B). A linear correlation between the amount of editosome and relative editing activity in the PC assays was observed (Figure 10.3C). The optimized ATP (100 nM) and UTP (10 µM) concentration with 50 fmol of

editosome were used in timepoint assay (Figure 10.3D), also exhibiting a linear relationship between time and activity, whereby overnight incubation led to the most substantial activity for all assays. Note that the timepoint initiation was after addition of ATP. The output of the optimized assays, as compared in Figure 10.3E against a standard curve of active HHR activity, indicates efficient activities from PC deletion and ligation.

As the PC assays use substrate ATP at 100 nM, 10,000-fold less than the conventional full-round ribozyme-based FRET assay, these assays are more sensitive to discovering competitive inhibitors of ATP. In Figure 10.3F, inhibition of the PC ligation assay by a nonhydrolyzable ATP analog, α, β-methylene ATP (ABMA), is observed. However, no such impact is seen on the "full-round" assay. The amenability of these assays in HTS applications was then determined through Z-factor calculation with at least 20 replicates in the presence and absence of a known inhibitor (suramin). The Z-factor determined for the PC ligation, deletion and insertion assays were 0.7, 0.82 and 0.84, respectively (data not shown).

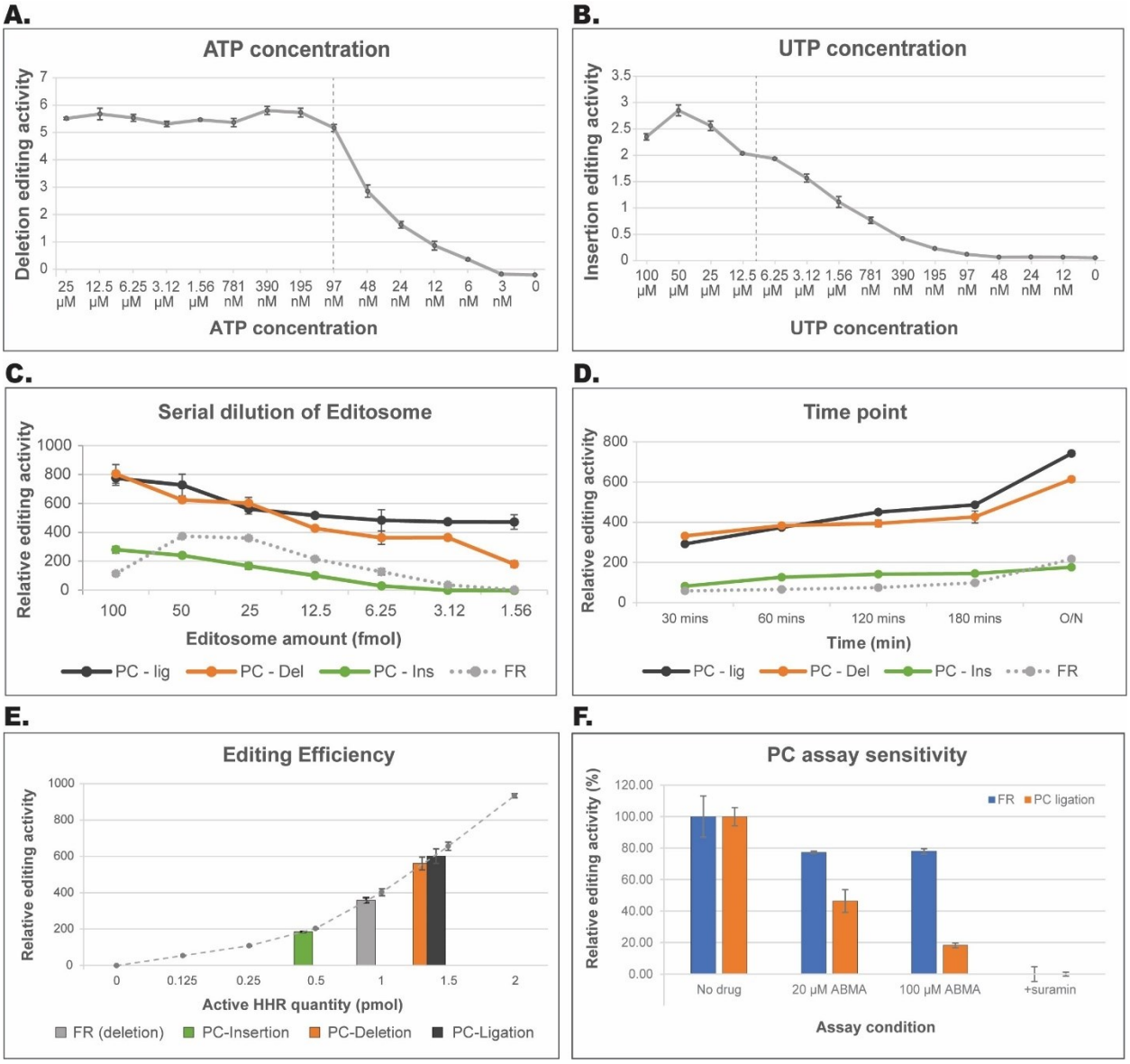


Figure 10.3. Optimized conditions for efficient editing in the pre-cleaved assays. *Varying concentrations of (A) ATP and (B) UTP substrates in the PC-deletion and insertion assays, respectively. (C) Relative RNA editing activities of different amounts of functional editosome in the PC-insertion, deletion, ligation, and the full-round assay. (D) Timepoint measurement of editing activities in the PC-insertion, deletion, ligation, and the conventional full-round assay. (E) Editing efficiencies of the different assays against a standard plot of active HHR. Each bar represents an editing assay and the corresponding activity of their edited product. (F) an ATP analog (α, β-methylene ATP) was used to compare the sensitivity of the ligation assay with a full-round assay to find competitive inhibitors. There was no difference between editing activity in full round assay in the presence of 20 μM versus 100 μM ABMA ($P > 0.05$) but the ligation assay inhibition was significant ($P < 0.05$) calculated by one-way ANOVA (GraphPad Prism). Means and standard deviations in all 6 plots were calculated and shown for at least two replicates.*

10.4.3 Ribozyme insertion/deletion editing (RIDE) assay

To monitor insertion and deletion editing activities simultaneously, the HHR sequence was slightly modified to recognize a different FRET substrate (Figure 10.4A). When tested in the same reaction, the active HHR1 and HHR2 reporters specifically cleave FRET1 (measured for FAM) and FRET2 (measured for Cy5) substrates respectively (Figure 10.4B). PC versions of these HHR substrates were thus designed to facilitate multiplexing U-insertion, and deletion editing activities in the RIDE assay (Figure 10.4C), where HHR1 is modified for use in U-insertion editing and HHR2 is modified for screening U-deletion editing. Figure 10.4D shows the edited products of U-insertion and U-deletion reactions to cleave their respective FRET1 and FRET2 substrates specifically. Consequently, multiplexing is feasible by combining these assays, where FRET signals representing both U-insertion and U-deletion activities are detected simultaneously, as corroborated on urea-PAGE (Supplemental Figure S10.6). Intriguingly, in the absence of UTP, a small amount of FRET1 cleavage (from the U-insertion HHR reporter) is observed, likely due to recycling of uridine residues obtained from the U-deletion HHR substrate catalysis.

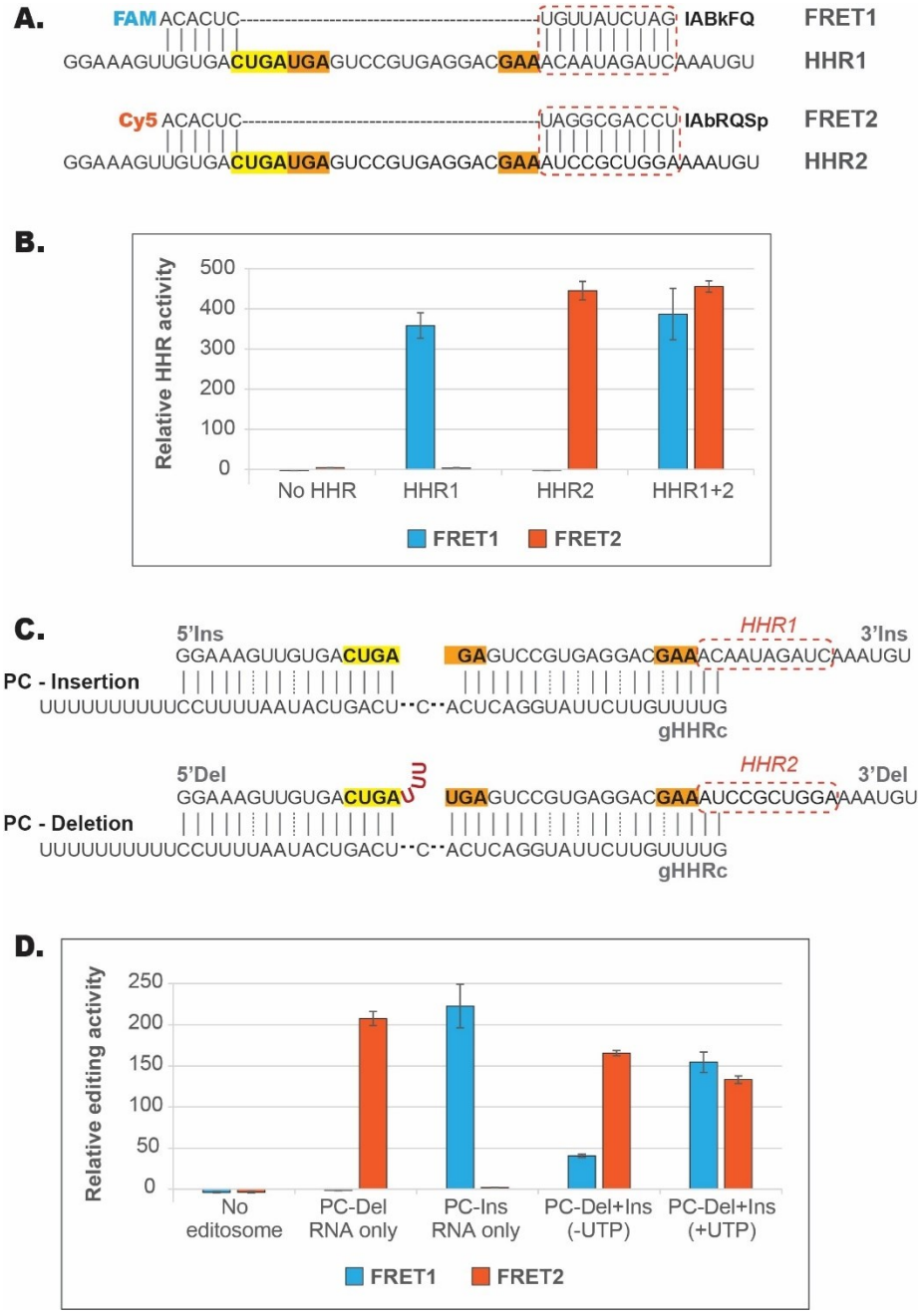


Figure 10.4. Multiplex measurement of U-insertion and deletion in the ribozyme-based insertion/deletion editing (RIDE) assay. *(A) Schematic representation of HHR1 and HHR2 bound to their respective target FRET substrates, FAM-FRET1- IABkFQ and Cy5-FRET2- IAbRQSp. The dissimilarity FRET substrate hybridizing region of the two HHRs is shown in red dashed boxes. Essential nucleotides in the catalytic site of ribozyme are highlighted in yellow and orange. (B) Specificity of HHR1 and HHR2 against their FRET substrates. Signals released because of FRET1 and FRET2 cleavage are shown in blue (FAM) and orange (Cy5) for four experimental conditions. All four lanes contain both FRET1 and FRET2 substrates. The X-axis determines which*

ribozyme is added to each lane. (C) Diagram of PC-insertion and PC- deletion trimolecular hybrids in the RIDE assay. HHR1 and HHR2 were fragmented to provide the PC RNA substrates for assaying insertion and deletion activities. (D) Measurement of U-insertion and deletion activities from the RIDE assay. FRET1 and FRET2 substrates are added post-editing. Means and standard deviations were obtained from three replicates and the values corresponding to each colored bar were statistically significant (P-value < 0.05) by one-way ANOVA (GraphPad Prism).

10.5 Discussion

Trypanosomatid RNA editing is suitable for target-based drug discovery and development (Salavati, Moshiri et al. 2012). While designing efficacious RNA editing assays is key to finding novel inhibitors, implementing an 800kDa multi-protein complex in HTS has previously led to discovering non-specific inhibitors. Apart from the recent finding that the editosome complex is prone to non-specific inhibition by negatively charged compounds, likely due to its reliance on the positively-charged MRP1/2 proteins for RNA chaperoning and editing initiation (Mehta, Moshiri et al. 2020), the assays presented in this paper have been optimized to overcome the other shortcomings with the assays currently in use. Bypassing the rate-limiting endonucleolytic cleavage step of RNA editing (Igo, Palazzo et al. 2000, Igo, Weston et al. 2002, Carnes and Stuart 2007) with the help of PC RNA substrates, not only improves editing efficiency as observed in Figure 10.3E, but also lowers the requirement of substrate ATP by 100 to 10,000-fold (Table 10.1), enabling heightened sensitivity of these assays in discovering ATP competitive inhibitors (Figure 10.3F). Moreover, with the development of the multiplexing RIDE assay, simultaneous measurements of TUTase, ExoUase and ligase activities further increase the odds of discovering inhibitors of these catalytic processes. Upon multiplexing the sensitivity for finding competitive inhibitors such as ABMA was maintained (Supplemental Figure S10.7).

During optimization of these assays, two factors played a role: (1) inclusion of a cytidine residue in the gRNA sequence and (2) delayed addition of ATP. The addition of a cytidine residue in the RNA editing site region of the gRNA, significantly enhances U-deletion and ligation activity (Supplemental Figure S10.2), as previously described (Cruz-Reyes, Zhelonkina et al. 2001, Wang, Salavati et al. 2002). As it does not affect the efficiency of the PC insertion assay (Supplemental Figure S10.2), either gRNA could be used for that assay, gHHR or gHHRc; therefore, to limit compromising the U-deletion efficiency in the RIDE assay, gHHRc is used in

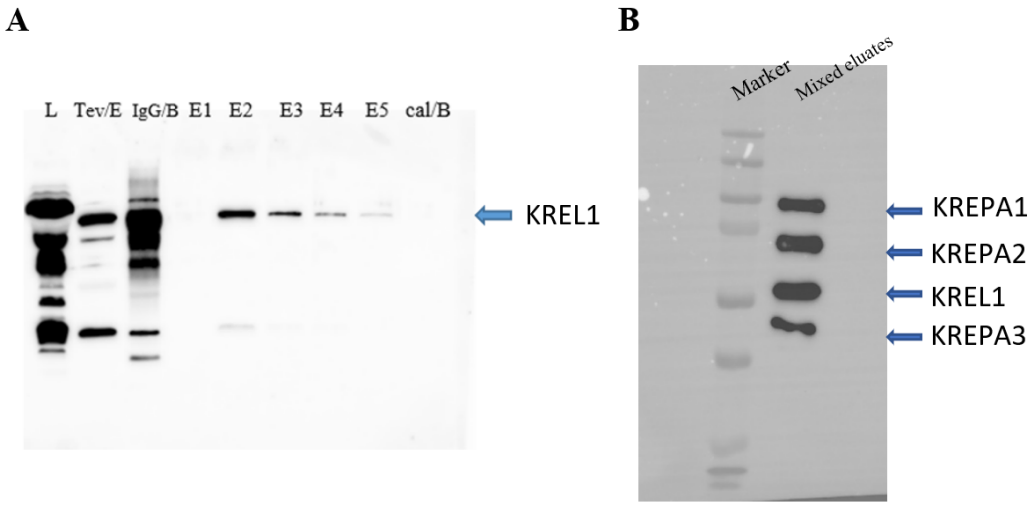
both tri-molecular hybrids (Figure 10.4A). The requirement of delayed addition of ATP stems from the observation in Figure 10.2 condition 7, where the ligated products do not appear to be completely edited on urea-PAGE (further resolved on a 6% urea-PAGE in Supplemental Figure S10.5). This indicates that the ligases are more efficient than the other enzymes in the RECC, which likely already contain pre-adenylated ligases (Figure 10.2 condition 6). This could presumably be why the inefficiency in the "full-round" assays, where the edited site is immediately ligated post-endonucleolytic cleavage. To circumvent this issue, the assay is designed to initiate in the absence of ATP for 1 h to allow complete insertion or deletion of uridine residues as per the gRNA sequence (Supplemental Figure S10.5), and significantly increase the activity of the final edited HHR (Figure 10.2 condition 8).

The assays presented exhibit a linear relationship with the amount of purified editosome, and robust activity is obtained from 1 h post incubation (1 h following ATP addition). Moreover, the RNA substrates do not require extensive chemical modifications to impart stability. Monitoring FRET substrate can be performed in real-time with kinetic readings on a quantitative RT-PCR machine or performed endpoint after incubation for 30-60 min with the FRET substrate, depending on the instrumentation available for HTS. Furthermore, preliminary testing establishes the feasibility of these assays for use in HTS applications with reconstituted catalytic editosomes with recombinant proteins, as performed earlier (Kang, Rogers et al. 2005, Kang, Gao et al. 2006). The major benefit of using reconstituted editosome is to obtain inhibitors only against the proteins involved, rather than obtaining non-specific inhibitors as previously observed, such as the sulfonated compounds that target the MRP1/2 proteins (Moshiri, Acoca et al. 2011, Mehta, Moshiri et al. 2020) In conclusion, this manuscript presents novel PC assays that are amenable for multiplexing in HTS applications and can also serve as secondary assays that are vital in determining the mechanism of known RNA editing inhibitors.

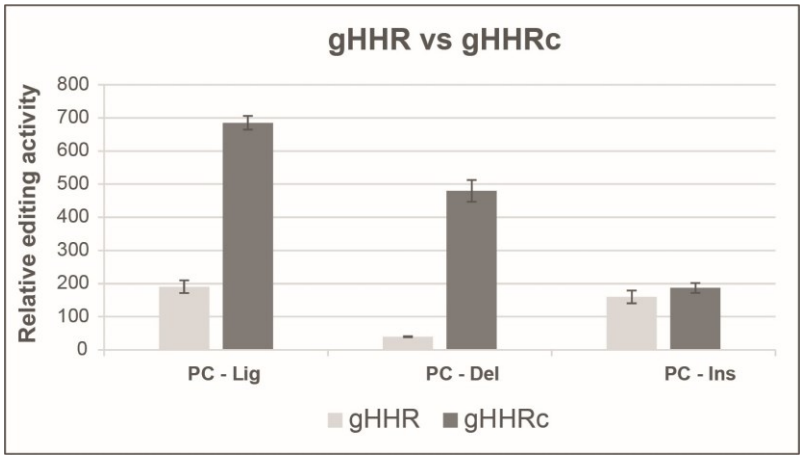
10.6 Acknowledgement

We thank Arezou Kamelshahroudi and Akshaya Srikanth for their technical assistance. This work was supported by the National Institutes of Health grant R01AI143593 to R.S.

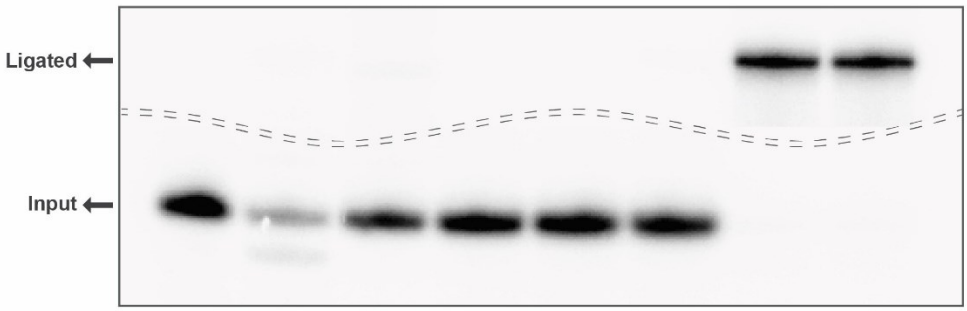
10.7 Supplementary data



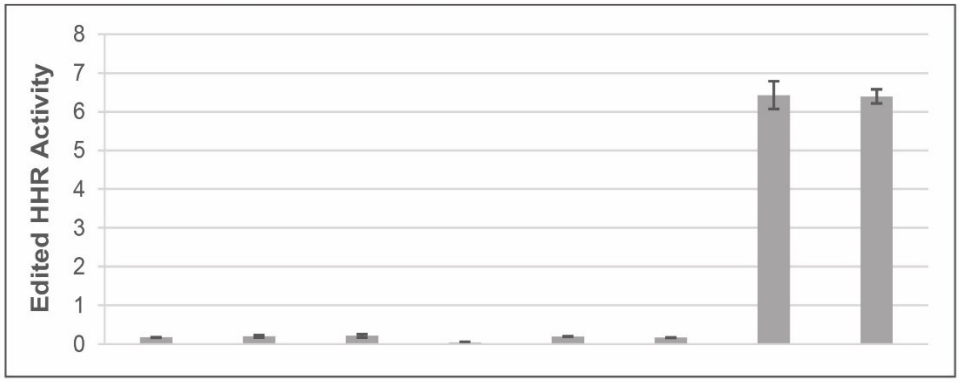
Supplemental Figure S10.1. Western blot analysis of isolation of TAP-tagged REL1 from T. brucei. *(A) Samples of cell lysate (lane 1), TEV eluate (eluate from first chromatography column, lane 2), IgG bead (lane 3), five final eluates from calmodulin chromatography column (lane 4-8) and calmodulin beads (lane 9) were analyzed using western blot with the anti-calmodulin Ab as the primary antibody and the anti-mouse Ab as the secondary antibody. (B) The final combined eluates from Tap-tag purification were probed against four essential proteins of the editosome (KREPA1-3 and KREL1) using antibodies developed for these proteins before.*



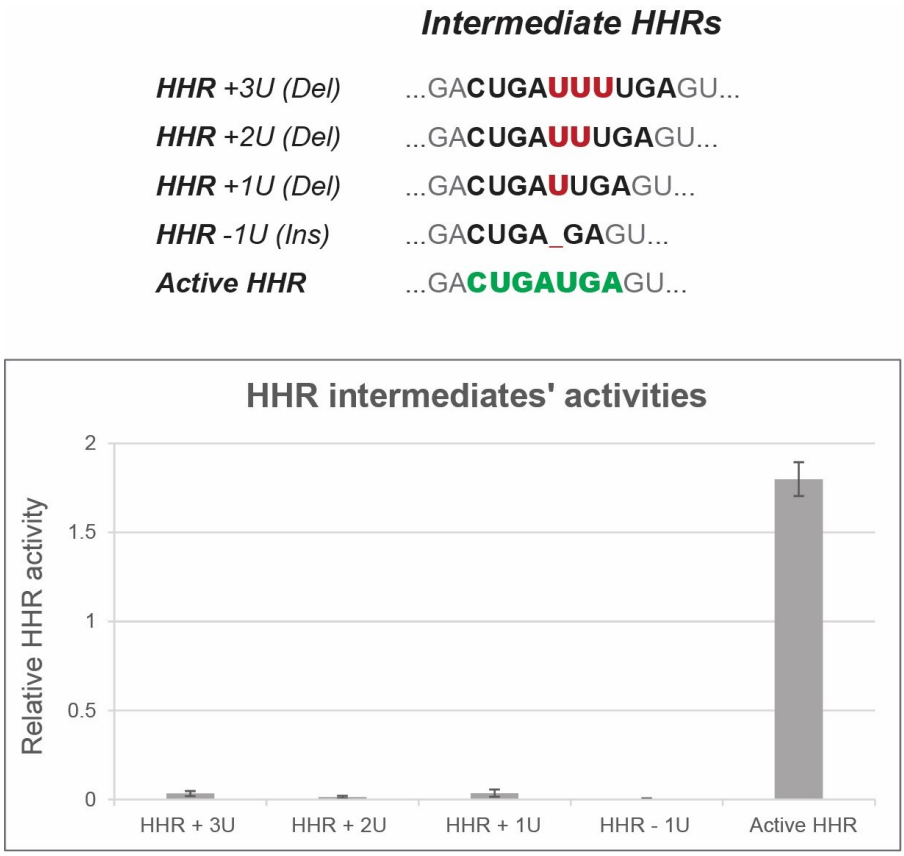
Supplemental Figure S10.2. Effect of using guide RNAs with and without a cytidine in the editing site. *Testing efficiency of RNA editing; PC-ligation, PC-Deletion, and PC-insertion using gHHR vs gHHRc. Means and standard deviation for at least two replicates are shown. Relative editing activity is measured in arbitrary units.*



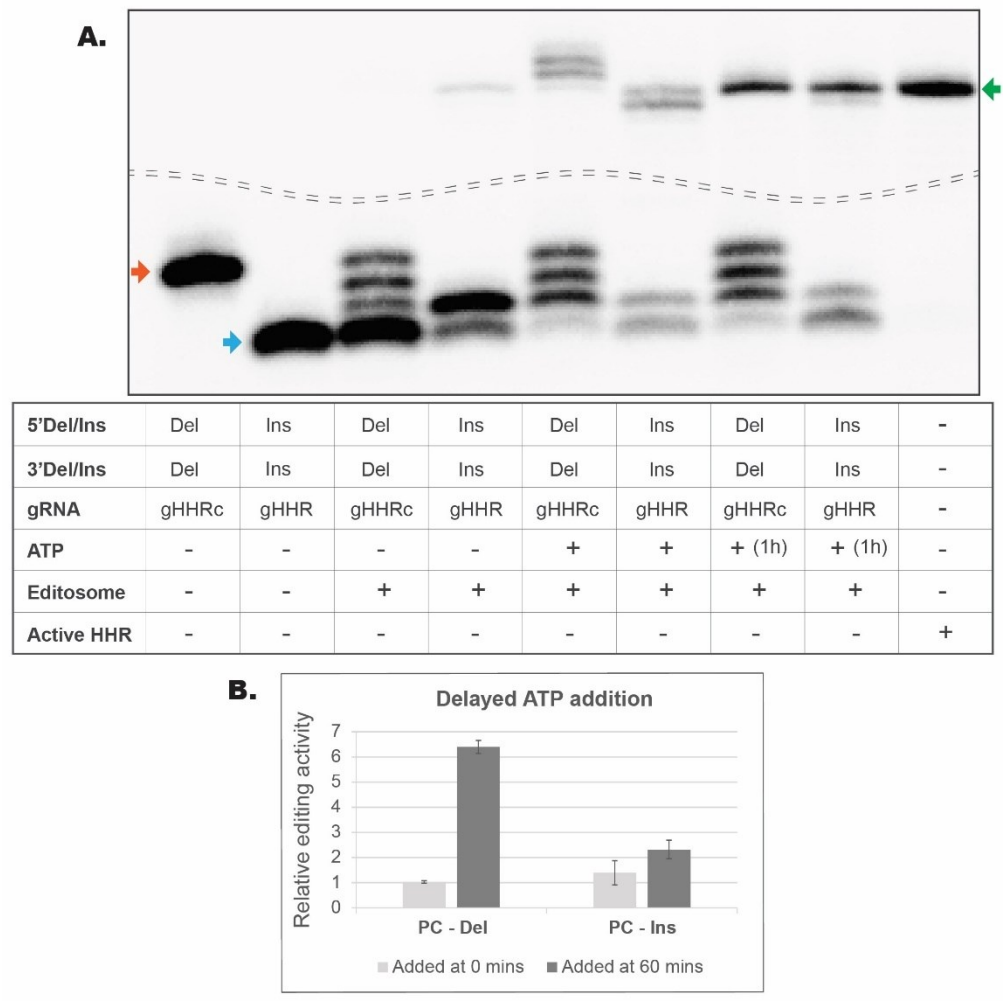
5'Ins	+	+	+	+	+	+	+	-
3'Del	+	+	-	+	+	+	+	-
gHHRc	+	-	+	+	+	+	+	-
ATP	+	+	+	+	+	-	+	-
Editosome	-	+	+	+	+	+	+	-
MrB	-	-	-	+	-	-	-	-
Suramin	-	-	-	-	+	-	-	-
Active HHR	-	-	-	-	-	-	-	+



Supplemental Figure S10.3. Development of PC-ligation assay. *Reactions are monitored using radiolabeled HHR RNA substrates (top panel) and FRET-based HHR substrates (bottom panel) with several controls (as described in the middle panel). PC-ligation assay with all components except functional editosome (lane 1), guide RNA (lane 2), 3' fragment (lane 3), and ATP (lane 6). Assay in the presence of RNA editing inhibitors (MrB and suramin, lanes 4 and 5). In the presence of all reaction components with discernible product/signal (lane 7). Active HHR serves as a size and activity control (lane 8). Means and standard deviations in the bottom plot were obtained from four replicates. Edited HHR activity is measured in arbitrary units.*

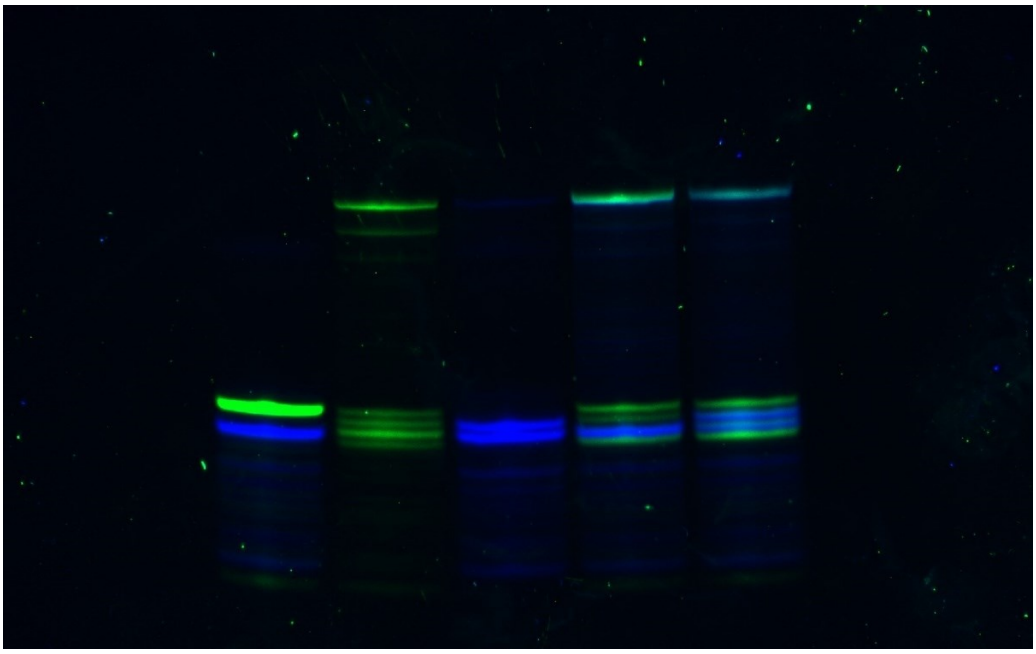


Supplemental Figure S10.4. FRET substrate cleavage activity of intermediate products in Precleaved deletion and insertion editing assays. *Partially edited RNA products of precleaved assays (colored in red) were investigated for FRET substrate cleavage activity. The fully edited sequence of the ribozyme's active site is colored green. Means and standard deviation for three replicates are shown on the bottom panel. Relative HHR activity is measured in arbitrary units.*

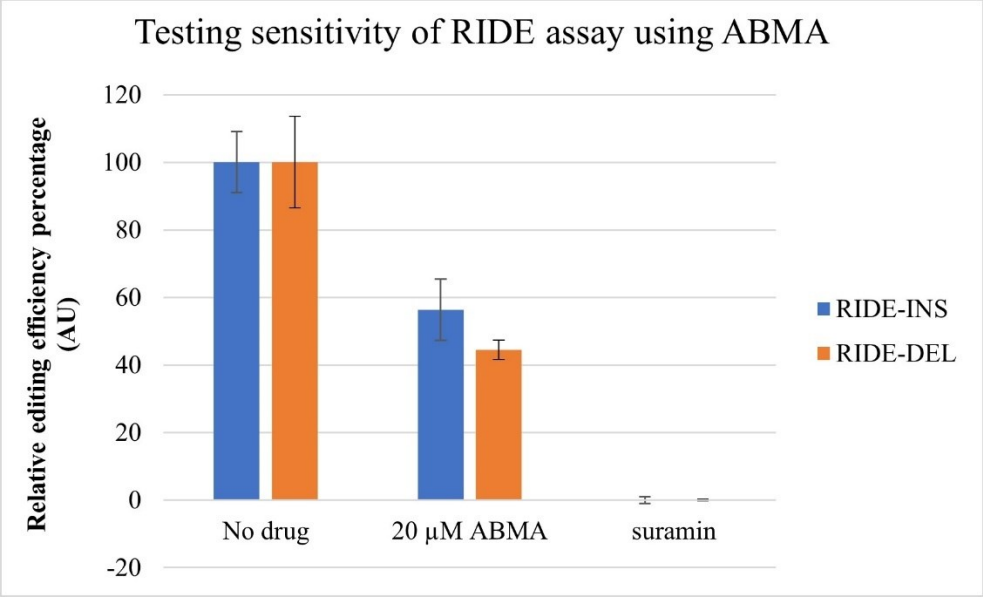


5'Del/Ins	Del	Ins	Del	Ins	Del	Ins	Del	Ins	-
3'Del/Ins	Del	Ins	Del	Ins	Del	Ins	Del	Ins	-
gRNA	gHHRc	gHHR	gHHRc	gHHR	gHHRc	gHHR	gHHRc	gHHR	-
ATP	-	-	-	-	+	+	+ (1h)	+ (1h)	-
Editosome	-	-	+	+	+	+	+	+	-
Active HHR	-	-	-	-	-	-	-	-	+

Supplemental Figure S10.5. Effect of delayed ATP addition on precleaved Insertion and deletion editing. *The impact of adding ATP to precleaved insertion and deletion editing reactions 1 hr post reaction outset was investigated using radiolabeled RNA substrates (A) and FRET-based assays (B). (A) In the absence of editosome (lane 1, 2), [5'Del] and [5'Ins] were not modified (shown by red and blue arrows, respectively. Editing in reactions lacking ATP, only contained ExoUase and TUTase activity on [5'Del] and [5'Ins], respectively (lane 3, 4). A significant difference can be observed among edited products when ATP is added to the reaction at the beginning (lanes 5, 6) compared to an addition 1 hr post reaction outset (lanes 7, 8). Lane 9 contains active HHR as a size control (shown by a green arrow). (B) Addition of ATP one hour after starting the reaction resulted in more edited HHR (measured by FRET cleavage activity) in both precleaved deletion and insertion assays. Relative editing activity is measured in arbitrary units.*



Supplemental Figure S10.6. Visualizing RIDE assay on the gel using fluorescent RNA substrates. *We monitored ExoUase, TUTase and ligase activity in the multiplex assay by labeling [5' Del] and [5' Ins] with Cy3 and Cy5, respectively (IDT, Coralville, IA). No modification is observed in the absence of editosome (lane1). Precursor RNA substrates of deletion and insertion editing were used in lanes 2 and 3, respectively. RIDE reactions without UTP/with UTP were loaded on lanes 4 and 5, respectively.*

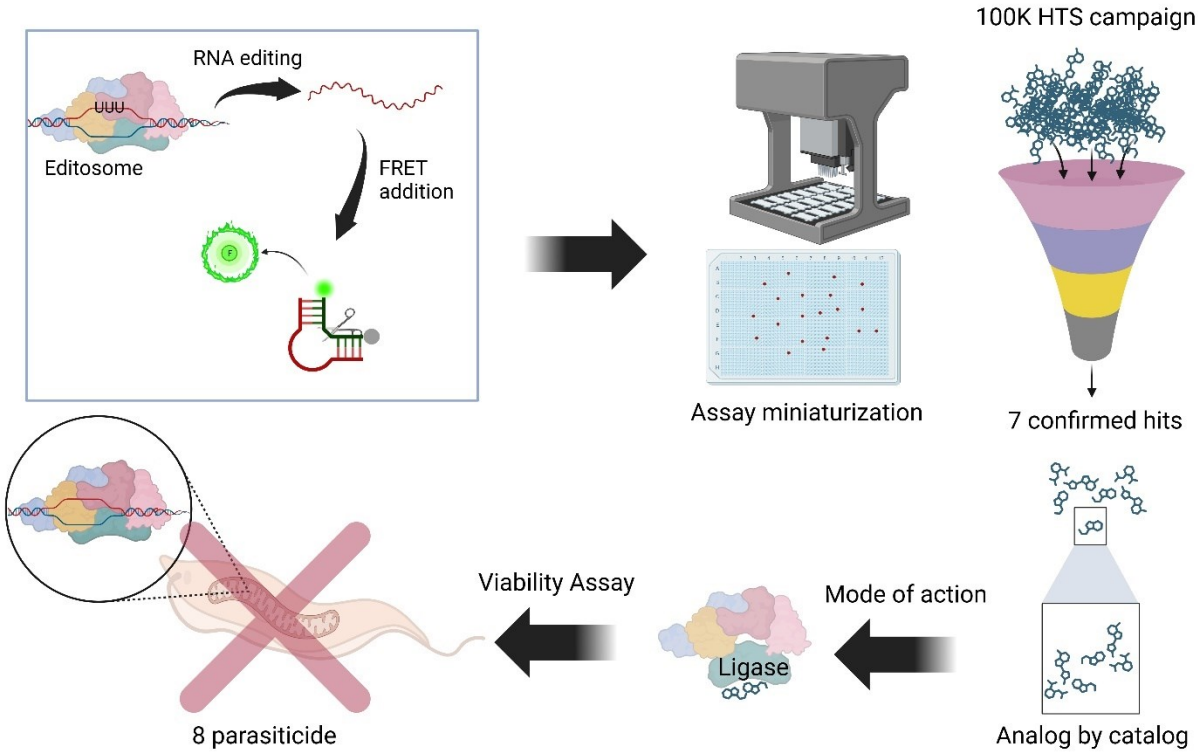


Supplemental Figure S10.7. Effect of ABMA on uridine insertion and deletion editing in multiplex assay. *The inhibitory effect of ABMA on uridine insertion and deletion editing was monitored simultaneously using RIDE assay. Suramin was used as a positive control. Means and standard deviations were obtained from two replicates. The inhibition in both deletion and insertion editing was significant (P value <0.05) by one-way ANOVA (GraphPad Prism).*

11. Connecting Statement I

Chapter III elaborated on a novel, efficient, HTS amenable, fluorescent-based precleaved RNA editing assay (Ribozyme Insertion Deletion Editing, or RIDE assay), which can monitor both uridine insertion and deletion editing in a one-pot reaction. This *in vitro* assay efficiently uses minimal amounts of ATP and UTP, facilitating the identification of competitive inhibitors. Additionally, we developed fluorescent FRET-based and gel-based precleaved assays to monitor each catalytic step of the editing process. These assays will be employed in the mode of action hits from a high-throughput screen of a large compound library, as detailed in Chapter IV. The HTS was conducted using a previously developed FRET-based assay from our lab, previously utilized in a pilot-scale screen (Moshiri and Salavati 2010, Moshiri, Mehta et al. 2015).

12. Chapter IV: High-throughput Screening of Compounds Targeting RNA Editing in *Trypanosoma brucei*: Novel Molecular Scaffolds with Broad Trypanocidal Effects



This chapter is a reprint of:

Rostamighadi M†, Kamelshahroudi A†, Mehta V, et al. High-throughput screening of compounds targeting RNA editing in Trypanosoma brucei: Novel molecular scaffolds with broad trypanocidal effects. *Biochem Pharmacol*. 2024;219:115937. doi:10.1016/j.bcp.2023.115937

†Mojtaba Rostamighadi and Arezou Kamelshahroudi contributed equally to this work and should be considered co-first authors.

12.1 Abstract

Mitochondrial uridine insertion/deletion RNA editing, catalyzed by a multiprotein complex (editosome), is essential for gene expression in trypanosomes and *Leishmania* parasites. As this process is absent in the human host, a drug targeting this mechanism promises high selectivity and reduced toxicity. Here, we successfully miniaturized our FRET-based full-round RNA editing assay, which replicates the complete RNA editing process, adapting it into a 1536-well format. Leveraging this assay, we screened over 100,000 compounds against purified editosomes derived from *Trypanosoma brucei*, identifying seven confirmed primary hits. We sourced and evaluated various analogs to enhance the inhibitory and parasiticidal effects of these primary hits. In combination with secondary assays, our compounds marked inhibition of essential catalytic activities, including the RNA editing ligase and interactions of editosome proteins. Although the primary hits did not exhibit any growth inhibitory effect on parasites, we describe eight analog compounds capable of effectively killing *T. brucei* and/or *Leishmania donovani* parasites within a low micromolar concentration. Whether parasite killing is - at least in part - due to inhibition of RNA editing in vivo remains to be assessed. Our findings introduce novel molecular scaffolds with the potential for broad antitrypanosomal effects.

12.2 Introduction

The three major groups of trypanosomatid pathogens ("the Tritryps"), namely *Trypanosoma brucei*, *Trypanosoma cruzi,* and *Leishmania* subspecies, are closely related and responsible for causing human African trypanosomiasis (and related diseases in animals), Chagas' disease, and the spectrum of diseases called Leishmaniases, respectively (Barrett, Burchmore et al. 2003, Hotez, Fenwick et al. 2009, Büscher, Cecchi et al. 2017, Alcântara, Ferreira et al. 2018, Burza, Croft et al. 2018, Pérez-Molina and Molina 2018). These flagellated protists belong to the order Kinetoplastea due to their unique DNA-containing structure (kinetoplast) within their single, large mitochondrion. The diseases they cause are predominantly found in tropical and subtropical regions across the globe. However, there is a growing concern due to their increasing prevalence in industrialized nations, including the USA (Manne-Goehler, Umeh et al. 2016, Curtin and Aronson 2021). The currently available medications are not highly effective and often have a range of adverse effects (Supuran 2023). Consequently, numerous research groups are dedicated to discovering improved treatments. This has led to the approval of new drugs and the progression of many candidates through preclinical and clinical trials (De Rycker, Wyllie et al. 2023). Notable examples include fexinidazole, acoziborole, oxaborole DNDI-6148, and GSK3494245 (DDD01305143) (Alcântara, Ferreira et al. 2018, Reguera, Pérez-Pertejo et al. 2019, De Rycker, Horn et al. 2020, Bernhard, Kaiser et al. 2022, Betu Kumeso, Kalonji et al. 2023, De Rycker, Wyllie et al. 2023, Melfi, Carradori et al. 2023)

Our efforts revolve around the exploration and development of pan-kinetoplastid drugs. We are particularly interested in a conserved pathway known as mitochondrial uridine insertion/deletion RNA editing, which is present in these parasites but absent in human hosts. This approach of targeting a conserved protein among kinetoplastids has previously proven successful; notable examples include the kinetochore, proteasome, and topoisomerase II (Khare, Nagle et al. 2016, Xie, Dick et al. 2019, Saldivia, Fang et al. 2020, Rao, Gould et al. 2023). However, these specific targets and their inhibitors were identified by deconvoluting the targets associated with hits from phenotypic screens.

In trypanosomes, RNA editing is critical for proper gene function. Specifically, this process places most mitochondrial mRNAs into the correct reading frame, thereby making these genes functional. This process is guided by small non-coding RNA molecules known as guide RNAs (gRNAs). These gRNAs are complementary to the regions surrounding the pre-edited mRNA (pre-mRNA) editing sites and serve as templates to direct the insertion or deletion of uridine residues (Goringer 2012, Aphasizhev and Aphasizheva 2014, Read, Lukes et al. 2016, Aphasizheva, Alfonzo et al. 2020).

The editing process relies on an ~20S (~800 kDa) RNA-editing catalytic complex (RECC), which executes the editing in a stepwise manner. The RECC is a modular assembly that allows editing functions on various RNA substrates. The precise positioning and number of the edits are determined by gRNAs (Blum, Bakalara et al. 1990, Blum and Simpson 1990). *In vivo*, the RECC potentially recognizes and binds its substrates directly. However, substrates are more likely to be delivered to RECC by another multiprotein complex, termed RNA editing substrate complex (RESC) (Liu, Wang et al. 2023). This complex recognizes a unique three-helix junction structure that forms when pre-mRNA hybridizes with gRNA, thus activating the editing process through its endonuclease activity. (Reifur and Koslowsky 2008, Goringer 2012). The subsequent steps involve adding or removing uridine residues by terminal uridyltransferase (TUTase) and ExoUase, respectively. Finally, the ligase enzyme joins the RNA substrates together, creating the edited final product. (Pollard, Harris et al. 1992, Piller, Decker et al. 1995, Corell, Read et al. 1996). The main proteins involved in U-insertion include KRET2 TUTase, KREPA1 zinc-finger protein, and KREL2 RNA ligase, and in U-deletion, KREX2 exonuclease, KREPA2 zinc-finger protein, and KREL1 RNA ligase. Crosslinking mass-spectrometry has revealed interactions involving RNase III domain dimerization between editing endonucleases (KREN1-3) and various partner proteins, orchestrating the precise editing of RNA in trypanosome mitochondria. While the modular nature of RECC is evident, the specific interactions that determine contacts between the core and distinct modules are not yet fully understood.

The inactivation of expression of the individual components involved in RNA editing demonstrates that most of them are essential for the growth and survival of the parasite

(Schnaufer, Panigrahi et al. 2001, Drozdz, Palazzo et al. 2002, Aphasizhev, Aphasizheva et al. 2003, Kang, Rogers et al. 2005, Kang, Gao et al. 2006, Guo, Ernst et al. 2008, Tarun, Schnaufer et al. 2008, Salavati, Moshiri et al. 2012). Therefore, the RNA-editing complex, due to its multitude of distinct proteins – including enzymes essential for the editing process, structural proteins that stabilize or position other components, and nucleic acid binding proteins – provides a rich set of potential drug targets.

Researchers have employed various approaches, including virtual (Amaro, Schnaufer et al. 2008, Durrant, Hall et al. 2010, Moshiri, Acoca et al. 2011, Demir, Labaied et al. 2014) and pilot scale screening with *in vitro* assays (Liang and Connell 2010, Moshiri, Mehta et al. 2015, Zimmermann, Hall et al. 2016), to find inhibitors targeting the enzymatic steps in RNA editing. Despite extensive efforts, the search has yielded only a limited number of confirmed hits, which exhibit relatively low efficiency. Our lab previously performed virtual screening on a large library of compounds and a pilot-scale screen on the library of pharmacologically active compounds (LOPAC®1280). From this, we found compounds that inhibited RNA editing by disrupting RNA-protein interactions (Moshiri, Acoca et al. 2011, Moshiri, Mehta et al. 2015). These findings helped us substantiate the role of mitochondrial RNA binding proteins 1 and 2 in the RNA editing process, thus demonstrating the effectiveness of chemical biology tools in analyzing the editosome complex (Mehta, Moshiri et al. 2020).

To discover new RNA editing inhibitors, we used our previously developed FRET-based RNA editing assay (Moshiri and Salavati 2010) to screen a library of 100,000 compounds from the SBP-curated screening collection of diverse compounds. This primary screen yielded 7 confirmed hits that inhibited RNA editing activities, with IC_{50} values in the low micromolar range without affecting parasite viability. Through fluorescence-based assays and structure-activity studies, we initiated mode-of-action studies that suggest potential mechanisms for some of the inhibitors and identified the critical functional groups on the chemical scaffold classes responsible for inhibiting RNA editing. Our screening identified a wide spectrum of inhibitors. These encompassed compounds targeting catalytic functions, those specific to subcomplexes, molecules broadly influencing RNA editing activities, and small molecules tailored to disrupt

protein-protein interactions within the editosome. Several compounds demonstrated parasiticidal activity against *T. brucei* and/or *L. donovani*, underscoring their potential as starting points for drug discovery in kinetoplastid diseases.

12.3 Materials and Methods

12.3.1 Preparation of RNA substrates

The RNA substrates used in the experiments were prepared using two methods: by large-scale *in vitro* transcription with the T7 polymerase RiboMAX transcription kit from Promega (Madison, WI) (Moshiri and Salavati 2010, Moshiri, Mehta et al. 2015) or by chemical synthesis and labeling performed by Integrated DNA Technologies (IDT, San Diego, CA). A summary of all sequences is provided in **Table 12.1**.

Full round	
PreA6	5'-/Phos/ACAUUUGAUCUAUUGUUUCGUCCUCACGGACUCAUCAAAAGUCACAACUUUC CCUUUCUCUCCUCCCCCUAACCUUUCC/OH/-3'
gA6	5'-/Phos/AAAAAAAAAAAAAAAAAUAAUUAUCAUAUCACUGUCAAGGGAAAGUUGUGA GGGUGAUGAGUCCGUGUAUAUCCC /OH/-3'
gA6 competitor	5'-/Phos/GGATATACACGGACTCATCACCCTCACAACTTTCCCTTGACAGTGATATGATAA TTATTTTTTTTTTTTTTTT/OH/-3'
FRET substrate	5'-/FAM/GAUCUAUUGUCUCACA/IABkFQ/-3'
TUTase activity (Mode of action assay)	
5′Ins	5'-/Cy5/GGAAAGUUGUGACUGA/OH/-3'
3′Ins	5'-/Phos/GAGUCCGUGAGGACGAAACAAUAGAUCAAAUGU/Phos/-3'

gHHR	5'-/OH/GUUUUGUUCUUAUGGACUCAUCAGUCAUAAUUUUCCUUUUUUUUUU/Phos/-3'
gHHR competitor	5’-AAAAAAAAAAGGAAAATTATGACTGATGAGTCCATAAGAACAAAAC-3'
ExoUase (Mode of action assay)	
5′Del	5'-/Cy3/GGAAAGUUGUGACUGAUUU/OH/-3'
3′Del	5'-/Phos/UGAGUCCGUGAGGACGAAACAAUAGAUCAAAUGU/Phos/-3'
gHHRc	5’-/OH/GUUUUGUUCUUAUGGACUCACUCAGUCAUAAUUUUCCUUUUUUUUUU/Phos/-3’
gHHRc competitor	5'-AAAAAAAAAAGGAAAATTATGACTGAGTGAGTCCATAAGAACAAAAC-3'
Interference Assay	
Active HHR	5'-/Cy5/GGAAAGUUGUGACUGAUGAGUCCGUGAGGACGAAACAAUAGAUCAAAUGU/Phos/-3'
EMSA	
gA6[14]	5'-/FAM/AUAUACUAUAACUCCAUAACGAAUCAGAUUUUGACAGUGAUAUGAUAAUUAUUUUUUUUUUUUUUU-3'

Table 12.1. List of RNA and DNA sequences used in the assays.

12.3.2 Large-scale Purification of Editosome complex

We prepared the editosome complex from a recombinant insect stage procyclic *T. brucei* cell line expressing tandem affinity purification (TAP)-tagged KREL1, using a previously described method (Stuart, Panigrahi et al. 2004, Rostamighadi, Mehta et al. 2023). The purification process, which involved immunoglobulin G and calmodulin chromatography steps, was scalable. To obtain enough editosomes for various studies — miniaturization, high-throughput screening (HTS), confirmation, mode of action, and structure-activity relationship (SAR) — we cultured fifty liters of *T. brucei* and purified the protein complex. The core editosome enzymes were confirmed to be present and active through Western blotting and enzymatic assays (data not shown)

12.3.3 In vitro translation of Recombinant RNA editing ligase 1

We used the TnT® Quick Coupled Transcription/Translation System (Promega, Madison, WI) to express the recombinant RNA editing ligase 1 protein (Moses, Mehta et al. 2023), which was then purified using magnetic nickel beads (Dynabeads, Invitrogen, Carlsbad, CA). This purified protein facilitated the study of compounds' inhibitory effects on an isolated single enzyme in contrast to the entire editosome complex, as assessed in the ligation assay.

12.3.4 Full-round deletion editing assay

For the full-round deletion editing assay (**Figure 12.1A**), facilitating the endonucleolytic cleavage, exonucleolytic removal of U residues, and subsequent ligation, we followed the procedure outlined in references (Moshiri and Salavati 2010, Moshiri, Mehta et al. 2015), with the substitution of TAP-tagged purified complexes for mitochondrial fractions. We combined 1 pmol of pre-edited RNA (PreA6) and 2.5 pmol of guide RNA (gA6), denatured at 70°C for 3 min, and cooled to room temperature (RT) for 10 min.

Then, we prepared a master mix comprising 25 mM HEPES (pH 7.9), 10 mM $Mg(OAc)_2$, 50 mM KCl, 1 mM EDTA, 1 mM adenosine triphosphate (ATP), 5 mM $CaCl_2$, and 0.1% Triton X-100, and introduced RNA, protein (50 fmol per each reaction), and compounds into the mix, respectively. The reaction mixture with the final volume of 20 µl was then incubated overnight at 27°C. Following this, 40 pmol of guide competitor (gA6 comp) was added, followed by incubation at 85°C for 10 min and further incubation at 25°C for 5 min. Finally, the FRET substrate (20 pmol) was introduced into the reaction, and the reaction mixture was kinetically measured at 37°C using a quantitative polymerase chain reaction (qPCR) machine.

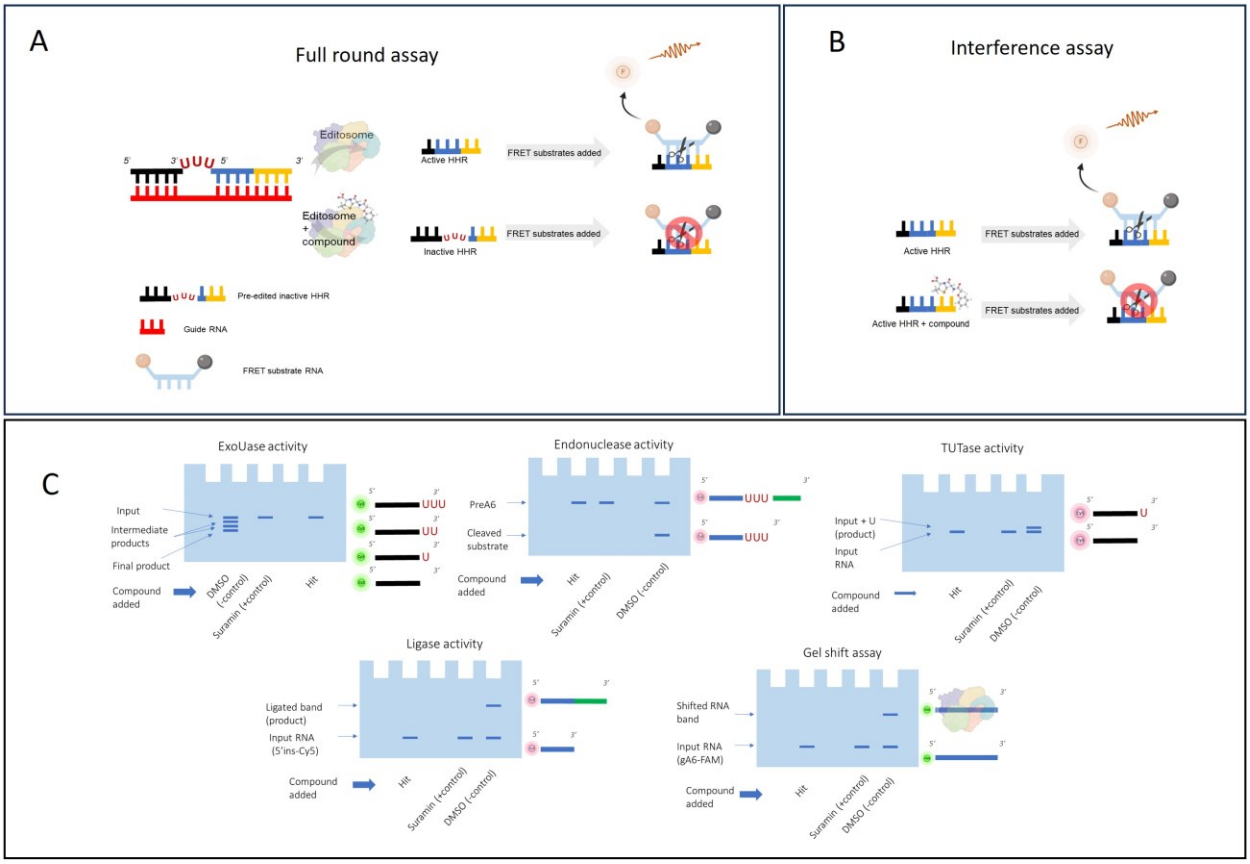


Figure 12.1. Schematic representation of the primary screening assay, interference assay, and mode of action assays. *(A) The primary ribozyme-based full-round assay workflow is depicted. If a compound inhibits editing, the inactive ribozyme cannot cleave the FRET substrate (B). The interference assay differentiates genuine editosome inhibition from ribozyme activity inhibition. (C) Each mode of action assay evaluates compounds against a single enzymatic step: ligase, endonuclease, TUTase, exoUase, and RNA-protein interaction inhibition. Each gel includes DMSO as a negative control and suramin (a known editosome inhibitor(Liang and Connell 2010, Mehta, Moshiri et al. 2020)) as a positive control. A hit may be inactive (similar to the negative control) or active (similar to the positive control). Inhibition in the assay is inferred when there is no change observed in the input RNA. A simple representation of each band (input and expected output/s) is illustrated on the right side of the gels. Suramin has been documented to inhibit a range of trypanosomal enzymes (Fairlamb and Bowman 1980, Willson, Callens et al. 1993, Liang and Connell 2010, Zimmermann, Hall et al. 2016, Albisetti, Halg et al. 2023), including RNA editing ligase, as shown in a study by Zimmermann et al. in 2016 (Zimmermann, Hall et al. 2016).*

12.3.5 High throughput screening

We adapted the full-round assay to fit a high-density 1536-well format (Corning 3724, Corning, NY). For a typical day of screening, we combined 3.6 nmol of pre-edited RNA (PreA6) and 7.2 nmol of guide RNA (gA6) in 350 µL of 25 mM HEPES, pH 7.9, then denatured this mixture at 70°C for 3 min, and cooled to RT for 10 min. This was then added to yield 60 mL of a

Master Mix containing 25 mM HEPES pH 7.5, 10 mM $Mg(OAc)_2$, 1 mM EDTA, 5 mM $CaCl_2$, 1 mM ATP, and 0.1% Triton-100, and 12 mL of the editosome protein to yield 10 fmol/µL editosome, 1.2 pmol/µL preA6, and 2.34 pmol/µL of gA6. We pre-dispensed 25 nL of DMSO, test compound, or reference ATA compounds into the appropriate wells of a 1536-well, as noted below. Then, we dispensed 5 µL of the Master Mix to each well of the 1536-well plate to yield a final 50 fmol editosome, 6 pmol preA6, and 11.7 pmol gA6 per well. We assessed the new conditions for Z' score (a statistical measure that assesses the quality of an assay by quantifying the separation between the positive and negative controls (Zhang, Chung et al. 1999)) and signal-to-background ratio (S/B) and conducted HTS at the Sanford Burnham Prebys (SBP) facility, screening 100,000 compounds from a curated internal library at SBP custom ordered and procured from multiple chemical vendors, at a final concentration of 10 µM in the reaction. The library was subjected to multiple chemi-informatics filters to eliminate **P**an **A**ssay **In**terfering Compound**S** (PAINS (Baell and Holloway 2010, Baell and Nissink 2018)), ensure structural diversity, and enrich for drug-like properties. We filled the plate's periphery with water to mitigate edge effects, utilizing only the central wells. We included negative controls consisting of DMSO in columns 6 and 7 and positive controls with 10 µM aurintricarboxylic acid (ATA, Millipore Sigma, St. Louis, CA), a known RNA editing inhibitor (Moshiri, Mehta et al. 2015)) in columns 4 and 5. We transferred both controls and compounds (988 compounds per plate) to the plate using a Beckman Coulter (Brea, CA) Echo ® acoustic drop ejector (2.5 nL per drop), then dispensed the reaction mix (containing master mix, protein, and RNA) onto the plate using a Beckman Coulter (Brea, CA) BioRAPTR dispenser. After centrifuging and placing a milled gasketed stainless steel Kalypsys lid (San Diego, CA – permanently closed) on top, we incubated the plate at RT overnight. After we incubated the plate at RT overnight, we added 1 µL of 10 µM guide competitor RNA using the BioRAPTR dispenser. We then incubated the plate at 85°C for 10 min, removed it from the incubator, and allowed it to cool down to RT while the lid was still in place. We subjected the plate to centrifugation at 1000 rpm for 1 min to facilitate the settling of the contents and finally, dispensed 1 µL of 7.5 µM FRET substrate diluted into 25 mM HEPES, pH 7.9. After spinning the plate again and covering it with a Kalypsys lid, we incubated it at 37°C for 2 hours to allow the enzymatic cleavage of the FRET substrate. Finally, we read the plate using a BMG LABTECH (Cary, NC) PHERAstar reader at 37°C, measuring the release

of fluorescein resulting from the cleavage of the FRET substrate. The resultant data, indexed against positive and negative controls, offered the inhibition percentage for each compound. This data underwent analysis via the Genedata software.

12.3.6 Interference assay

Each compound was tested in an interference assay (**Figure 12.1B**) to differentiate between non-specific inhibitors of ribozyme activity, such as ethidium bromide; artifacts that reduce the emission of fluorescent signal, such as ruthenium red; and genuine editosome inhibitors. This assay gauges the compound's impact on editosome activity, thereby eliminating potential confounding effects, such as non-specific ribozyme binding that could block activity. Only the active ribozyme (A6) at 1 pmol is included, excluding preA6 and guide RNA. The reaction lacks proteins, but all other conditions are consistent with those in the full-round assay. After incubating the samples for 30-min at 27°C, we kinetically assessed the cleavage of the FRET substrate to evaluate ribozyme catalytic activity.

12.3.7 Mode of action studies

Fluorescent gel-based assays were developed to examine the effect of compounds on individual RNA editing enzymatic steps, including ligase, TUTase, ExoUase, and endonuclease activities. Our primary assay was designed to discover inhibitors targeting activities associated with deletion editing, specifically those of the endonuclease, ExoUase, and ligation activities. It is crucial to ensure that these inhibitors do not impact the insertion activity of TUTase. Therefore, we incorporated the TUTase assay into our study, given that the purified editosome includes both TUTase and its associated activities. These assays were adapted from previous radiation-based *in vitro* RNA editing assays (Igo, Palazzo et al. 2000, Igo, Weston et al. 2002, Wang, Salavati et al. 2002, Moshiri, Mehta et al. 2015, Rostamighadi, Mehta et al. 2023).

For each assay, RNA substrates were annealed at 70 °C for 5 min, followed by a cooldown to RT. ([5’ Ins, 3’ Ins, gHHR], [5’ Del, 3’ Del, gHHRc], [5’ Ins, 3’ Del, gHHRc] and [PreA6, gA6] for TUTase, ExoUase, ligase, and endonuclease activity assays, respectively)(Moshiri, Mehta et al. 2015, Rostamighadi, Mehta et al. 2023). PreA6, used in the

endonuclease assay, was labeled with Cy5 by IDT (labels are depicted in **Figure 12.1C**). All assays were performed under the same conditions as the full-round assay. However, the ligation assay used 100 nM ATP, while the TUTase activity reaction included 100 μM UTP. To stop the ligase from modifying the RNA substrates in the mode of action studies (except in the ligation assay), ATP was excluded from the reactions. For these reactions, 50 fmol of the KREL1 TAP-tag purified editosome protein was used in all assays, except for the endonuclease assay, which required 250 fmol. After completion of the editing reaction, the entire reaction mixture was combined with gel loading buffer (7 M Urea in TBE buffer) and then loaded onto a 20% polyacrylamide gel for electrophoresis with 7 M urea. This was subjected to a run of 2 hours at 18 watts. Finally, the bands were visualized using the respective fluorescent dyes with a ChemiDoc mp system (bio-rad).

Suramin was used as a positive control in each mode of action assay. Suramin has been shown to inhibit a variety of trypanosomal enzymes (Fairlamb and Bowman 1980, Willson, Callens et al. 1993, Liang and Connell 2010, Zimmermann, Hall et al. 2016, Albisetti, Halg et al. 2023), including RNA editing ligase, as detailed in Zimmermann et al.'s 2016 study (Zimmermann, Hall et al. 2016), suggesting it may have global effects on the editing complex. This aligns with Suramin's broad impact on the editosome (Liang and Connell 2010), potentially through its interaction with mitochondrial binding proteins (Mehta, Moshiri et al. 2020). This interaction inhibits each enzymatic step of RNA editing, similar to the mechanism of other sulfonated naphthalene group inhibitors like Mordant Black and C35 (Mehta, Moshiri et al. 2020). These findings underscore Suramin's utility as an inhibitor in RNA editing studies.

For the FRET-based ligation assay, a modified version of [5’ Ins, 3’ Del, gHHRc] RNA substrates (without fluorescent label), 100 nM ATP, and a desired protein source (either recombinant (Moses, Mehta et al. 2023) or editosome (Rostamighadi, Mehta et al. 2023)) were used in the Full round assay condition.

12.3.8 Electrophoretic mobility shift assay

A fluorescent-based gel electrophoretic mobility shift assay (EMSA) was used to investigate the compound's effect on the RNA binding activity of the complex. This assay was developed based on previously described radioactive-based EMSA methods (Kala and Salavati 2010, Nikpour and Salavati 2019, Mehta, Moshiri et al. 2020). This assay used gA6[14] RNA labeled with fluorescein maleimide (FAM, IDT, Coralville, IA) to bind to the protein. Before the reaction, this RNA was heated at 95°C for 5 min and then cooled to RT. The reaction mixture had a total volume of 20 µl, containing 10 pmol of the labeled gA6[14] RNA (FAM), along with 20 mM Tris-HCl (pH 7.9), 150 mM KCl, 5 mM $MgCl_2$, 100 µg/ml BSA, 10% glycerol, 1 mM DTT, 20 units of RNasin ribonuclease inhibitor (Promega, Madison, WI), and 5 µl of 10-fold concentrated KREL1 TAP-tag editosome complex (2.5 nmol per each reaction). Before being added to the reaction mixture, the compound was incubated with the concentrated protein for 10 min. The reaction mixture was then incubated at RT for 30 min to allow for the binding between the labeled RNA and the protein complex. The reaction mixture was loaded onto a 4% native polyacrylamide gel and subjected to electrophoresis for 15 min at 150 V in 0.5X TBE buffer (Tris, boric acid, EDTA), maintained at 4°C. The bands were then visualized directly using the FAM fluorophore with a ChemiDoc mp imaging system (Bio-Rad, Hercules, CA).

12.3.9 Viability assay on parasites

We utilized the Alamar blue assay to test the viability of *T. brucei* PRA-382 (Lister 427 VSG 221 bloodstream form) and Axenic *L. donovani* strain 1S2D (MHOM/SD/62/1S-CL2D (Vacchina, Norris-Mullins et al. 2016)) promastigote parasites, using 96-well plates, following established protocols (Räz, Iten et al. 1997, Sykes and Avery 2009). On day one, the bloodstream from *T. brucei* cells was seeded at a density of 2000 cells/ml, allowing adaption and growth in an HMI-9 medium. The following day, we introduced the compounds of interest into the plate's wells. After 48 hours of incubation at 37°C, we added Alamar blue reagent (from Life Technologies, Carlsbad, CA) to each well and continued incubation for an additional four hours at 37°C. This period allowed viable cells to interact with the reagent. We then measured the plate's fluorescence at 590 nm using a fluorescence reader or plate reader. Elevated fluorescence

values indicated increased cell metabolic activity, which served as a proxy for cell viability. Based on these values, we calculated the minimum inhibitory concentration (MIC) for each compound. The MIC represents the lowest concentration of the compound at which noticeable changes in the parasites' metabolic activity and population growth occur, indicating its potency. While this assay is a valuable tool for assessing *in vitro* activity, it's important to recognize that its primary measurement is the reduction in metabolic activity, serving as an indicator of parasite viability. Notably, it may not differentiate between static and trypanocidal effects.

The rationale for selecting a 48-hour duration for the Alamar Blue assay is based on the potentially higher efficiency of chemical ablation compared to genetic ablation (i.e., RNAi effects) on RNA editing, which can take as long as 3 days to manifest. Chemical interventions, due to their intrinsic properties, tend to elicit responses more rapidly. This distinction is evident in prior studies on trypanosomes. For instance, in research on topoisomerase II, RNAi-induced effects took 3-4 days to impact viability (Kulikowicz and Shapiro 2006), whereas chemical compounds achieved parasite eradication in just a day (Rao, Gould et al. 2023). Similarly, a study on phosphofructokinase showed a 50% reduction in viability over 2 days with RNAi [14], but a chemical inhibitor attained a 99% kill rate in under 30 minutes [15]. These examples highlight the varying timeframes required for RNAi-induced effects and chemical inhibition in different contexts (for a comprehensive review (Weiss, Taylor et al. 2007)), emphasizing the importance of understanding the unique characteristics and kinetics of the compounds being evaluated. We believe that the chosen assay conditions, while relatively short, are suitable for assessing the initial viability effects of the compounds under investigation.

12.3.10 Time-to-kill assay

The efficacy of each compound was assessed against *T. brucei* PRA-382 (Lister 427 VSG 221 bloodstream form). On day one, we seeded 2000 cells into each well of a 96-well plate. Following a 24-hour incubation period, we introduced the compounds at their MIC concentration. At predetermined intervals post-incubation, we counted the live cells using a Neubauer chamber. The percentage of viability was calculated by comparing the live cell count to that of a “no drug” control well. Suramin, a medication currently used for treating sleeping

sickness, served as the positive control for the assay. Suramin was selected for use in our study based on its well-established activity against *T. brucei*. It has been reported to have an EC50 of approximately 27-68 nM against this parasite in previous studies (Jones, Hallyburton et al. 2010, Thomas, Baker et al. 2018). To ensure robust and detectable results in our time-to-kill assay, we opted for a suramin concentration of 10 µM. This concentration is at least 100 times higher than the reported EC50, providing a substantial safety margin to ensure that the compound effectively inhibits parasite growth and viability.

Furthermore, our choice of 10 µM suramin aligns with the findings of a previous study [13], which demonstrated a slow killing rate of 11 µM suramin with a time-to-kill of over 20 hours. Therefore, the selected concentration provides a clear margin of safety and is consistent with the observed kinetics of suramin's effect on parasite viability in the literature.

12.3.11 In silico ADME prediction

We used the SWISSADME online tool to predict important properties of various compounds, including physicochemical characteristics, water solubility, pharmacokinetics, drug-likeness, and medicinal chemistry compatibility for different compounds (Daina, Michielin et al. 2017). To begin, we converted each compound’s 2D structure into its SMILES format using OSRA. The resultant computed descriptors were retrieved directly from the server. Additionally, we employed the BOILED-Egg (Brain Or IntestinaL EstimateD permeation predictive) model to predict gastrointestinal absorption and brain access for each compound. These predictions are important factors to consider when developing a drug to combat human African trypanosomiasis (Daina and Zoete 2016, Daina, Michielin et al. 2017).

12.4 Results

12.4.1 Improved assay efficiency through miniaturization

After conducting multiple tests, we have successfully miniaturized the assay into a 1536-well format. We achieved this by using custom-milled aluminum gasketed microplate lids, which helped reduce temperature gradients across the microplates during the denaturation/annealing step. Our analysis of the HTS experiment showed that the 1536-well format is superior to the 384-well format in terms of accuracy and precision in detecting the intended target (**Table 12.2**). This led to a significant reduction (4-fold) in the amount of editosome and RNA substrates needed for the HTS campaign on SBPs curated diverse chemical library described in the Methods.

Parameter	384-well	1536-well
(-) Control (DMSO) a.u.	152,056 ± 14882	140,751 ± 8,576
(+) Control (10 μM ATA) a.u.	77,372 ± 3,605	59,234 ± 3,035
S/B	1.97	2.38
Z’	0.26	0.57

Table 12.2. Full round assay amenability to miniaturization. *Full-round assay was performed in two different plate formats (384 and 1536 wells). Positive and negative control conditions included ATA (full inhibition of RNA editing) and DMSO (no inhibition in RNA editing), respectively. The mean of the final fluorescence readouts for each condition was reported in arbitrary units (a.u.). Signal to background ratio (S/B) and Z’ parameters were assessed to compare the assay’s performance in the two formats. Z’ is a statistical parameter that judges whether an assay is qualified for HTS. S/B >3 and 0.5 < Z’ < 1 are desired for HTS assays. Means and standard deviations were calculated from at least ten replicates for each condition.*

12.4.2 HTS: Screening of 100,000 compounds using a full-round deletion RNA editing assay

Our rigorous testing procedures ensured the consistent and reliable evaluation of both the RNA and the extracts used in the primary screening assay. We completed a pilot-scale screening of 10,000 compounds that demonstrated the assay's robustness under full-scale HTS conditions

(**Table 12.3**) with a mean Z-factor (Z') and mean S/B ratio within the acceptable range of >0.8 and >3, respectively. This supported our preparation of sufficient RNA substrates and editosomes to embark on a large-scale screen of 100,000 compounds from the SBP library using the full-round RNA editing assay at a final concentration of 10 μM. Our results **(Table 12.3)** show that the robustness of the assay (Z'>0.8 and S/B > 3) is maintained throughout the full 100,000 compound HTS. The frequency distribution of hits exhibited a classical Gaussian "normal" distribution (**Figure 12.2A**), and the scatterplot highlighted hit compounds (**Figure 12.2B**). And though some plates (X-axis indexes between 100000-130000 **Figure 12.2B**) showed a slight drift in the scatterplot, the Z' and S/B were still within the acceptable range. We obtained between 0.5-1 % hit rate using 50-30% inhibition cut-offs (**Table 12.3**). We considered 30% inhibition activity as the threshold to identify positive hits. This resulted in 1066 initial hits, that upon retesting their stock solutions in triplicate at 10 μM, resulted in confirmation of only 71 (6.7%) compounds. This low confirmation rate possibly reflected the lower initial threshold selected and the propensity of the FAM label to suffer from optical interferences. These confirmed hits were then assessed at 5 points dose-response (80, 40, 20, 10, and 5 μM) in triplicate also from their original stock solutions. The 36 hits (51%) that showed dose-dependent activity were passed through Pan Assay Interference Compounds (PAINS) (Baell and Holloway 2010, Baell and Nissink 2018) filters to rule out potential promiscuous hits and artifacts. This resulted in the identification of 18 final compounds. We then ordered fresh powders of 16 of these dose-responsive hit compounds from vendors and two close analogs of an unavailable scaffold. We tested the 18 compounds for RNA editing inhibition at 10 different concentrations in duplicate, confirming 7 final compounds that showed RNA editing inhibitory effects, with low to moderate micromolar IC_{50}s (**Figure 12.3**).

	10K Pilot Screen	**100K Full HTS** *(including 10K pilot)*
(-) Control (DMSO) a.u.	174,841 ± 4,746	164,332 ± 20,739
(+) Control (10 μM ATA) a.u.	36,875 ± 1,218	53,810 ± 12,263
S/B	4.76 ± 0.48	3.19 ± 0.75

Z factor (Z')	0.89 ± 0.03	0.84 ± 0.07
No. Hit (≥50%)	63 (0.64%)	517 (0.52%)
No. Hit (≥40%)	78 (0.79%)	715 (0.72%)
No. Hit (≥30%)	100 (1.01%)	1066 (1.07%)

Table 12.3: HTS performance. *These results show that the pilot screen performance was maintained during the full 100K HTS, with the parameters (S/B and Z') in the acceptable range. ATA (previously known RNA editing inhibitor) and DMSO were used as positive and negative controls, respectively. The mean of the final fluorescence readout for each control conditions in full round assay was reported in arbitrary unit (a.u.). Z' is a statistical parameter used to determine whether an assay is qualified for HTS [52]. Desired values in HTS are S/B >3 and 0.5 < Z' < 1. The means and standard deviations for each parameter were calculated.*

Table 12.4 summarizes the data for the 7 final hits. It compares the results from two technical replicates of the full-round assays performed using new dry powders with the assay results obtained from testing original compounds on library plates.

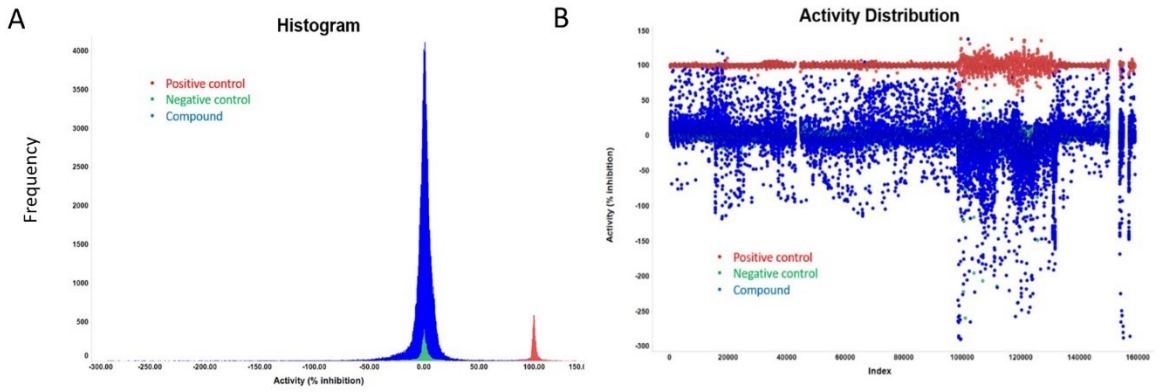


Figure 12.2: HTS campaign of a 100k tranche. *A) Frequency distribution of inhibitory activity: The x-axis represents the percentage of inhibition, while the y-axis denotes the number of compounds showing corresponding inhibition percentage B) Activity distribution of compounds: The x-axis shows the number of tested compounds, and the y-axis indicates the percentage of inhibition. Compounds showing more than 30% inhibition activity are considered hits.*

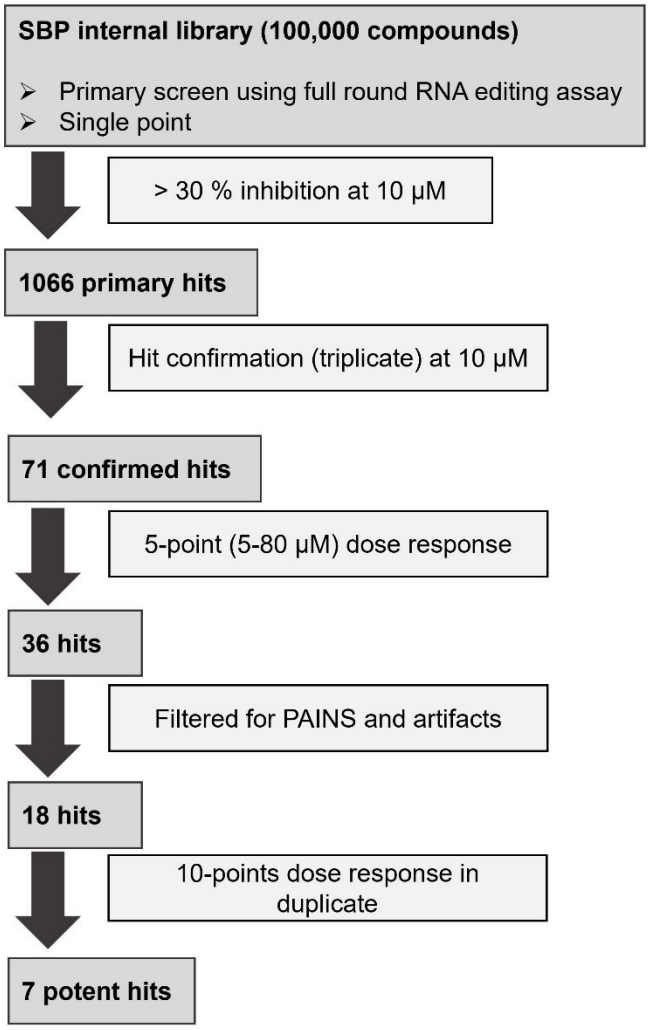


Figure 12.3. High-throughput screening cascade. *This figure depicts the workflow of the high throughput screen and further hit confirmation steps. It also summarized the attrition from initial primary hits to confirmed hits and the final 7 potent confirmed hits.*

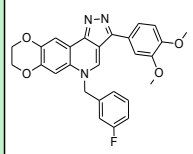
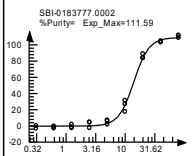
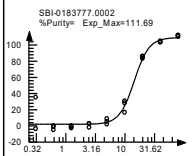
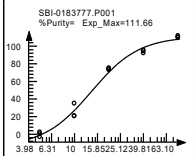
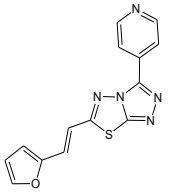
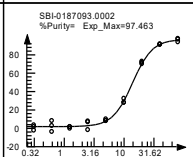
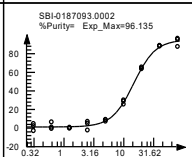
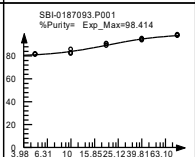
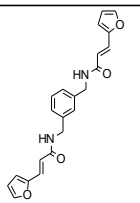
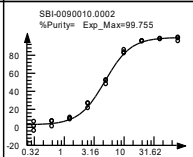
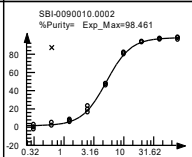
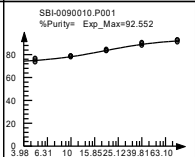
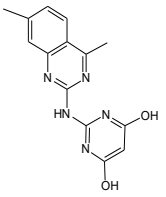
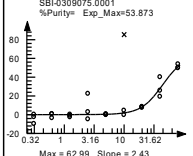
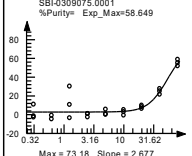
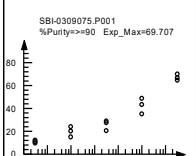
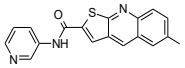
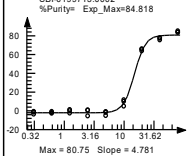
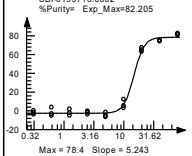
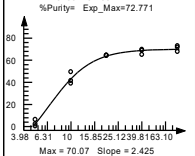
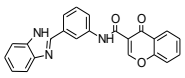
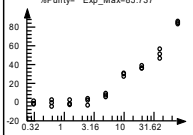
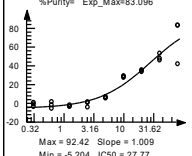
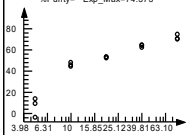
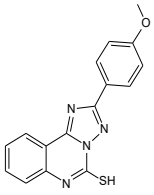
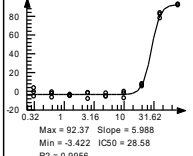
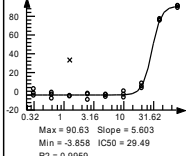
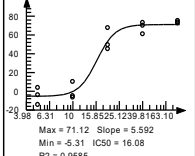
Compound ID	Structure	Dry Powders - Assay 1		Dry Powders - Assay 2		Library Plates (previous data)	
		IC_{50} (μM)	Curve	IC_{50} (μM)	ResponseCurve	IC_{50} (μM)	ResponseCurve
1		13.97		14.36		14.07	
2		13.85		14.67		20.32	
3		4.79		5.12		19.23	
4		43.71		53.85		> 80.00	
5		14.89		14.77		6.51	
6		> 80.00		27.77		> 80.00	
7		28.58		29.49		16.08	

Table 12.4: Activity summary of HTS final hits in 1536-well format full-round assays. *Information on the 7 final hits, along with the result of testing new dry powders using the full-round assay in a 10-point dose-response manner, its technical replicate, and the original compound from library plates. We note a >80 μM IC_{50} indicates data were insufficient for refinement to a 4-parameter non-linear least squares fitted curve. Estimated IC50s by inspection are noted in parentheses for Cpd 4 and 6.*

12.4.3 Mode of action studies and anti-trypanosomal efficacy of the 7 confirmed potent hits

The 7 confirmed potent hits were counter-screened using an interference assay to discern specific inhibitors. None inhibited ribozyme activity at concentrations effective for RNA editing, suggesting specificity. Fluorescent gel-based assays probed the compounds' inhibitory mechanisms against editosomes' catalytic activities, including endonuclease, exonuclease, TUTase, and ligase. Although deletion RNA editing does not involve TUTase activity, by testing TUTase activity, we aimed to determine if inhibiting other proteins impacts it, providing a comprehensive understanding of the compounds' effects within the context of the editosome's intricate network of enzymatic activities. Furthermore, an electrophoretic mobility shift assay (EMSA) was used to screen for inhibitors that could disrupt RNA-protein interactions.

Figure 12.1 illustrates the primary screening and interference assays and all related modes of action assays. **Table 12.5** summarizes the results for these assays and their hypothesized modes of action against the editosome. Despite the limitations of testing three concentrations without replicates, this approach suggests potential dose-dependent effects needed for initial screenings. **Figure 12.4** is an illustrative example, detailing the gel-based assay results for all seven parent compounds. The anti-trypanosomal efficacy of these 7 hits was also assessed through in vitro assays. However, none of these compounds effectively eliminated the parasites within the tested concentration ranges (data not shown).

Interestingly, compounds #4 and #6 appear to inhibit specific enzymatic activities: endonuclease and RNA ligase, respectively. For compound #6, the RNA ligase inhibitory action was observed at 100 μM, a much higher concentration than its IC_{50} of 28 μM in the full round assay. Despite using five times more protein in the endonuclease assay compared to the full-round assay and considering that compound #4 has an IC50 of approximately 50 μM in the full-round assay, compound #4 still inhibited the endonuclease assay by 50-80% at a concentration of 50 μM. This indicates that the inhibitory effect of compound #4 was greater in the endonuclease assay, even with a higher protein concentration. This higher protein concentration may contribute to the increased inhibitor concentration required to inhibit activity. While the biochemical activities being measured (endonuclease activity) are related, the assay systems have differences

that influence their sensitivity and the apparent efficacy of compounds. The full-round assay, being more complex, might have slower dynamics or additional rate-limiting steps that are not present in the simpler endonuclease-specific assay. If the compound's inhibition dynamics are slower than the assay's rate-limiting step, it might appear less effective in the full-round assay. Compound #5 affected both ligase and endonuclease, while #1, #2, #3, and #7 had complex effects, disrupting RNA-protein interactions and multiple enzymatic activities. We cannot rule out the possibility that some compounds, such as compound 7, may exhibit nucleophilic behavior due to the presence of the SH group. Such groups can engage in various interactions, and we plan to further evaluate this aspect in future studies.

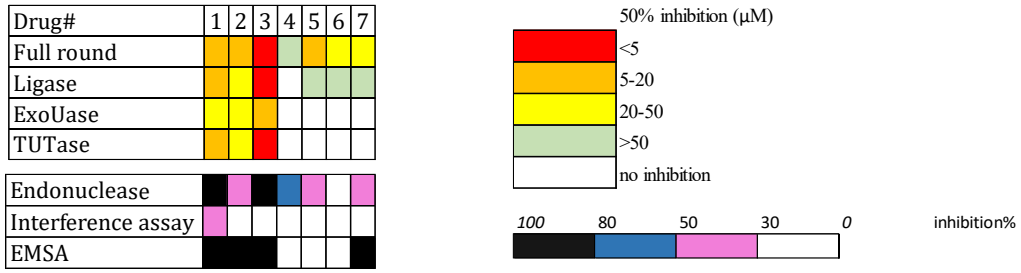


Table 12.5 Mode of action of the primary hits *Each compound was tested in secondary assays to determine its mode of action against editosome. The activity of compounds against TUTase, exoUase, and ligase was assessed at three different concentrations: 1.5, 25, and 100 µM. A line was plotted using the obtained data, and the concentration at which 50% inhibition (IC_{50}) occurred was derived. The data is visually represented in a heatmap format. The interference assay was done at two concentrations (50 and 200 µM), with the percentage inhibition at 50 µM depicted in the heatmap. The compounds were also subjected to EMSA and endonuclease assays at a concentration of 50 µM. The heatmap format visually represents the percentage inhibition, indicated by the lower legend. The effect of each compound shown in the heatmap for the full-round assay is based on IC_{50} values detailed in Table 12.4.*

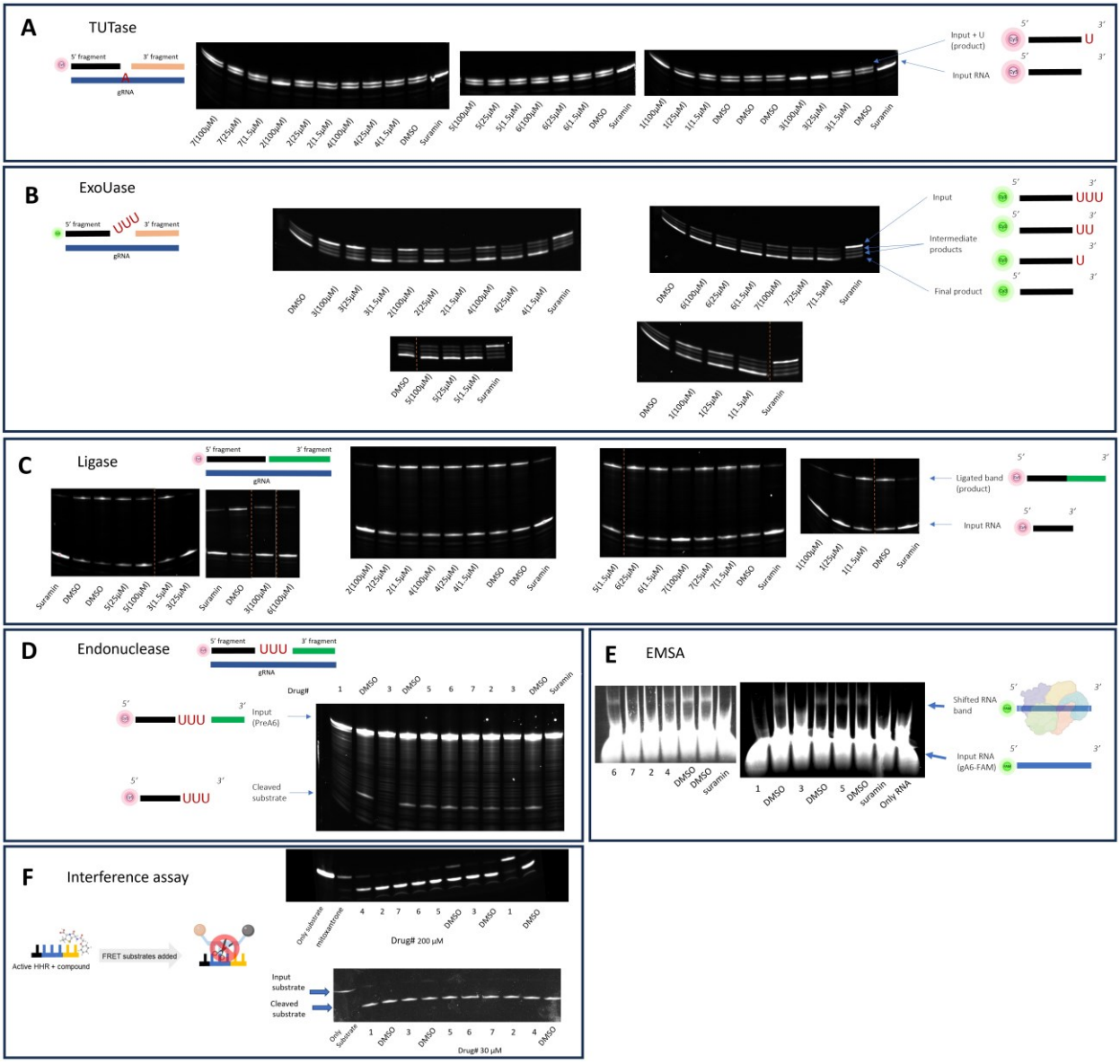


Figure 12.4. ***Mode of action studies of parent compounds***. *Each compound was tested against single enzymatic steps of editing, namely, TUTase (**A**), exoUase (**B**), ligase (**C**), and endonuclease (**D**). Schematics and descriptions of the RNA substrates in the reactions were depicted on top of the gels, illustrating the addition/deletion of uridines and pinpointing cleavage or ligation sites. Reactions were performed using the methods explained in the "Materials and Methods" section. After the completion of each reaction, samples were loaded on 20% polyacrylamide gel, and the results were visualized using their respective fluorescent label attached to the inputs. The arrows indicate inputs, intermediate products, and final products on the gels. (**E**) Gel shift assays were performed to investigate the potential for interference with RNA-protein interactions. Each assay included positive controls (demonstrating 100% inhibition by suramin, a known RNA editing inhibitor) and negative controls (showing 0% inhibition with DMSO). We determined the inhibition percentage of each compound by comparing their effects with these controls. (**F**) Moreover, the compounds were subjected to an interference assay to assess their impact on ribozyme activity and to rule out the possibility of non-specific inhibition. Mitoxantrone served as a positive control in this interference assay.*

12.4.4 Probing structure-activity relationship in editing inhibition by analogs of primary hits

Following the analysis of the primary hits, we obtained 31 commercially available analogs of compounds 1,2,4,5 & 6 to assess their inhibitory effects on RNA editing using the full-round assay. We did not find available analogs for compounds 3 and 7. **Table 12.6** groups the analogs using Markush representations against their parent compounds, with substituents highlighted in light blue. The potencies of the parent compounds are in bold blue font. The results suggest that analogs from compound group 2 show improved potency. However, the SAR is steep for group 5 compounds, indicating that minor molecular substitutions in the parent compound can lead to significant changes in their potency, either beneficial or detrimental. Compounds in group 6 showed shallow inhibition curves with an IC_{50} value greater than 8 μM. Subsequent mode of action assays further tested these analogs, with results categorized based on a compound group (**Table 12.7**). For clear representation, we adopted a systematic numbering scheme. For example, analog #2 of the primary hit #1 is denoted as 1-2, while analog #7 of the primary hit #5 as 5-7, and so forth.

12.4.4.1 Enzyme-specific inhibitors for the editosome: Targeting individual catalytic steps

Targeting specific steps in large protein complexes, such as the editosome, offers precise perturbation of biochemical pathways. Through SAR studies, we discovered compounds that potentially target one enzymatic step in the RNA editing process.

The endonuclease activity initiates the editing process. We discovered three small molecules that potentially disrupt this step: at 50 μM, compounds #4, #4-1, and #1-2 inhibited the activity by 75%, 60%, and 100%, respectively. Notably, replacing the hydroxyl group at R_1 in #4 with a methyl group in #4-1 increased its potency five-fold, reducing the IC_{50} from 50 μM to 9 μM, confirmed by a full-round assay (**Table 12.6 and Table 12.7**). Given the inherent limitations of testing three concentrations without replicates, as presented in **Table 12.7**, it's worth noting that the selection of three distinct concentrations provides a spectrum of responses essential for preliminary assessments. Following endonuclease activity, the editing continues

with uridine deletion by ExoUase or addition by TUTase. Analogs #2-1 and #4-5 potentially inhibit uridine addition, showing moderate potency (IC_{50}: 20-50 μM, **Table 12.7**). In contrast, analog #4-6 potentially prevents ExoUase-mediated uridine deletion but is less potent (IC_{50} > 50 μM **Table 12.7**). We speculate that #2-1, #4-5, and #4-6 may influence protein-protein interactions in the editing complex, as evidenced by their effects in the full-round assay.

Finally, we found four compounds inhibiting the final ligation step of the editing among all modes of action assays (parent compound #6 and analogs #6-2, #6-4, and #1-4).

12.4.4.2 Editosome inhibitors with broad-spectrum impacts

1. Broad-spectrum editosome inhibitors affect all editing assays, revealing potential complex mechanisms and cross-regulations. We identified two *in vitro* inhibitor types: RNA-protein interaction inhibitors: Compounds #1, #2, and #3, along with analog #1-1, affected both editing and EMSA, likely interacting with editosome's RNA binding proteins, known as mitochondrial RNA binding proteins.
2. Complex integrity inhibitors: Analogs #2-3, 2-4, and 2-6 impacted all editing without altering EMSA, suggesting they may affect the complex's structure or a shared essential factor.

12.4.4.3 Subcomplex (SC)-specific inhibitors

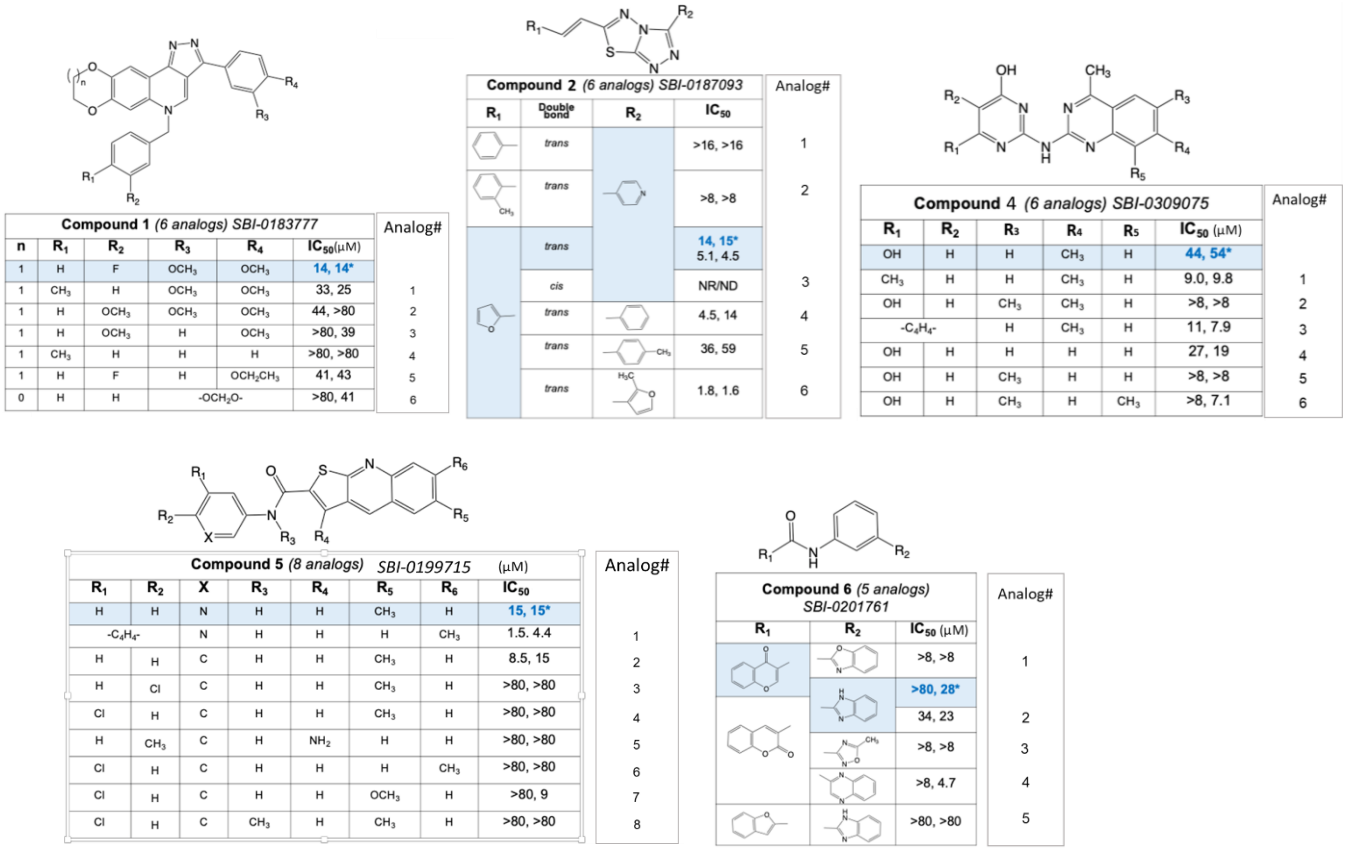
The RNA editing catalytic complex (RECC) is proposed to partition into two separate subcomplexes (SCs): the insertion SC, composed of KRET2, KREPA1, and KREL2; and the deletion SC, which includes KREX2, KREPA2, and KREL1 (Schnaufer, Ernst et al. 2003) (note KRE denotes kinetoplastid RNA editing).

Within this framework, some compounds, such as #1-5, #5-2, #5-6, and #5-7, potentially impact the insertion subcomplex, with #5-2 being the most potent. Compound #1-5 also displayed significant anti-parasitic effects against *T. brucei* and *L. donovani*.

12.4.4.4 Other types of inhibitors

A group of compounds mainly affects the full-round assay without impacting other action mode assays, e.g., #1-6, #2-5, #5-5, and #5-8. Most of these show moderate inhibitory potency except for #2-5.

Another set of compounds (#4-4, #5-1, #5-3, #5-4) suppress ligase, exoUase, and TUTase activities but not endonuclease activity. The lack of impact on endonuclease activity could be due to its unique assay requirements or the inhibitors acting later in the editing process. Yet another group impacts several action mode assays. For instance, Compound #5 and analog #1-3 affect both ligase and endonuclease activities, possibly due to their interaction with shared proteins. Compound #4-2 affects ExoUase and endonuclease activities with a mild impact on the full-round assay. Compound #4-3 strongly inhibits ExoUase, TUTase, and endonuclease activities, particularly in the full-round assay. Compounds #1-3 and #4-3 show strong parasiticidal effects from this last group. However, their IC50 values in the full-round assay and their efficacy against cells vary, with #4-3's IC50 closely matching its parasiticidal activity concentration.



Compound 1 *(6 analogs) SBI-0183777*

n	R_1	R_2	R_3	R_4	IC_{50}(μM)	Analog#
1	H	F	OCH_3	OCH_3	**14, 14***	
1	CH_3	H	OCH_3	OCH_3	33, 25	1
1	H	OCH_3	OCH_3	OCH_3	44, >80	2
1	H	OCH_3	H	OCH_3	>80, 39	3
1	CH_3	H	H	H	>80, >80	4
1	H	F	H	OCH_2CH_3	41, 43	5
0	H	H	-OCH_2O-		>80, 41	6

Compound 2 *(6 analogs) SBI-0187093*

R_1	Double bond	R_2	IC_{50}	Analog#
	trans		>16, >16	1
	trans		>8, >8	2
	trans		**14, 15*** 5.1, 4.5	
	cis		NR/ND	3
	trans		4.5, 14	4
	trans		36, 59	5
	trans		1.8, 1.6	6

Compound 4 *(6 analogs) SBI-0309075*

R_1	R_2	R_3	R_4	R_5	IC_{50} (μM)	Analog#
OH	H	H	CH_3	H	**44, 54***	
CH_3	H	H	CH_3	H	9.0, 9.8	1
OH	H	CH_3	CH_3	H	>8, >8	2
-C_4H_4-		H	CH_3	H	11, 7.9	3
OH	H	H	H	H	27, 19	4
OH	H	CH_3	H	H	>8, >8	5
OH	H	CH_3	H	CH_3	>8, 7.1	6

Compound 5 *(8 analogs) SBI-0199715* (μM)

R_1	R_2	X	R_3	R_4	R_5	R_6	IC_{50}	Analog#
H	H	N	H	H	CH_3	H	**15, 15***	
-C_4H_4-		N	H	H	H	CH_3	1.5. 4.4	1
H	H	C	H	H	CH_3	H	8.5, 15	2
H	Cl	C	H	H	CH_3	H	>80, >80	3
Cl	H	C	H	H	CH_3	H	>80, >80	4
H	CH_3	C	H	NH_2	H	H	>80, >80	5
Cl	H	C	H	H	H	CH_3	>80, >80	6
Cl	H	C	H	H	OCH_3	H	>80, 9	7
Cl	H	C	CH_3	H	CH_3	H	>80, >80	8

Compound 6 *(5 analogs) SBI-0201761*

R_1	R_2	IC_{50} (μM)	Analog#
		>8, >8	1
		>80, 28*	
		34, 23	2
		>8, >8	3
		>8, 4.7	4
		>80, >80	5

Table 12.6 Activity summary of analogs in the full-round assay *IC_{50}s and chemical structures for each are provided. Markush diagrams are paired with their respective parent compounds, with specific substituents emphasized in a light blue shade. The parent compounds' potencies are highlighted using a bold blue typeface. The two IC50 values are independent repeats from fresh powders of the analogs obtained, with the parent compounds highlighted in blue. NR/ND means – while ordered, it was ultimately Not Received (depleted), and therefore IC50 was Not Determined (ND)*

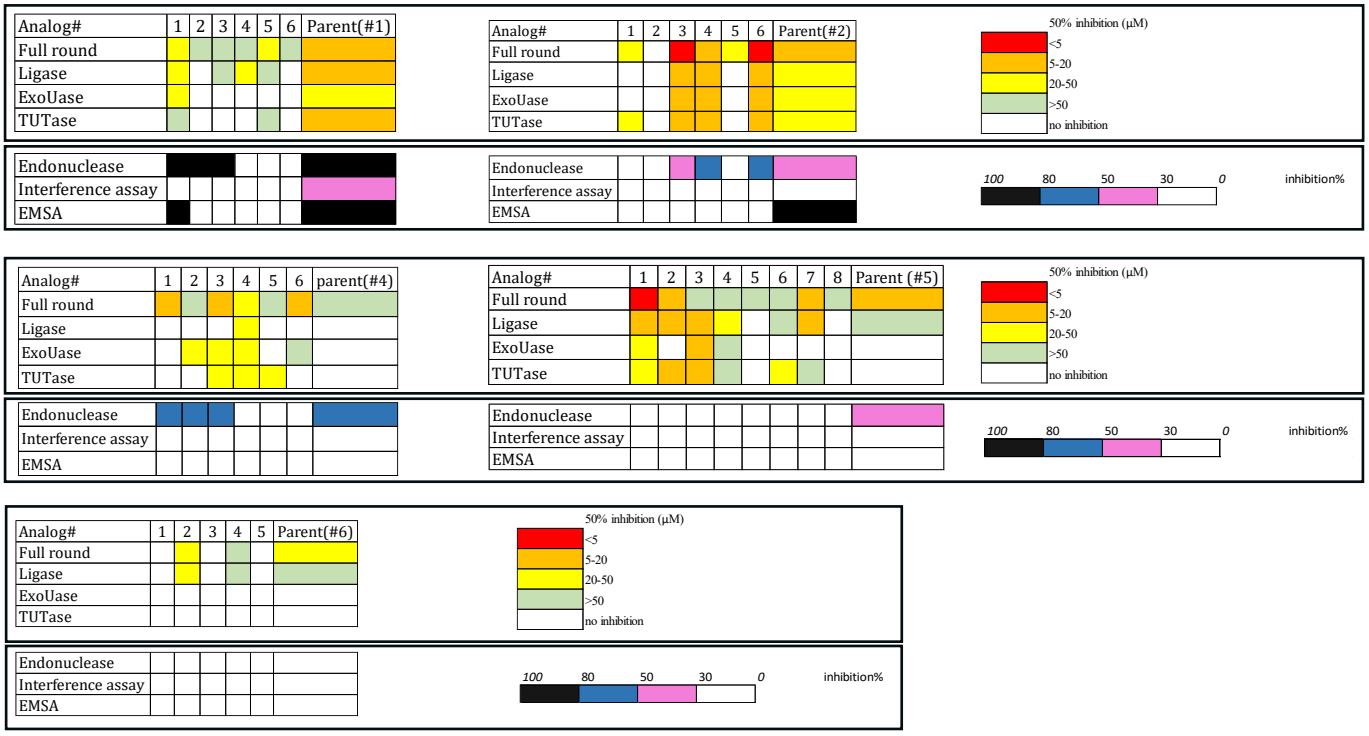


Table 12.7 Summary of the mode of action of different Parent compound groups *Each compound was tested in secondary assays to define its mode of action against the editosome. The activity of compounds against TUTase, exoUase, and ligase was assessed at three different concentrations (3, 12.5, and 50 μM) using gels as described in the materials and methods. Data were plotted, and the concentration at which 50% inhibition (IC_{50}) occurred was determined in micromolar. The resulting information is presented in a heatmap demonstration. The interference assay was performed at two concentrations (50 and 200 μM), with the percentage of inhibition at 50 μM shown in the heatmap. The compounds were also subjected to EMSA and endonuclease assays at a concentration of 50 μM. The resulting percentage of inhibition is also presented in the heatmap format. The IC_{50} of each compound in the full-round assay was added into the heatmap, based on Table 12.6.*

12.4.5 Testing compounds against ligation with the recombinant RNA editing ligase 1 and editosome

By substituting the protein source in the ligation assay from editosome to recombinant ligase, we aimed to discern the specificity of the identified hits on the individual enzyme. We tested the seven confirmed primary hits and their analogs against the recombinant ligase at a concentration of 50 μM. Interestingly, confirmed primary hits #1, #2, and #3 exhibited concentration-dependent inhibition of ligation activity, with IC_{50}s ranging from 4-17 μM (**Figure 12.5**). Additionally, we tested these seven hits in a dose-response manner (0.3-200 μM), using

our previously developed precleaved ligation assay against the editosome (**Table 12.8**) (Rostamighadi, Mehta et al. 2023). The results showed that parent compounds #1 and #3 could effectively inhibit ligation with the editosome. However, both compounds also inhibit EMSA (**Table 12.7**) and affect both the recombinant ligase and the editosome in the ligation assay.

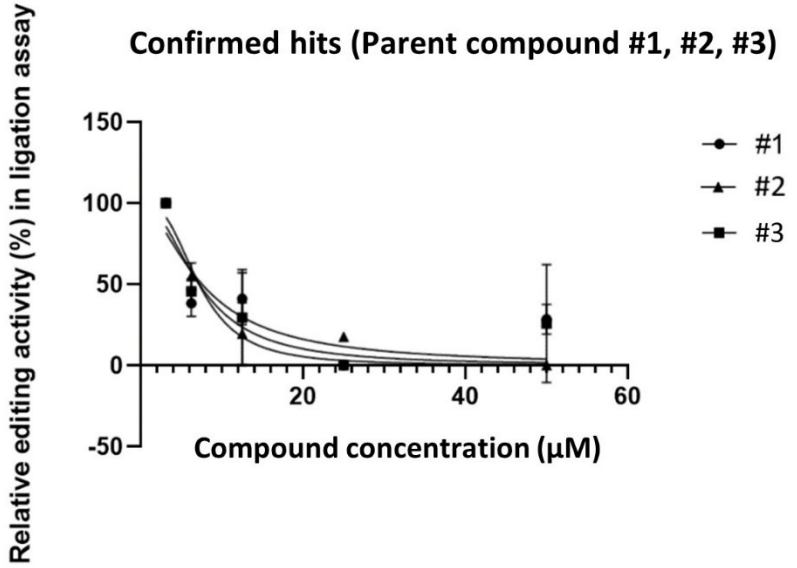


Figure 12.5. Testing confirmed hits against recombinant ligase in a dose-response manner. *The IC_{50} for each compound, determined using recombinant protein, ranged from 4 to 17 µM in the low micromolar range. Means and standard deviations were obtained from two replicates.*

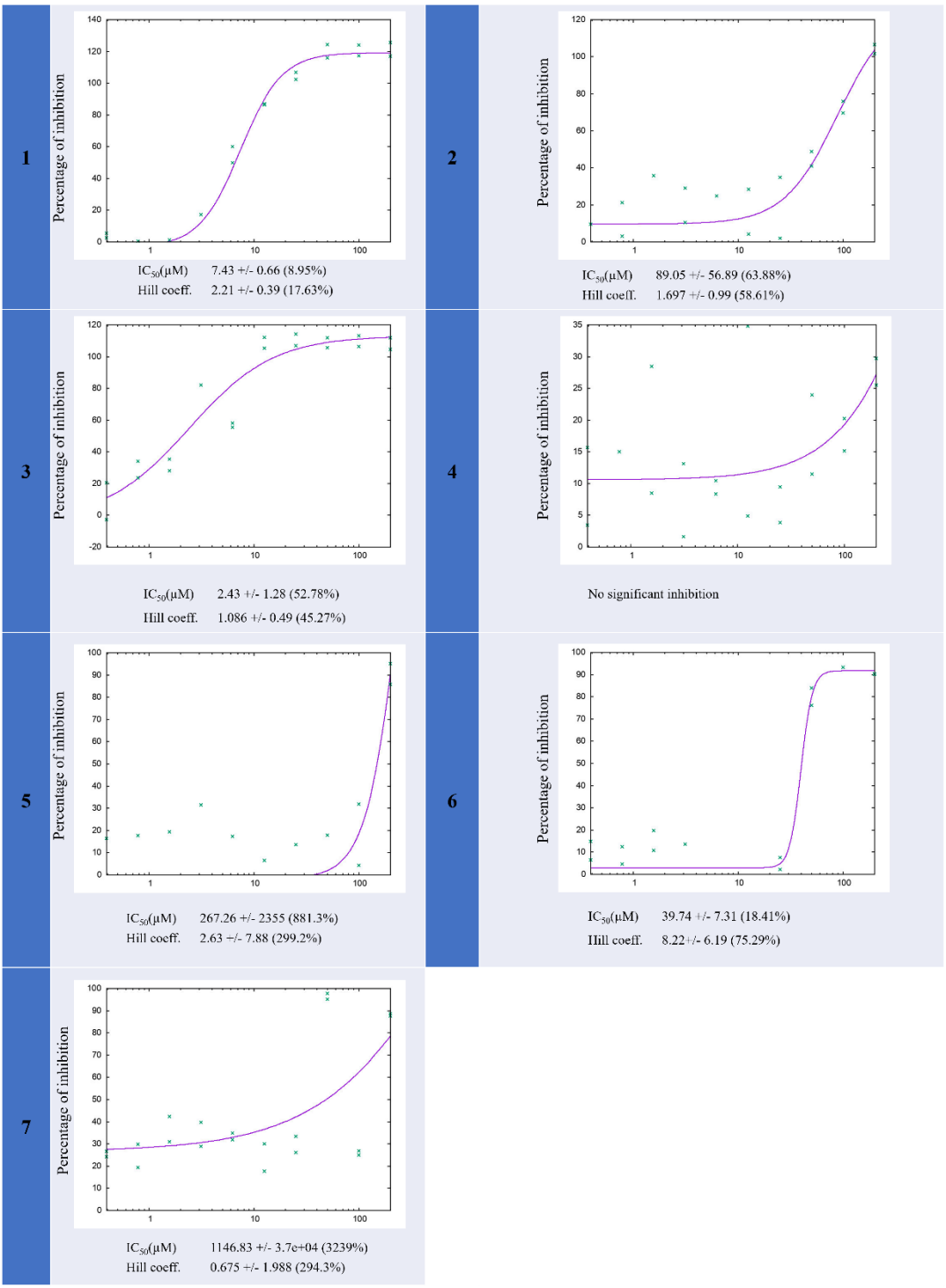


Table 12.8: Activity summary of HTS final hits in FRET-based ligation assay. *Each initially confirmed hit was tested in a dose-response manner (0.3-200 μM) in the fluorescent-based ligation assay to determine if the hits impacted this catalytic step of RNA editing.*

12.4.6 Determination of minimum inhibitory concentration (MIC) and time-to-kill for parasiticidal compounds

Using the Alamar blue assay (Räz, Iten et al. 1997, Sykes and Avery 2009), we tested all identified hits and their analogs against *T. brucei* and *L. donovani* in a dose-dependent manner, ranging from 0.1-20 μM. Some analogs showed growth inhibitory effects against either one (#1-1, 1-2, 1-3, 5-1, 6-3) or both parasites (#1-5, #4-3, and #4-1), as detailed in **Table 12.9**. Due to solubility issues and the toxic effect of DMSO on parasite viability, some analogs could not be tested at concentrations exceeding 6 μM. **Table 12.9** summarizes the MIC values of each compound against *T. brucei* and *L. donovani.*

Among the potential catalytic specific inhibitors, hits #1-2 and #4-1 showed trypanocidal activity (when tested in 100 nM-20 μM range) against *T. brucei* with minimum inhibitory concentrations of 3 μM and 6 μM, respectively (**Table 12.9**). However, while both compounds exhibited parasiticidal activity at concentrations within the low single-digit micromolar range, their efficacy in the full-round assay – the most representative of *in vivo* conditions – was relatively subdued. Specifically, #4-1 had an IC_{50} of approximately 9 μM, and #1-2 had an IC_{50} of about 44 μM. This suggests that #1-2 may potentially target additional cellular functions, although it's important to note that *in vivo* activity on RNA editing has not been assessed for any of the compounds.

Among broad-spectrum inhibitors, only #1-1 had notable efficacy against cells at low micromolar levels (**Table 12.9**), differing from its 25-33 μM IC_{50} in the full round assay.

We evaluated the time required to eliminate parasites for each compound (**Figure 12.6**), except for compounds 5-1 and 6-3 due to insufficient quantities of these compounds. The findings indicate that four derivatives of the parent compound #1 efficiently eradicated all *T. brucei* parasites within a 6-hour timeframe, achieving this at their respective minimum inhibitory concentrations (MIC) within the single-digit micromolar range. This highlights their rapid parasite-killing capability, resulting in a considerably quicker elimination than the control

compound (suramin). However, the rapid cidal activity of some compounds occurs in less time than one cell division cycle, indicating that it cannot be solely attributed to the loss of de novo synthesis of the oligomycin-sensitive component of the F0/F1-ATPase, the functional protein synthesized in the mitochondria of bloodstream-form *T. brucei*. Notably, sterile cultures for compound #1-2 were achieved within a timeframe spanning from 6 to 24 hours. In contrast, compounds #4-3 and #4-1 took 24 and 48 hours, respectively, to eliminate the parasites when tested at their MIC concentrations.

cpd#		1-1	1-2	1-3	1-5	4-1	4-3	5-1	6-3
MIC (µM)	*T. brucei*	3	3	6	20	6	6	N/A	6
	L. donovani	N/A	N/A	N/A	6	3	3	6	N/A

Table 12.9 MIC values for compounds against T. brucei and L. donovani *the minimum concentration (in micromolar) at which each compound kills all the parasites. N/A denotes that the compound did not impact the parasite at the tested concentrations. Data shown are mean values from two independent experiments. The numbering convention is as explained before; for instance, #1-2 refers to analog #2 of parent compound #1.*

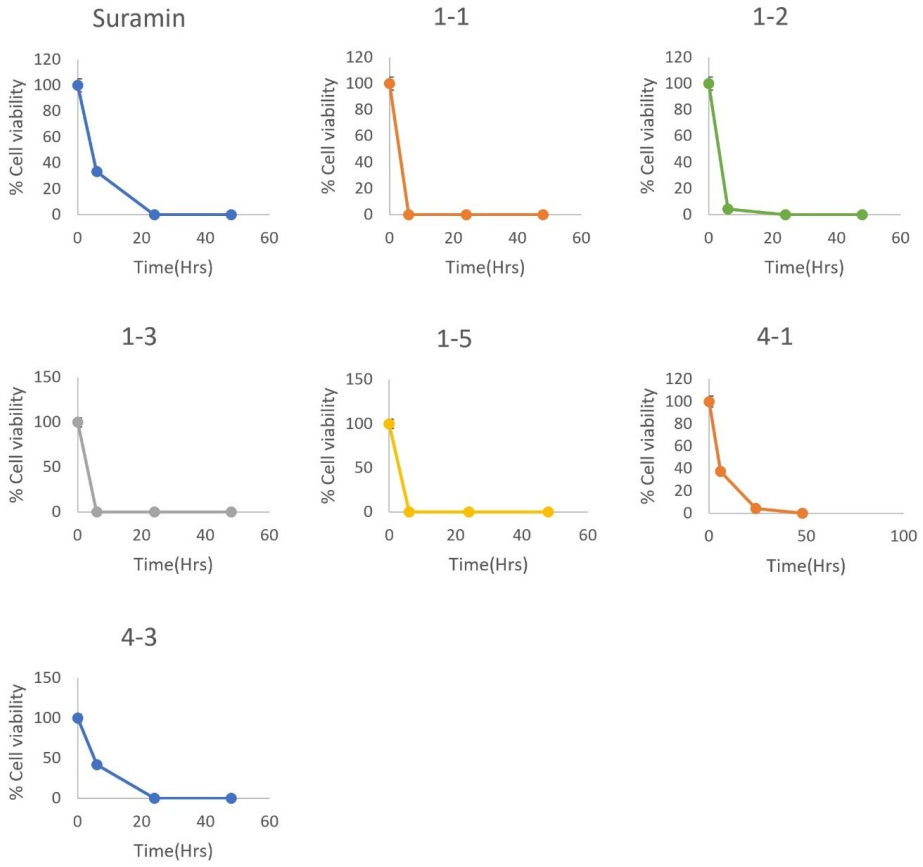


Figure 12.6. Time-to-kill assay performed on the trypanocidal hits. *The assay was conducted at different time intervals and compared with the controls, where no drug and suramin served as negative and positive controls, respectively. Results show that parent compound #1 analogs eradicated all parasites within the first 6-hour incubation period, showing faster cidality than suramin, the current drug used for sleeping sickness. Compounds 4-3 and 4-1 completely eradicate parasites after 24 and 48 hours, respectively. The data represents the mean and standard deviation values from three independent technical replicates.*

12.4.7 Drug-likeness and pharmacokinetics of the hits

We employed the SWISSADME computational tool for predicting compound properties (**Table 12.10**) (Daina, Michielin et al. 2017). Except for compound #4 and analogs (#4-2, #4-4, #4-5, #4-6) with topological polar surface area (TPSA) nearing 100 angstroms, others stayed below this blood-brain barrier (BBB) penetration threshold.

SWISSADME solubility predictions indicate that parent compounds #1, #5, and #6 and their corresponding analogs tend to have either poor or moderate solubility. In contrast, primary hits #2 and #4, along with their analogs, exhibit a significantly higher level of water solubility.

Parent compounds #1, #5, and #6, as well as their analogs, are expected to be more lipophilic, while parent compounds #2, #4, and their analogs are less so due to solubility considerations. Gastrointestinal (GI) absorption analysis suggests that all compounds have a high absorption rate, as determined by the white component of the BOILED-Egg methodology (Daina and Zoete 2016). Interestingly, two compounds (analogs #1-4 and #1-6) were classified in the yolk element, suggesting effective blood-brain barrier penetration potential. These predictions are based on computed lipophilicity (WLOGP) and polarity (TPSA) values of the compounds.

Each compound was assessed for its potential as a permeability glycoprotein substrate or its ability to inhibit cytochrome P450, using established support vector machine models (Daina, Michielin et al. 2017). All compounds were predicted to exhibit non-substrate characteristics for permeability glycoprotein except for parent compound #1 and its entire cohort of analogs and analogs #5-1 and #6-5.

Compounds were evaluated for drug-like properties using five filters: Lipinski, Ghose, Veber, Egan, and Muegge. Except for analogs #1-5 and #5-8, all compounds (primary hits and remaining analogs) displayed promising drug-like qualities by at least four filtering methods used. Notwithstanding the rigorous screening for structural alerts in the primary hits, analogs #6-2 and #6-3 contained coumarin, which may relate to the trypanocidal efficacy of analog #6-3. Additionally, an appraisal of lead-likeness was executed for each compound. Notably, it was observed that the compounds' lack of lead-likeness was attributed to their molecular weight and XLOGP3 (an indicator of lipophilicity). Nevertheless, many compounds successfully fell within the lead-like classification, highlighting their potential suitability for further lead optimization endeavors.

Table 12.10
Predicting pharmacokinetic properties and drug-likeness of compounds using SWISSADME tool.

Molecule	Canonical SMILES	Formula	MW	#Heavy atoms
parent 1	COc1cc(ccc1OC)c1nnc2c1cn(Cc1cccc(c1)F)c1c2cc2c(c1)OCCO2	C27H22FN3O4	471.48	35
1.1	COc1cc(ccc1OC)c1nnc2c1cn(Cc1ccc(cc1)C)c1c2cc2c(c1)OCCO2	C28H25N3O4	467.52	35
1.2	COc1cccc(c1)Cn1cc2c(nnc2c2c1cc1OCCOc1c2)c1ccc(c(c1)C)OC	C28H25N3O4	467.52	35
1.3	COc1ccc(cc1)c1nnc2c1cn(Cc1cccc(c1)OC)c1c2cc2c(c1)OCCO2	C27H23N3O4	453.49	34
1.4	Cc1ccc(cc1)Cn1cc2c(nnc2c2c1cc1OCCOc1c2)c1ccccc1	C26H21N3O2	407.46	31
1.5	CCOc1ccc(cc1)c1nnc2c1cn(Cc1cccc(c1)F)c1c2cc2c(c1)OCCO2	C27H22FN3O3	455.48	34
1.6	C1OC2C(O1)C = c1c(=C2)n(Cc2ccccc2)cc2c1nnc2c1ccc2c(c1)OCO2	C25H19N3O4	425.44	32
parent 2	n1ccc(cc1)c1nnc2n1nc(s2)/C = C/c1ccco1	C14H9N5OS	295.32	21
2.1	c1ccc(cc1)/C = C/c1sc2n(n1)c(nn2)c1ccncc1	C16H11N5S	305.36	22
2.2	Cc1ccccc1/C = C/c1sc2n(n1)c(nn2)c1ccncc1	C17H13N5S	319.38	23
2.3	n1ccc(cc1)c1nnc2n1nc(s2)/C = C/c1ccco1	C14H9N5OS	295.32	21
2.4	c1ccc(cc1)c1nnc2n1nc(s2)/C = C/c1ccco1	C15H10N4OS	294.33	21
2.5	Cc1ccc(cc1)c1nnc2n1nc(s2)/C = C/c1ccco1	C16H12N4OS	308.36	22
2.6	Cc1occc1c1nnc2n1nc(s2)/C = C/c1ccco1	C14H10N4O2S	298.32	21
parent 3	O = C(/C = C/c1ccco1)NCc1cccc(c1)CNC(=O)/C = C/c1ccco1	C22H20N2O4	376.41	28
parent 4	Cc1ccc2c(c1)nc(nc2C)Nc1nc(O)cc(n1)O	C14H13N5O2	283.29	21
4.1	Cc1ccc2c(c1)nc(nc2C)Nc1[nH]c(C)cc(=O)n1	C15H15N5O	281.31	21
4.2	Oc1cc(O)nc(n1)Nc1nc(C)c2c(n1)cc(c(c2)C)C	C15H15N5O2	297.31	22
4.3	Cc1ccc2c(c1)nc(nc2C)Nc1nc(O)c2c(n1)cccc2	C18H15N5O	317.34	24
4.4	Oc1cc(O)nc(n1)Nc1nc(C)c2c(n1)cccc2	C13H11N5O2	269.26	20
4.5	Cc1ccc2c(c1)c(C)nc(n2)Nc1nc(O)cc(n1)O	C14H13N5O2	283.29	21
4.6	Cc1cc(C)c2c(c1)c(C)nc(n2)Nc1nc(O)cc(n1)O	C15H15N5O2	297.31	22
parent 5	Cc1ccc2c(c1)cc1c(n2)sc(c1)C(=O)Nc1ccenc1	C18H13N3OS	319.38	23
5.1	Cc1ccc2c(c1)nc1c(c2)cc(s1)C(=O)Nc1cnc2c(c1)cccc2	C22H15N3OS	369.44	27
5.2	Cc1ccc2c(c1)cc1c(n2)sc(c1)C(=O)Nc1ccccc1	C19H14N2OS	318.39	23
5.3	Clc1ccc(cc1)NC(=O)c1cc2c(s1)nc1c(c2)cc(cc1)C	C19H13ClN2OS	352.84	24
5.4	Cc1ccc2c(c1)cc1c(n2)sc(c1)C(=O)Nc1cccc(c1)Cl	C19H13ClN2OS	352.84	24
5.5	Cc1ccc(cc1)NC(=O)c1sc2c(c1N)cc1c(n2)cccc1	C19H15N3OS	333.41	24
5.6	Cc1ccc2c(c1)nc1c(c2)cc(s1)C(=O)Nc1cccc(c1)Cl	C19H13ClN2OS	352.84	24
5.7	COc1ccc2c(c1)cc1c(n2)sc(c1)C(=O)Nc1cccc(c1)Cl	C19H13ClN2O2S	368.84	25
5.8	Cc1ccc2c(c1)cc1c(n2)sc(c1)C(=O)N(c1cccc(c1)Cl)C	C20H15ClN2OS	366.86	25
parent 6	O = C(c1coc2c(c1 = O)cccc2)Nc1cccc(c1)c1nc2c([nH]1)cccc2	C23H15N3O3	381.38	29
6.1	O = C(c1coc2c(c1 = O)cccc2)Nc1cccc(c1)c1nc2c(o1)cccc2	C23H14N2O4	382.37	29
6.2	O = C(c1cc2ccccc2oc1 = O)Nc1cccc(c1)c1nc2c([nH]1)cccc2	C23H15N3O3	381.38	29
6.3	Cc1onc(n1)c1cccc(c1)NC(=O)c1cc2ccccc2oc1 = O	C19H13N3O4	347.32	26
6.4	O = C(c1cc2CCC = Cc2oc1 = O)Nc1cccc(c1)c1cnc2c(n1)cccc2	C24H17N3O3	395.41	30
6.5	O = C(c1cc2c(o1)cccc2)Nc1cccc(c1)c1nc2c([nH]1)cccc2	C22H15N3O2	353.37	27
parent 7	COc1ccc(cc1)c1nn2c(n1)c1ccccc1nc2S	C16H12N4OS	308.36	22

Molecule	#Aromatic heavy atoms	Fraction Csp3	#Rotatable bonds	#H-bond acceptors	#H-bond donors	MR	TPSA	iLOGP	XLOGP3
parent 1	25	0.19	5	7	0	130.03	67.63	3.89	3.96
1.1	25	0.21	5	6	0	135.04	67.63	3.99	4.22
1.2	25	0.21	5	6	0	135.04	67.63	3.9	4.22
1.3	25	0.19	5	6	0	130.07	67.63	3.85	3.86
1.4	25	0.15	3	4	0	122.05	49.17	3.63	4.28
1.5	25	0.19	5	6	0	128.35	58.4	3.97	4.36
1.6	21	0.2	3	6	0	117.43	67.63	3.53	3.17
parent 2	19	0	3	5	0	79.53	97.35	2.56	2.26
2.1	20	0	3	4	0	87.26	84.21	2.71	3.16
2.2	20	0.06	3	4	0	92.23	84.21	2.9	3.52
2.3	19	0	3	5	0	79.53	97.35	2.56	2.26
2.4	19	0	3	4	0	81.73	84.46	2.94	3.33
2.5	19	0.06	3	4	0	86.7	84.46	3.19	3.69
2.6	18	0.07	3	5	0	78.96	97.6	2.93	2.79

(*continued on next page*)

Table 12.10 (*continued*)

Molecule	#Aromatic heavy atoms	Fraction Csp3	#Rotatable bonds	#H-bond acceptors	#H-bond donors	MR	TPSA	iLOGP	XLOGP3
parent 3	16	0.09	10	4	2	105.36	84.48	3.59	2.52
parent 4	16	0.14	2	6	3	78.65	104.05	2.59	2.75
4.1	16	0.2	2	4	2	82.4	83.56	1.89	1.75
4.2	16	0.2	2	6	3	83.62	104.05	2.68	3.12
4.3	20	0.11	2	5	2	94.13	83.82	3.02	4.11
4.4	16	0.08	2	6	3	73.68	104.05	2.25	2.45
4.5	16	0.14	2	6	3	78.65	104.05	2.45	2.75
4.6	16	0.2	2	6	3	83.62	104.05	2.85	3.12
parent 5	19	0.06	3	3	1	94.1	83.12	2.47	4
5.1	23	0.05	3	3	1	111.61	83.12	3.06	5.34
5.2	19	0.05	3	2	1	96.31	70.23	2.89	5.07
5.3	19	0.05	3	2	1	101.32	70.23	3.19	5.47
5.4	19	0.05	3	2	1	101.32	70.23	3.11	5.7
5.5	19	0.05	3	2	2	100.71	96.25	2.7	4.72
5.6	19	0.05	3	2	1	101.32	70.23	3.13	5.7
5.7	19	0.05	4	3	1	102.84	79.46	3.12	5.31
5.8	19	0.1	3	2	0	106.22	61.44	3.54	5.89
parent 6	25	0	4	4	2	111.79	87.99	3.03	3.82
6.1	25	0	4	5	1	109.7	85.34	3.47	4.09
6.2	25	0	4	4	2	111.79	87.99	2.81	4.18
6.3	21	0.05	4	6	1	94.96	98.23	3.03	3.23
6.4	22	0.08	4	5	1	115.47	85.09	3.38	3.42
6.5	24	0	4	3	2	105.52	70.92	2.83	4.72
parent 7	19	0.06	2	4	0	87.47	91.11	3.01	3.55

Molecule	WLOGP	MLOGP	Silicos-IT Log P	Consensus Log P	ESOL Log S	ESOL Solubility (mg/ml)	ESOL Solubility (mol/l)
parent 1	5.65	3.01	5.39	4.38	−5.46	1.65E-03	3.50E-06
1.1	5.4	2.84	5.5	4.39	−5.6	1.19E-03	2.54E-06
1.2	5.4	2.84	5.5	4.37	−5.6	1.19E-03	2.54E-06
1.3	5.09	2.64	4.98	4.08	−5.3	2.29E-03	5.04E-06
1.4	5.38	3.52	5.4	4.44	−5.46	1.41E-03	3.46E-06
1.5	6.03	3.54	5.73	4.73	−5.62	1.08E-03	2.37E-06
1.6	2.19	2.28	3.57	2.95	−4.76	7.35E-03	1.73E-05
parent 2	2.79	1.1	2.93	2.33	−3.57	8.02E-02	2.71E-04
2.1	3.2	2.38	3.52	2.99	−4.2	1.93E-02	6.33E-05
2.2	3.51	2.63	4.01	3.31	−4.48	1.05E-02	3.29E-05
2.3	2.79	1.1	2.93	2.33	−3.57	8.02E-02	2.71E-04
2.4	3.4	2.17	3.48	3.06	−4.23	1.72E-02	5.83E-05
2.5	3.71	2.83	3.97	3.48	−4.52	9.36E-03	3.04E-05
2.6	3.3	1.54	3.38	2.79	−3.88	3.90E-02	1.31E-04
parent 3	3.01	0.9	4.06	2.82	−3.52	1.13E-01	2.99E-04
parent 4	2.19	0.62	1.56	1.94	−3.76	4.92E-02	1.74E-04
4.1	2.38	1.06	3.17	2.05	−3.12	2.14E-01	7.61E-04
4.2	2.5	0.87	2.06	2.25	−4.06	2.62E-02	8.81E-05
4.3	3.64	2.08	3.05	3.18	−4.88	4.17E-03	1.31E-05
4.4	1.88	0.35	1.07	1.6	−3.51	8.27E-02	3.07E-04
4.5	2.19	0.62	1.56	1.91	−3.76	4.92E-02	1.74E-04
4.6	2.5	0.87	2.06	2.28	−4.06	2.62E-02	8.81E-05
parent 5	4.21	2.34	4.7	3.54	−4.75	5.63E-03	1.76E-05
5.1	5.37	3.6	5.71	4.62	−5.93	4.37E-04	1.18E-06
5.2	4.82	3.42	5.26	4.29	−5.42	1.21E-03	3.79E-06
5.3	5.47	3.92	5.88	4.79	−5.86	4.85E-04	1.38E-06
5.4	5.47	3.92	5.88	4.82	−6.01	3.48E-04	9.85E-07
5.5	4.41	2.84	4.53	3.84	−5.27	1.80E-03	5.39E-06
5.6	5.47	4.19	5.88	4.88	−6.01	3.48E-04	9.85E-07

(*continued on next page*)

Table 12.10 (*continued*)

Molecule	WLOGP	MLOGP	Silicos-IT Log P	Consensus Log P	ESOL Log S	ESOL Solubility (mg/ml)	ESOL Solubility (mol/l)
5.7	5.17	3.33	5.42	4.47	−5.77	6.26E-04	1.70E-06
5.8	5.69	4.14	5.82	5.02	−6.19	2.37E-04	6.46E-07
parent 6	4.4	2.21	4.61	3.61	−4.99	3.95E-03	1.03E-05
6.1	4.66	2.21	4.48	3.78	−5.16	2.64E-03	6.90E-06
6.2	4.4	3.02	4.61	3.8	−5.21	2.34E-03	6.14E-06
6.3	3.21	2.05	3.43	2.99	−4.36	1.51E-02	4.35E-05
6.4	4.16	2.63	4.47	3.61	−4.72	7.45E-03	1.88E-05
6.5	5.04	2.91	4.56	4.01	−5.4	1.41E-03	4.00E-06
parent 7	3.24	3.12	2.63	3.11	−4.5	9.85E-03	3.20E-05

Molecule	ESOL Class	Ali Log S	Ali Solubility (mg/ml)	Ali Solubility (mol/l)	Ali Class	Silicos-IT LogSw
parent 1	Moderately soluble	−5.08	3.92E-03	8.30E-06	Moderately soluble	−9.25
1.1	Moderately soluble	−5.35	2.09E-03	4.46E-06	Moderately soluble	−9.36
1.2	Moderately soluble	−5.35	2.09E-03	4.46E-06	Moderately soluble	−9.36
1.3	Moderately soluble	−4.98	4.78E-03	1.05E-05	Moderately soluble	−8.99
1.4	Moderately soluble	−5.03	3.85E-03	9.44E-06	Moderately soluble	−9.16
1.5	Moderately soluble	−5.3	2.27E-03	4.99E-06	Moderately soluble	−9.54
1.6	Moderately soluble	−4.26	2.33E-02	5.48E-05	Moderately soluble	−6.74
parent 2	Soluble	−3.94	3.38E-02	1.15E-04	Soluble	−4.57
2.1	Moderately soluble	−4.6	7.69E-03	2.52E-05	Moderately soluble	−5.35
2.2	Moderately soluble	−4.97	3.40E-03	1.07E-05	Moderately soluble	−5.73
2.3	Soluble	−3.94	3.38E-02	1.15E-04	Soluble	−4.57
2.4	Moderately soluble	−4.78	4.88E-03	1.66E-05	Moderately soluble	−4.94
2.5	Moderately soluble	−5.15	2.16E-03	7.01E-06	Moderately soluble	−5.32
2.6	Soluble	−4.5	9.52E-03	3.19E-05	Moderately soluble	−4.54
parent 3	Soluble	−3.94	4.32E-02	1.15E-04	Soluble	−6.79
parent 4	Soluble	−4.59	7.28E-03	2.57E-05	Moderately soluble	−4.77
4.1	Soluble	−3.12	2.12E-01	7.55E-04	Soluble	−6.22
4.2	Moderately soluble	−4.97	3.16E-03	1.06E-05	Moderately soluble	−5.16
4.3	Moderately soluble	−5.58	8.42E-04	2.65E-06	Moderately soluble	−7.01
4.4	Soluble	−4.28	1.42E-02	5.26E-05	Moderately soluble	−4.39
4.5	Soluble	−4.59	7.28E-03	2.57E-05	Moderately soluble	−4.77
4.6	Moderately soluble	−4.97	3.16E-03	1.06E-05	Moderately soluble	−5.16
parent 5	Moderately soluble	−5.45	1.14E-03	3.57E-06	Moderately soluble	−7.16
5.1	Moderately soluble	−6.84	5.36E-05	1.45E-07	Poorly soluble	−8.8
5.2	Moderately soluble	−6.29	1.64E-04	5.16E-07	Poorly soluble	−7.53
5.3	Moderately soluble	−6.7	7.00E-05	1.98E-07	Poorly soluble	−8.12
5.4	Poorly soluble	−6.94	4.04E-05	1.15E-07	Poorly soluble	−8.12
5.5	Moderately soluble	−6.47	1.13E-04	3.39E-07	Poorly soluble	−7.16
5.6	Poorly soluble	−6.94	4.04E-05	1.15E-07	Poorly soluble	−8.12
5.7	Moderately soluble	−6.73	6.87E-05	1.86E-07	Poorly soluble	−7.85
5.8	Poorly soluble	−6.95	4.08E-05	1.11E-07	Poorly soluble	−7.79
parent 6	Moderately soluble	−5.36	1.65E-03	4.34E-06	Moderately soluble	−9.1
6.1	Moderately soluble	−5.59	9.88E-04	2.59E-06	Moderately soluble	−9.1
6.2	Moderately soluble	−5.74	6.99E-04	1.83E-06	Moderately soluble	−9.1
6.3	Moderately soluble	−4.97	3.76E-03	1.08E-05	Moderately soluble	−7.46
6.4	Moderately soluble	−4.89	5.13E-03	1.30E-05	Moderately soluble	−8.47
6.5	Moderately soluble	−5.94	4.07E-04	1.15E-06	Moderately soluble	−8.79
parent 7	Moderately soluble	−5.15	2.19E-03	7.11E-06	Moderately soluble	−5.76

Molecule	Silicos-IT Solubility (mg/ml)	Silicos-IT Solubility (mol/l)	Silicos-IT class	GI absorption	BBB permeant	Pgp substrate	CYP1A2 inhibitor
parent 1	2.67E-07	5.66E-10	Poorly soluble	High	No	Yes	No
1.1	2.05E-07	4.38E-10	Poorly soluble	High	No	Yes	No
1.2	2.05E-07	4.38E-10	Poorly soluble	High	No	Yes	No
1.3	4.67E-07	1.03E-09	Poorly soluble	High	No	Yes	Yes
1.4	2.81E-07	6.89E-10	Poorly soluble	High	Yes	Yes	Yes

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Table 12.10 (*continued*)

Molecule	Silicos-IT Solubility (mg/ml)	Silicos-IT Solubility (mol/l)	Silicos-IT class	GI absorption	BBB permeant	Pgp substrate	CYP1A2 inhibitor
1.5	1.31E-07	2.88E-10	Poorly soluble	High	No	Yes	Yes
1.6	7.80E-05	1.83E-07	Poorly soluble	High	Yes	Yes	Yes
parent 2	8.00E-03	2.71E-05	Moderately soluble	High	No	No	Yes
2.1	1.37E-03	4.49E-06	Moderately soluble	High	No	No	Yes
2.2	5.97E-04	1.87E-06	Moderately soluble	High	No	No	Yes
2.3	8.00E-03	2.71E-05	Moderately soluble	High	No	No	Yes
2.4	3.39E-03	1.15E-05	Moderately soluble	High	No	No	Yes
2.5	1.48E-03	4.79E-06	Moderately soluble	High	No	No	Yes
2.6	8.62E-03	2.89E-05	Moderately soluble	High	No	No	Yes
parent 3	6.10E-05	1.62E-07	Poorly soluble	High	No	No	No
parent 4	4.78E-03	1.69E-05	Moderately soluble	High	No	No	Yes
4.1	1.70E-04	6.05E-07	Poorly soluble	High	No	No	Yes
4.2	2.08E-03	7.00E-06	Moderately soluble	High	No	No	Yes
4.3	3.12E-05	9.82E-08	Poorly soluble	High	No	No	Yes
4.4	1.09E-02	4.06E-05	Moderately soluble	High	No	No	Yes
4.5	4.78E-03	1.69E-05	Moderately soluble	High	No	No	Yes
4.6	2.08E-03	7.00E-06	Moderately soluble	High	No	No	Yes
parent 5	2.22E-05	6.97E-08	Poorly soluble	High	No	No	Yes
5.1	5.86E-07	1.58E-09	Poorly soluble	High	No	Yes	Yes
5.2	9.40E-06	2.95E-08	Poorly soluble	High	No	No	Yes
5.3	2.65E-06	7.50E-09	Poorly soluble	High	No	No	Yes
5.4	2.65E-06	7.50E-09	Poorly soluble	High	No	No	Yes
5.5	2.30E-05	6.89E-08	Poorly soluble	High	No	No	Yes
5.6	2.65E-06	7.50E-09	Poorly soluble	High	No	No	Yes
5.7	5.16E-06	1.40E-08	Poorly soluble	High	No	No	Yes
5.8	6.00E-06	1.64E-08	Poorly soluble	High	No	No	Yes
parent 6	3.06E-07	8.02E-10	Poorly soluble	High	No	No	Yes
6.1	3.04E-07	7.96E-10	Poorly soluble	High	No	No	Yes
6.2	3.06E-07	8.02E-10	Poorly soluble	High	No	No	Yes
6.3	1.19E-05	3.44E-08	Poorly soluble	High	No	No	Yes
6.4	1.35E-06	3.41E-09	Poorly soluble	High	No	No	Yes
6.5	5.69E-07	1.61E-09	Poorly soluble	High	No	Yes	Yes
parent 7	5.40E-04	1.75E-06	Moderately soluble	High	No	No	Yes

Molecule	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	log Kp (cm/s)	Lipinski #violations	Ghose #violations	Veber #violations
parent 1	Yes	Yes	Yes	Yes	−6.36	0	2	0
1.1	Yes	Yes	Yes	Yes	−6.16	0	1	0
1.2	Yes	Yes	Yes	Yes	−6.16	0	1	0
1.3	Yes	Yes	Yes	Yes	−6.33	0	1	0
1.4	Yes	Yes	No	Yes	−5.75	0	0	0
1.5	Yes	Yes	Yes	Yes	−5.98	0	1	0
1.6	Yes	Yes	Yes	Yes	−6.64	0	0	0
parent 2	Yes	Yes	No	Yes	−6.5	0	0	0
2.1	Yes	Yes	No	No	−5.92	0	0	0
2.2	Yes	Yes	No	No	−5.75	0	0	0
2.3	Yes	Yes	No	Yes	−6.5	0	0	0
2.4	Yes	Yes	No	No	−5.73	0	0	0
2.5	Yes	Yes	No	No	−5.56	0	0	0
2.6	Yes	Yes	No	No	−6.14	0	0	0
parent 3	Yes	Yes	Yes	Yes	−6.81	0	0	0
parent 4	No	No	Yes	Yes	−6.08	0	0	0
4.1	No	No	Yes	Yes	−6.77	0	0	0
4.2	No	No	Yes	Yes	−5.9	0	0	0
4.3	No	Yes	Yes	Yes	−5.32	0	0	0
4.4	No	No	Yes	Yes	−6.2	0	0	0
4.5	No	No	Yes	Yes	−6.08	0	0	0

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Table 12.10 (*continued*)

Molecule	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	log Kp (cm/s)	Lipinski #violations	Ghose #violations	Veber #violations
4.6	No	No	Yes	Yes	−5.9	0	0	0
parent 5	Yes	Yes	Yes	Yes	−5.41	0	0	0
5.1	Yes	Yes	No	Yes	−4.76	0	0	0
5.2	Yes	Yes	No	Yes	−4.64	0	0	0
5.3	Yes	Yes	No	Yes	−4.57	0	0	0
5.4	Yes	Yes	No	Yes	−4.41	0	0	0
5.5	Yes	Yes	Yes	Yes	−4.98	0	0	0
5.6	Yes	Yes	No	Yes	−4.41	1	0	0
5.7	Yes	Yes	Yes	Yes	−4.78	0	0	0
5.8	Yes	Yes	No	Yes	−4.36	0	1	0
parent 6	Yes	Yes	Yes	Yes	−5.91	0	0	0
6.1	Yes	Yes	Yes	Yes	−5.73	0	0	0
6.2	Yes	Yes	Yes	No	−5.66	0	0	0
6.3	No	Yes	No	Yes	−6.13	0	0	0
6.4	Yes	Yes	No	Yes	−6.28	0	0	0
6.5	Yes	Yes	Yes	Yes	−5.1	0	0	0
parent 7	Yes	Yes	No	Yes	−5.66	0	0	0

Molecule	Egan #violations	Muegge #violations	Bioavailability Score	PAINS #alerts	Brenk #alerts	Leadlikeness #violations	Synthetic Accessibility
parent 1	0	0	0.55	0	0	2	3.44
1.1	0	0	0.55	0	0	2	3.54
1.2	0	0	0.55	0	0	2	3.58
1.3	0	0	0.55	0	0	2	3.42
1.4	0	0	0.55	0	0	2	3.17
1.5	1	0	0.55	0	0	2	3.35
1.6	0	0	0.55	0	0	1	4.64
parent 2	0	0	0.55	0	0	0	3.34
2.1	0	0	0.55	0	0	0	3.24
2.2	0	0	0.55	0	0	1	3.36
2.3	0	0	0.55	0	0	0	3.34
2.4	0	0	0.55	0	0	0	3.42
2.5	0	0	0.55	0	0	1	3.52
2.6	0	0	0.55	0	0	0	3.58
parent 3	0	0	0.55	0	1	2	3.16
parent 4	0	0	0.55	0	0	0	2.3
4.1	0	0	0.55	0	0	0	2.35
4.2	0	0	0.55	0	0	0	2.41
4.3	0	0	0.55	0	0	1	2.4
4.4	0	0	0.55	0	0	0	2.17
4.5	0	0	0.55	0	0	0	2.29
4.6	0	0	0.55	0	0	0	2.46
parent 5	0	0	0.55	0	0	1	2.62
5.1	0	1	0.55	0	0	2	2.85
5.2	0	1	0.55	0	0	1	2.53
5.3	0	1	0.55	0	0	2	2.54
5.4	0	1	0.55	0	0	2	2.56
5.5	0	0	0.55	0	0	1	2.82
5.6	0	1	0.55	0	0	2	2.58
5.7	0	1	0.55	0	0	2	2.56
5.8	0	1	0.55	0	0	2	2.67
parent 6	0	0	0.55	0	0	2	3.06
6.1	0	0	0.55	0	0	2	3.38
6.2	0	0	0.55	0	1	2	3.11
6.3	0	0	0.55	0	1	0	3.16
6.4	0	0	0.55	0	0	1	3.7
6.5	0	0	0.55	0	0	2	2.76
parent 7	0	0	0.55	0	1	1	2.36

Table 12.10 Predicting pharmacokinetic properties and drug-likeness of compounds using SWISSADME tool *SMILES format of all initial hits and analogs were entered in SWISSADME predictor tool and their physicochemical and pharmacokinetic properties were predicted.*

12.5 Discussion

Discovering compounds to modulate the editosome, the molecular machine responsible for RNA editing in trypanosomatids, holds promise for treating TriTryps diseases. Uridine insertion/deletion RNA editing is crucial for gene expression in these parasites but absent in their hosts, making it a promising drug target in the quest for new TriTryps diseases therapies (Liang and Connell 2010, Moshiri and Salavati 2010, Salavati, Moshiri et al. 2012, Zimmermann, Hall et al. 2016, Aphasizheva, Alfonzo et al. 2020, Rostamighadi, Mehta et al. 2023).

Here, we modified our established RNA editing assay for HTS in a 1536-well plate format. We identified and confirmed RNA editing inhibitors, assessed their potency (IC_{50}), and examined their ability to hinder the growth of *T. brucei* and *L. donovani*. Furthermore, we utilized SAR among diverse chemical structures to enhance the specificity and the potency of the inhibitors.

We found 7 potent compounds that effectively inhibited the RNA editing assay. It appears that two of the compounds may have affected either endonuclease or ligase activity. Three others seemed to impact RNA-protein interactions, which might have influenced all secondary RNA editing assays. The other two compounds appeared to have some influence on both ligase and endonuclease activities, with one also showing an effect on EMSA. Further research is needed to confirm and understand these effects better. Our study also revealed several analogs that demonstrated modest improvements *in vitro* against RNA editing. Moreover, eight compounds showed trypanocidal activity at low micromolar concentrations, underscoring their therapeutic potential. Importantly, two of these compounds (#1-2, #4-1) appeared to impact the single endonuclease activity.

Given the complex biology of the RNA Editing Core Complex (RECC), our results offer intriguing avenues for drug development. The multiple functionalities inherent to the editosome suggest that a single compound could potentially modulate several interactions within the complex, including enzyme activities, RNA binding, or protein interactions. Our primary hits #1, #2, and #3 showed some pleiotropic impact in this regard, as they inhibited the ligase enzyme and had some effects on EMSA and secondary assays.

Considering that each secondary assay focuses on a specific enzymatic step and all three compounds disrupt the gel shift assay, it's likely that these compounds primarily interfere with RNA binding to the complex. This could result from the compounds binding to the protein or inhibiting protein-protein interactions (PPI) rather than individual enzyme inhibition. Our study investigated the influence of compound modifications on the activity. We found that altering the functional groups in compound #1 could reduce its effectiveness against RNA editing, while adding a fluorine group at specific positions appeared to enhance its inhibitory action. Previous studies (Berkowitz, Karukurichi et al. 2008, Otake, Ubukata et al. 2023) have shown that adding a fluorine group can have a positive effect on inhibition in PPI and enzyme processes. When comparing the parent compound #1 to analog #1-2, we found that introducing a fluorine group at the R_2 position seemed to improve RNA editing inhibition due to its higher electronegativity and hydrophobicity compared to a methoxy group. Conversely, when we replaced the R_2, R_3, and R_4 functional groups with hydrogen, we observed a loss of the inhibitory effect on the editosome. This suggests these functional groups may play a role in the required molecular interactions for inhibition. Replacing them with hydrogen likely disrupted or weakened these binding interactions, leading to reduced activity. This highlights the potential significance of these specific functional groups in influencing the interactions and overall inhibitory potency on the editosome. Similar modifications were studied in parent compounds #2 and #5, leading to insights into how alterations like replacing pyridine with 2-methylfuran (#2-6) or adding a chloride (#5-3 vs #5-2) could affect their inhibitory effect on RNA editing.

In the case of parent compound #2, certain analogs (e.g., #2-3, 2-4, and 2-6) showed improved effectiveness in the full-round assay while maintaining their ability to inhibit all secondary editing assays, especially when the R_2 group was modified from pyridine to 2-methylfuran (#2-6). Pyridine has a nitrogen atom that can potentially interact with certain protein residues (Bissantz, Kuhn et al. 2010), and its aromatic ring can also engage with aromatic amino acids. In contrast, 2-methylfuran, although similar in its aromatic nature, lacks the nitrogen atom for hydrogen bonding with protein residues but can still interact with aromatic protein residues. The change in the electrophilic behavior might have affected the compound’s hydrophobicity and polarity, as suggested by our ADME predictions. However, it is important to consider factors

like steric hindrance and electronic effects. When we modified the R1 group, substituting benzyne or toluene groups with a more hydrophilic and polar five-membered heterocyclic ring, like furan, appeared to increase activity.

While we acknowledge the potential electrophilicity of the styryl group in compound 2, there are precedents for such groups in approved pharmaceuticals. Istradefylline serves as an example, indicating that such groups can be safe and efficacious when present in the right molecular context .

For parent compound #5, adding a chloride reduced its activity in the full-round assay (#5-3 vs. #5-2), but its inhibitory effect in the mode of action assays remained. This reduction in activity for compound # 5-3 may be due to changes in hydrophobicity and polarity, which can affect how well it dissolves and interacts with the target. Also, modifying R_1 and R_2 by changing pyridine to quinoline in analog compound #5-1 made it ten times more active than the parent compound. This enhanced efficacy is likely due to structural changes. Replacing pyridine with benzene in analog #5-2 increased hydrophobicity and improved inhibitory effects in the mode of action assays.

Testing analogs derived from parent compound #6 did not result in potent inhibitors. However, it’s worth noting that the presence of coumarin in analog #6-3 might have played a role in its observed trypanocidal efficacy. Interestingly, while compound #6 effectively blocked RNA editing activity when the editosome complex was the protein source, it did not inhibit the recombinant ligase protein when tested in isolation. This suggests that the difference in inhibition between the editosome complex and recombinant ligase might be due to varying interacting partners present in the complex setting, which could possibly affect the enzyme's affinity or activity. Further investigations are required to elucidate these complex interactions and optimize the compounds for targeted inhibition.

It's worth noting that although compounds such as #4-3 and #4-1 showed trypanocidal activity and inhibited the editosome, their specific cellular targets remain uncertain, warranting further investigation.

The behavior of a multiprotein complex can also differ significantly from that of a single enzyme, which can affect the observed Hill slope in a dose-response curve (**Table 12.4**). For instance, cooperativity often plays a role, with multiprotein complexes exhibiting either positive or negative cooperativity, influencing the steepness of the dose-response curve. Multiple binding sites within these complexes, each with varying affinities and functionalities, could contribute to complex dose-response patterns. Allosteric effects, where conformational changes occur upon ligand binding, further complicate the relationship. Additionally, the variability in subunit composition among multiprotein complexes results in diverse functional outcomes and different sensitivities to ligands. These complexities can result in Hill slopes that deviate from those observed in single enzyme-ligand interactions.

We also conducted an analysis to determine the time required for each compound to eradicate all *T. brucei* in the media (**Figure. 12.6**). Some analog compounds, such as #1-1, 1-2, 1-3, 1-5, achieved rapid eradication within six hours. This suggests their effectiveness and potential multitarget capabilities, which could reduce the risk of drug resistance. In contrast, compounds #4-3 and #4-1 required 24 and 48 hours, respectively, for parasite elimination. While they act more slowly, these compounds may have advantages like lower toxicity or specific targeting, which deserve further investigation. We acknowledge that the lone pairs of pyrimidine nitrogens in compound 4 may have the potential to be bidentate chelators of metal ions, which, in principle could contribute to its potency and that of its analogs (**Table 12.6**). However, as the full-round assay contains 1 mM EDTA and 5 mM CaCl2, the effect of chelation should be minimized.

Interestingly, some compounds could eliminate parasites at lower concentrations than required for inhibiting *in vitro* RNA editing assays. This could be attributed to two hypotheses: polypharmacology and specialized transporters (McNae, Kinkead et al. 2021, de Vries, Jansen et al. 2022, Napolitano, Mróz et al. 2022). Polypharmacology suggests that a single compound may have multiple modes of action, making it effective against parasites. Alternatively, specialized transporters may provide an alternative means for these compounds to reach their intracellular targets, potentially circumventing solubility and permeability limitations. However, the target

engagement of the compounds was not investigated in this paper, and the rapid cidal activity of some compounds is inconsistent with mere inhibition of RNA editing, which could lead to the parasite's death, as at least one cell cycle is needed for the loss of a functional F0/F1-ATPase component due to a compound's effect on RNA editing. Further research is needed to validate and elucidate the underlying mechanisms responsible for these outcomes.

The compounds identified in our studies are not just potential therapeutic agents, they also offer valuable insights into the mechanistic aspects of the RNA editing process. They could serve as molecular probes to dissect the intricate dynamics and interactions that govern RNA editing, thereby contributing to a more comprehensive understanding of this crucial biological process.

Overall, we have discovered several promising candidates that warrant further analysis, with the potential to serve as effective treatments for TriTryps diseases. The ideal therapeutic candidate would be a compound capable of either disrupting the function or assembly of the editosome. Successful interference with this complex would effectively block the lifecycle of infectious trypanosomatids, thereby neutralizing the disease-causing agents without inducing significant toxicity in the host cells.

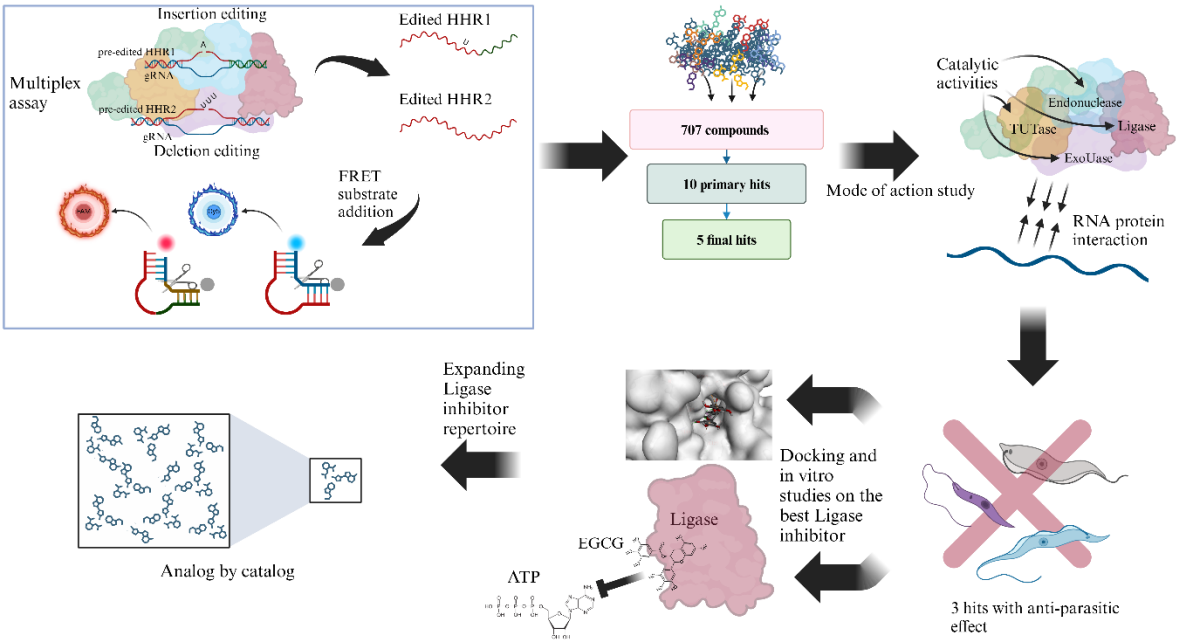
12.6 Acknowledgement

We would like to thank Daniel Moses and Norma Bautista Lopez for their technical assistance. This work was supported by the National Institutes of Health grant R01AI143593 to R.S.

13. Connecting Statement II

The high-throughput screening of compounds detailed in Chapter IV resulted in the identification of several novel scaffolds exhibiting diverse modes of action. These initial hits necessitate extensive optimization processes to evolve into lead compounds. Alternatively, another strategy involves the repurposing of drugs already deemed safe for human use and previously optimized. Additionally, to validate the robustness of the novel RIDE assay developed in Chapter III and demonstrate its suitability for HTS, we conducted a pilot-scale screening of the NIH Clinical Collection. This collection includes current FDA-approved drugs and compounds that have undergone clinical trials, as presented in Chapter V.

14. Chapter V: A pilot-scale screening of clinically approved drugs to identify uridine insertion/deletion RNA editing inhibitors in *Trypanosoma brucei*



This chapter is a reprint of:

Rostamighadi M, Kamelshahroudi A, Pitsitikas V, Jacobson KA, Salavati R. Pilot-Scale Screening of Clinically Approved Drugs to Identify Uridine Insertion/Deletion RNA Editing Inhibitors in *Trypanosoma brucei*. *ACS Infect Dis*. Published online August 9, 2024. doi:10.1021/acsinfecdis.4c00394

14.1 Abstract

RNA editing pathway is a validated target in kinetoplastid parasites (*Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* spp.) that cause severe diseases in humans and livestock. An essential large protein complex, the editosome, mediates uridine insertion and deletion in RNA editing through a stepwise process. This study details the discovery of editosome inhibitors by screening a library of widely used human drugs using our previously developed *in vitro* biochemical Ribozyme Insertion Deletion Editing (RIDE) assay. Subsequent studies on the mode of action of the identified hits and hit expansion efforts unveiled compounds that interfere with RNA-editosome interactions and novel ligase inhibitors with IC_{50} values in the low micromolar range. Docking studies on the ligase demonstrated similar binding characteristics for ATP and our novel epigallocatechin gallate inhibitor. The inhibitors demonstrated potent trypanocidal activity and are promising candidates for drug repurposing due to their lack of cytotoxic effects. Further studies are necessary to validate these targets using more definitive gene-editing techniques and to enhance the safety profile.

14.2 Introduction

African and American trypanosomiasis and various forms of leishmaniasis are caused by *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* species. They belong to the order Kinetoplastea due to their distinctive kinetoplast, a DNA-containing structure within their single, large mitochondrion (Stuart, Brun et al. 2008, Alcântara, Ferreira et al. 2018). Currently, there are no vaccines and few highly effective drugs without side effects available for these diseases, underscoring the pressing need for new drug development efforts (Álvarez-Rodríguez, Jin et al. 2022, Dinc 2022, De Rycker, Wyllie et al. 2023, Ramponi, Aerts et al. 2023).

We employed a drug-centric drug repositioning strategy (Parisi, Adasme et al. 2020), to identify novel treatments, linking a known drug to a new target with its associated indication, specifically antiparasitic activity. Among several targets validated as unique and conserved among these kinetoplastids (Field, Horn et al. 2017), we focus on mitochondrial uridine insertion/deletion editing. This target is particularly compelling for drug discovery due to its essential role and extensive drug-binding landscape (Salavati, Moshiri et al. 2012). These microorganisms possess a cryptic mitochondrial gene expression system. The genes transcribed from the mitochondrial genome necessitate decryption and editing to acquire functionality. This process, termed mitochondrial uridine insertion/deletion editing, is executed by a large protein complex known as the editosome. Comprising over 70 distinct proteins, the holoenzyme editosome has been described as an RNA editing catalytic complex (RECC), RNA-editing substrate-binding complex (RESC), and RNA-editing helicase 2 complex (Aphasizheva, Alfonzo et al. 2020). The RECC catalyzes the enzymatic activities and has been targeted in various screens (Liang and Connell 2010, Moshiri, Mehta et al. 2015, Zimmermann, Hall et al. 2016, Del Campo, Leeder et al. 2020, Rostamighadi, Kamelshahroudi et al. 2024) as most of the catalytic core components are essential for the survival of the parasites (Salavati, Moshiri et al. 2012). Editing is directed by untranslatable short RNAs (30–60 nt) known as the guide RNAs (gRNAs), mostly transcribed from mitochondria minicircles. Initially, a hybrid of gRNA and pre-edited mRNA forms through

the complementarity between the 5′ anchor region of the gRNA and the pre-edited mRNA. This results in the protrusion of single-stranded uridines within the mRNA (deletion sites) or purine nucleotides within the gRNA (insertion sites). An endonuclease in the complex then recognizes and cleaves at the first unpaired nucleotide adjacent to the 5′ anchor duplex. The specific isoform of RECC containing KREN1+KREPB8+KREX1 cleaves U-deletion sites, whereas the isoforms with KREN2+KREPB7 or KREN3+KREPB6 cleaves U-insertion sites (Carnes, Trotter et al. 2008, Ernst, Panicucci et al. 2009, Carnes, Lewis Ernst et al. 2012, Guo, Carnes et al. 2012, Carnes, McDermott et al. 2017). Depending on the type of editing, either the exonuclease KREX2 removes uridine residues or the terminal uridylyl transferase KRET2 adds them, guided by sequence complementarity with the gRNA. Finally, the ligase (KREL1 for deletion or KREL2 for insertion) joins the two RNA fragments to produce the final edited mRNA. Although the two ligases are spatially separated in two different subcomplexes and perform distinct functions, only KREL1 is shown to be essential for the parasite's viability, and it can compensate for the lack of KREL2 (Huang, Cruz-Reyes et al. 2001, Schnaufer, Panigrahi et al. 2001, Cruz-Reyes, Zhelonkina et al. 2002, Gao and Simpson 2003).

Different methods have been employed to discover new inhibitors of this pathway (Amaro, Schnaufer et al. 2008, Durrant, Hall et al. 2010, Liang and Connell 2010, Moshiri, Acoca et al. 2011, Demir, Labaied et al. 2014, Moshiri, Mehta et al. 2015, Zimmermann, Hall et al. 2016, Del Campo, Leeder et al. 2020, Rostamighadi, Kamelshahroudi et al. 2024), including both virtual and *in vitro* screens. Our laboratory has developed a FRET-based full-round deletion RNA editing assay (Moshiri and Salavati 2010), which has been utilized in both pilot-scale (Moshiri, Mehta et al. 2015) and high-throughput screening (HTS) (Rostamighadi, Kamelshahroudi et al. 2024) to discover new inhibitors of this pathway. Further studies are underway to evaluate the hits from these screenings. We enhanced our FRET-based assay, now termed the Ribozyme Insertion Deletion Editing (RIDE) assay, by modifying it to bypass the rate-limiting step of endonuclease activity. This assay simultaneously monitors both insertion and deletion editing within the same reaction, as shown in Figure 14.1 (Rostamighadi, Mehta et al. 2023). The RIDE assay uses ribozymes that bind and cleave a specific FRET substrate. Post-editing, these ribozymes cleave their specific substrates to release a detectable signal. This assay is adaptable

for high-throughput applications and requires significantly lower ATP concentrations—10,000 times less than the full-round assay —thereby facilitating the discovery of competitive inhibitors.

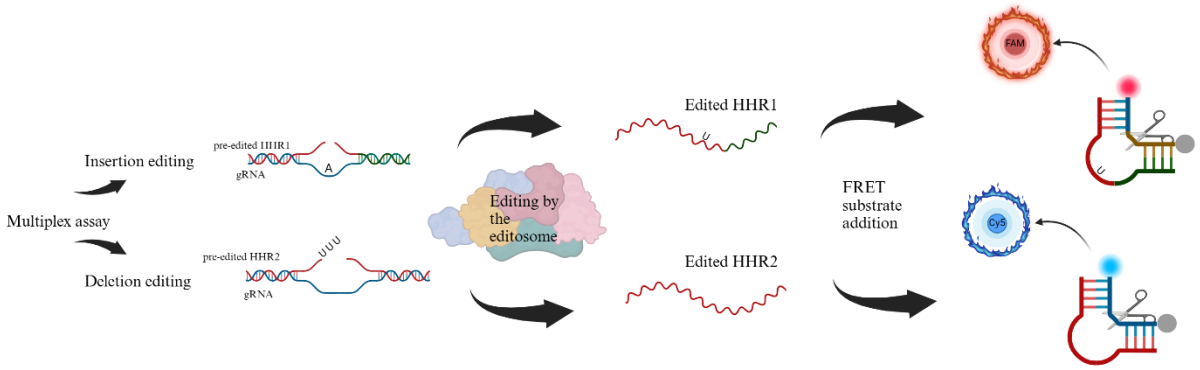


Figure 14.1. Schematic representation of the Ribozyme Insertion Deletion Editing (RIDE) assay. *This assay integrates both uridine insertion and deletion RNA editing processes (post-endonucleolytic activity) into a single multiplex reaction. The pre-edited RNA sequence, guided by the gRNA, undergoes editing by the editosome, activating two hammerhead ribozymes (HHR1 for insertion and HHR2 for deletion) from a catalytically inactive state. These activated ribozymes are then capable of cleaving their respective FRET substrates. Fluorescent signals emitted upon cleavage of these FRET substrates indicate the quantity of fully edited RNA molecules present.*

In our search for potential drug candidates that can suppress RNA editing, we conducted an *in vitro* biochemical screening of the National Institutes of Health Clinical Collections (NCC) library. This library comprises 707 small molecules, including FDA-approved drugs and late-stage development candidates, which have been tested against various targets and disease agents previously (Cao, Forrest et al. 2015, Lariosa-Willingham, Rosler et al. 2016, Trader, Simanski et al. 2017, Widmeier, Tan et al. 2017, Abrahams, Mosebi et al. 2018, Bowden, Land et al. 2018, Kozisek, Hamann et al. 2020, Bezemer, van Cleef et al. 2022, Johnson, Wang et al. 2022, Bond, Crocker et al. 2024). Using our refined assay, we identified five compounds inhibiting *in vitro* RNA editing and four exhibiting antiparasitic activity. Further investigations and hit expansion efforts revealed that these compounds interfere with multiple catalytic steps in our *in vitro* mode

of action assays and include novel ligase inhibitors with IC50 values in the low micromolar range, with two compounds disrupting RNA-protein interactions. Significantly, these compounds have shown potent anti-parasitic effects at micromolar concentrations, with minimal cytotoxicity to host cells. Our findings suggest new potential targets for these compounds. By leveraging existing preclinical and/or clinical data on these molecules, we can optimize these hits into lead compounds, advancing the development of effective anti-kinetoplastid drugs. Future studies must confirm their target specificity and rigorously enhance their safety profiles.

14.3 Results

14.3.1 Screening for RNA editing inhibitors using the RIDE assay

To discover new RNA editing inhibitors, we utilized the RIDE assay to screen the NIH Clinical Collection (Rostamighadi, Mehta et al. 2023) (Figure 14.1). This HTS method includes both insertion and deletion editing in a single reaction, omitting endonuclease activity. We employed a KREL1-tagged tandem affinity purified editosome from *T. brucei* as the protein source and conducted the screening at 20 μM concentration. Compounds reducing RNA editing activity by more than 50% compared to the control were considered hits, resulting in an acceptable screen performance with a signal-to-background ratio greater than 3 and a mean Z' factor of 0.8 ± 0.1.

We identified eleven primary hits from the library. These hits represent a 1.5% hit rate, with ten exhibiting reproducible inhibitory effects (Figure 14.2)—a 0.14% false-positive discovery rate after confirmation using the multiplex assay and individual precleaved assays. The hits included four drugs from the anthracycline family, three from the flavonoids, and three singleton compounds.

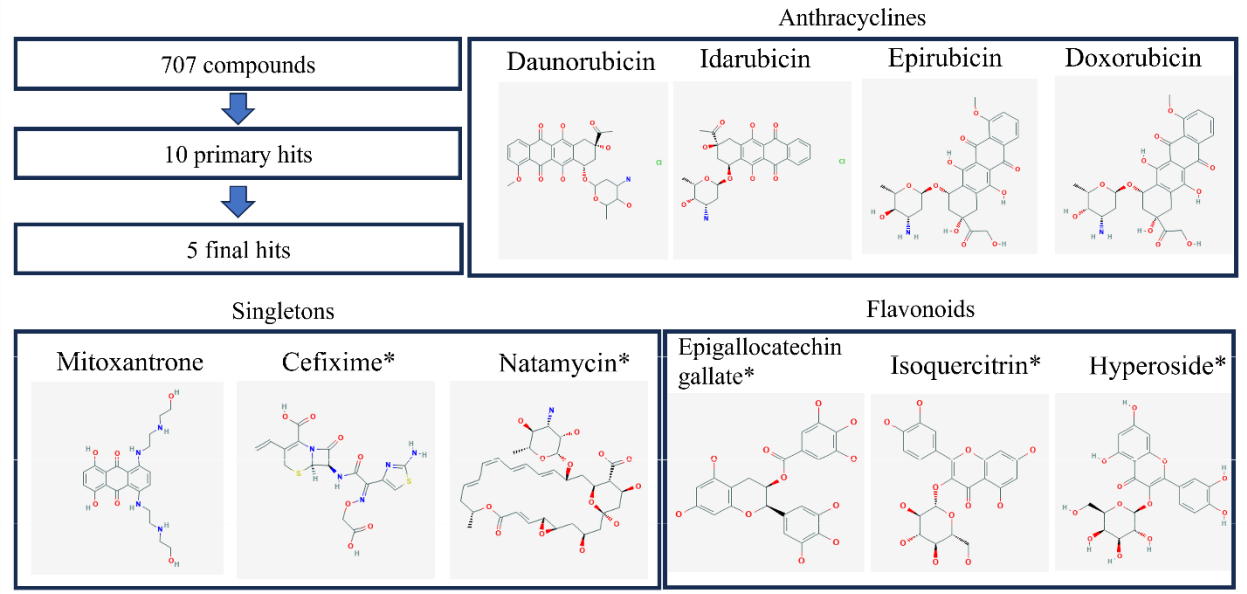


Figure 14.2. Screening cascade and chemical structures of initial hits*. The pilot-scale screening identified ten primary hits: two compound groups (flavonoids and anthracyclines) and three distinct compounds (mitoxantrone, cefixime, and natamycin). After excluding non-specific hits, five final hits remained, including selected flavonoids, cefixime, and natamycin. These final hits are marked with an asterisk.*

14.3.2 Counter screening for specificity (interference assay)

A counter screen was conducted to identify compounds that hindered ribozyme activity independent of the editosome to ascertain the specificity of the inhibition observed in the initial screening. This validation process involved assessing the impact of potential inhibitors on edited hammerhead ribozymes in the absence of the editosome. Among the ten hits, five inhibitors were found to non-specifically impede ribozyme activity at 40 µM concentration (Figure 14.3). Notably, members of the anthracyclines family (Daunorubicin, Idarubicin, Doxorubicin, epirubicin) and Mitoxantrone exhibited this non-specific inhibition. Additionally, the activity of these compounds was assessed by monitoring ribozyme cleavage activity on a gel, which revealed distinct effects—some impeded FRET cleavage (Doxorubicin, Epirubicin, and Mitoxantrone), while others interfered with fluorescence detection (Daunorubicin and Idarubicin). This approach enabled the characterization of each compound's non-specific

mechanism of action in the RIDE assay and led to the elimination of promiscuous hits from the initial hits.

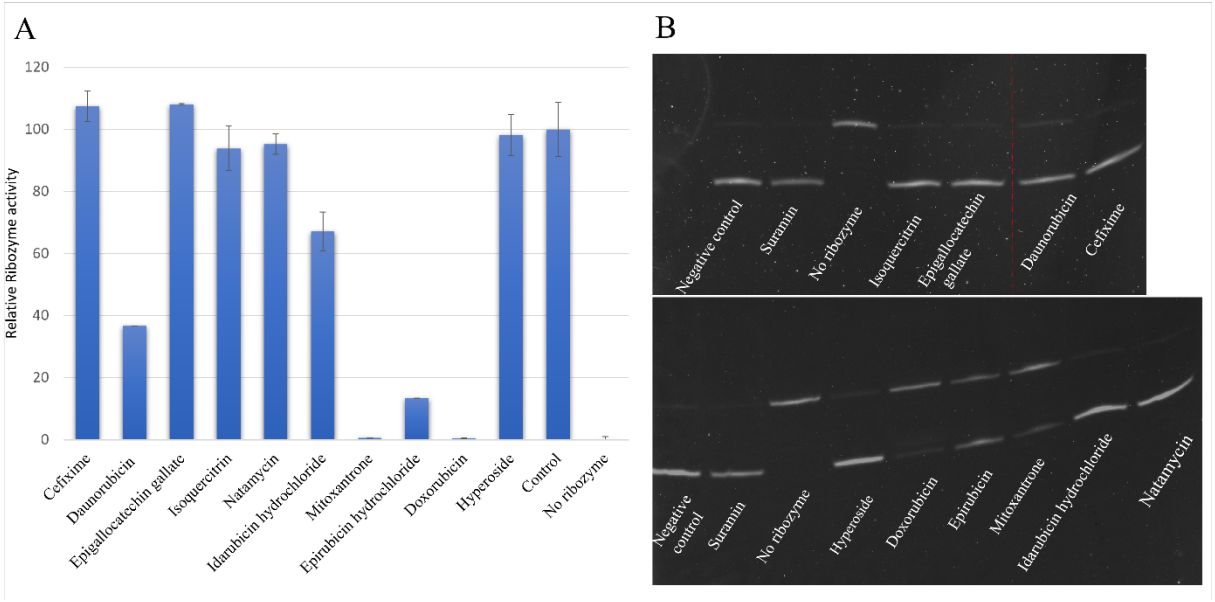


Figure 14.3. Counter-screening initial hits for elimination of false positives. *The ten primary hits were subjected to counter-screening against the active ribozyme to determine potential interference with (A) fluorescence in the FRET-based assay and (B) cleavage of the FRET substrate in a gel-based format. Controls without the ribozyme and without drugs were included to establish baseline activity. Suramin served as an additional negative control within the assay. Mean values and standard deviations were calculated from the FRET-based assay data and are shown for two independent replicates.*

14.3.3 RNA editing inhibitors' efficacy in RIDE assay and their effect on different parasite's viability: Dose-response analysis and comparative efficacy

Each confirmed hit was tested in a dose-response manner in the multiplex assay, and their IC_{50}s were determined using a three-parameter non-linear regression model in GraphPad (Figure 14.4). Among flavonoids, epigallocatechin gallate (EGCG) showed its inhibitory effects even at 100 nM, whereas isoquercitrin and hyperoside were less potent, with IC_{50} values in the low to moderate micromolar range. Cefixime and natamycin impaired the FRET assay in the moderate micromolar range, with their IC_{50}s ranging from 6 to 55 µM. Cefixime, isoquercitrin, and EGCG

demonstrated effects on both insertion editing and deletion editing within a similar concentration range. This suggests that they may target proteins involved in regulating both types of editing. Conversely, natamycin and hyperoside exhibited stronger activity against insertion and deletion, respectively. This indicates that the proteins they target may influence both types of editing, but with a greater impact on one over the other.

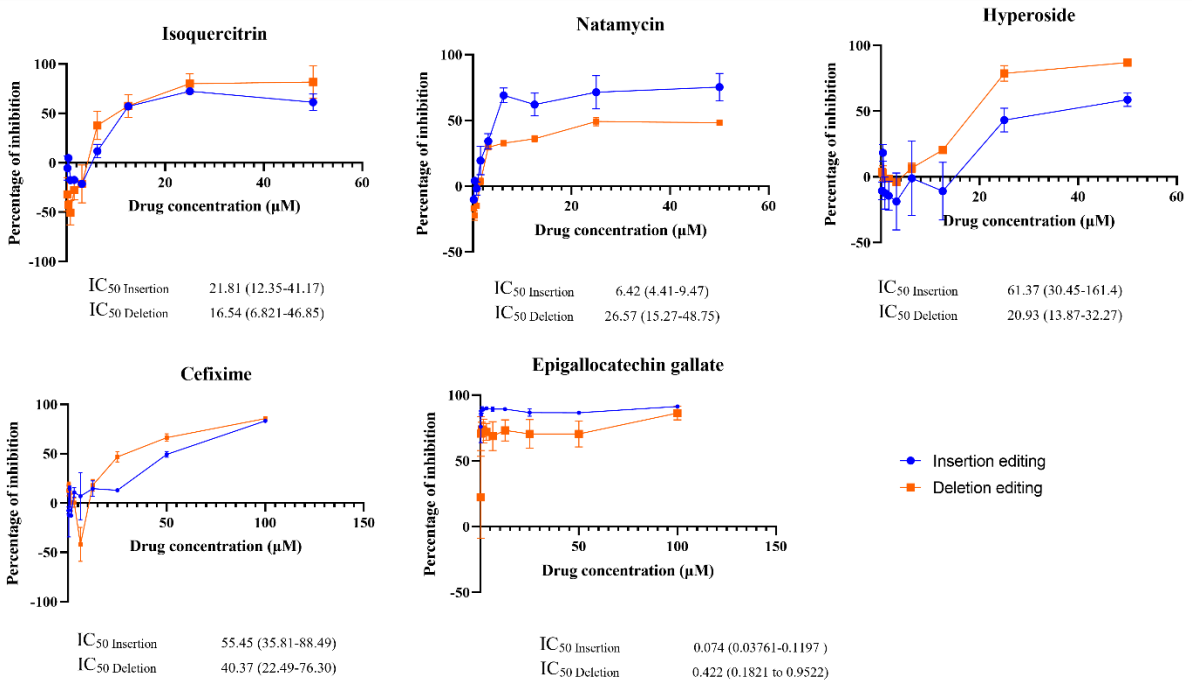


Figure 14.4. Dose-response curves for confirmed RNA editing inhibitors*. Each confirmed hit was tested in the RIDE assay under a range of concentrations to establish dose-response profiles. The IC_{50} for each inhibitor was determined for both insertion and deletion editing. The IC_{50} values are presented in µM with a 95% confidence interval in parentheses. Mean values and standard deviations are depicted for each data set based on a minimum of two independent experiments.*

Subsequently, we tested the hits against *T. brucei* using the Alamar blue assay, and EC_{50} values against other kinetoplastids, such as *T. cruzi* and *leishmania* species, were added to Table 14.1 (data sourced from the literature). The results from other sources were consistent with our finding for *T. brucei*. Cefixime showed no effects on *T. brucei* viability or growth in our experiments, and no data was available for its impact on other kinetoplastids). Flavonoids showed varying anti-trypanosomatid effects in the moderate micromolar range, with EGCG being the most potent among them, displaying EC_{50} of 20 µM, 41 µM and 200 µM against *T.*

brucei, *leishmania,* and *T. cruzi*, respectively. Hyperoside showed mediocre efficacy against all three parasite species, with EC_{50} values ranging from 50-65 µM.

Isoquercitrin had no observable effects on *T. brucei* viability at the tested concentrations. However, data for its analog, quercitrin, showed it impacting parasite viability within the 39.5 - 67 µM range. We predict, if present, the isoquercitrin EC_{50} for *T. brucei* will be more than 20 µM. No data was found for isoquercitrin against *T. cruzi* and *leishmania*.

Apart from EGCG, which had a very low IC_{50} in the RIDE assay, and Cefixime, which showed no effect on the viability of *T. brucei*, the other compounds (natamycin, hyperoside, and isoquercitrin) affected both editing and the viability of the parasites in the same concentration ranges, suggesting the editing as one of their probable targets which warrants further investigation.

We also compiled data on the effects of hits on different human cells and included this in Table 14.1 to provide insight into their selectivity towards the parasites. Given that these compounds are selected from the NIH clinical collection, they were expected to show low or no effects on mammalian cells. Their selectivity and safety for human use make these hits promising candidates for repurposing in treating kinetoplastid diseases. Further studies will be needed to enhance the selectivity index for these compounds.

Drug name	EC50			
	T. brucei	*T. cruzi*	*leishmania*	Human cells
Cefixime	NI	ND	ND	>200 µM (MT-4) (Abrahams, Mosebi et al. 2018)

EGCG	20 µM	200 µM (Baldim, de Alcântara et al. 2017)	41 µM (Baldim, de Alcântara et al. 2017)	246 µM (HGF-1) (Weisburg, Weissman et al. 2004)
Isoquercitrin (Quercitrin)	>20 µM (62 µM) (Baldim, de Alcântara et al. 2017)	ND (67 µM) (Baldim, de Alcântara et al. 2017)	ND (39.5 µM) (Baldim, de Alcântara et al. 2017)	500 µM (CHO)(Haskins, Su et al. 2016) (3.3 mM (Vero E6)) (Yi, Li et al. 2004)
Natamycin	12 µM	ND	15 µM (Awasthi and Mitra 2018)	100 µM (macrophage) (Awasthi and Mitra 2018)
Hyperoside	50 µM	64.7 µM (Baldim, de Alcântara et al. 2017)	64.7 µM (Baldim, de Alcântara et al. 2017)	194 µM (L6) (Baldim, de Alcântara et al. 2017)

Table 14.1. Antitrypanosomal activity of confirmed hits. *This This table presents the EC_{50} values for each compound against T. brucei parasites, as measured using the Alamar blue assay. Suramin (one of the current drugs for human African trypanosomiasis) and no-drug served as the positive and negative controls in our viability assay, respectively. Additional data from the literature are included where available (see references). Information on the effects of these compounds on human cells is also provided. ND and NI abbreviations indicate "not determined" and "no inhibition" within the tested range, respectively. IC_{50} values against RNA editing assays were added from Figure 4 to compare with their effect on cell viability. The number of replicates for each data is mentioned below.*

** Values are averages of at least three separate experiments.*

*** Mean values from at least two replicates (variation is a maximum of 20%).*

**** Independent experiment with a minimum of three replicates for each endpoint.*

***** The number of replicates was not indicated.*

****** Conducted at least three times in triplicates.*

14.3.4 Mode of action analysis for confirmed hits

Each confirmed hit (EGCG, isoquercitrin, natamycin, cefixime, and hyperoside) was tested in the *in vitro* mode of action assays to evaluate its effect on individual catalytic steps of uridine insertion/deletion RNA editing and its impact on RNA-protein interaction (Figure 14.5). We previously developed fluorescent gel-based assays to monitor each catalytic step—endonuclease, exoUase, TUTase, and ligase (Rostamighadi, Kamelshahroudi et al. 2024). Additionally, EMSA was utilized to determine if a hit impairs the editosome's ability to bind the RNA substrate. Suramin served as the positive control, known to inhibit all enzymatic steps and interfere with RNA protein interactions, which is presumed to be its mode of action against the editosome (Liang and Connell 2010, Mehta, Moshiri et al. 2020, Rostamighadi, Kamelshahroudi et al. 2024).

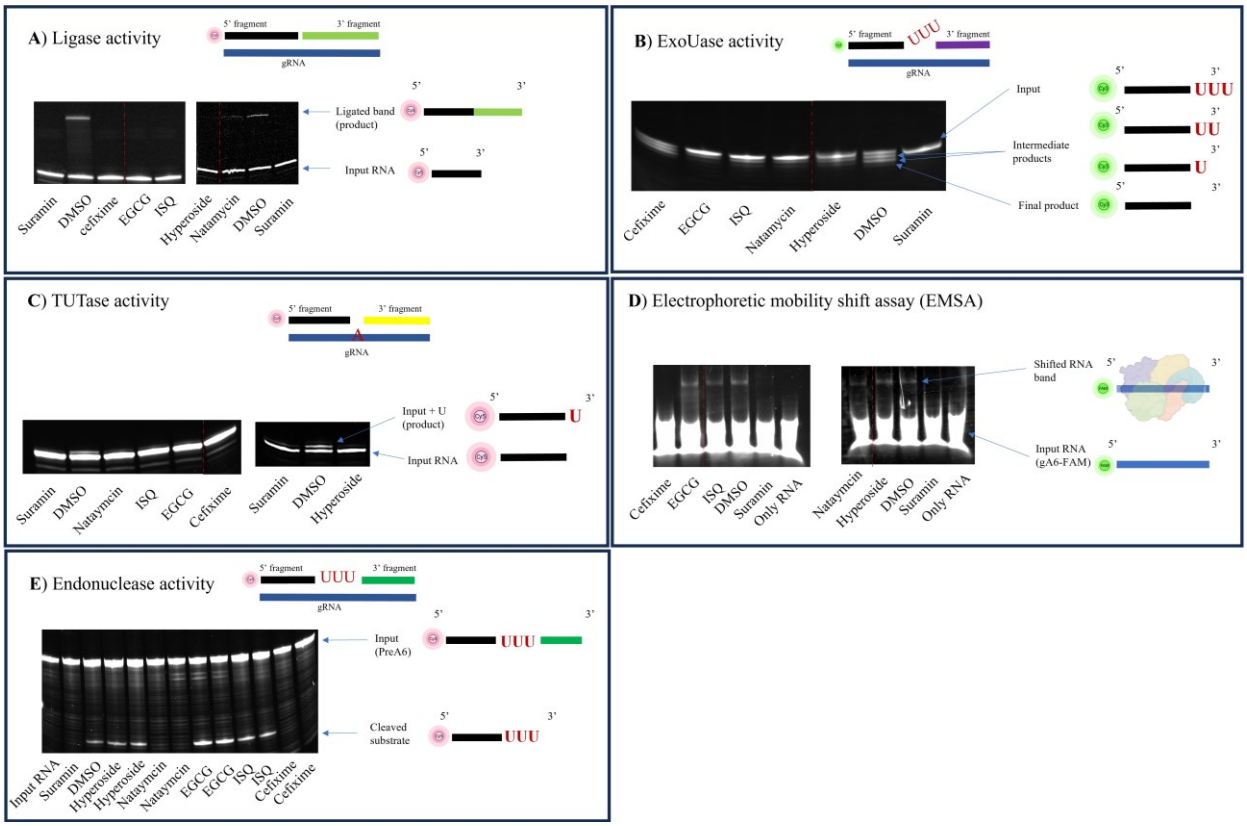


Figure 14.5. Characterization of the hits' mode of action. *Each compound was assessed for its effect on the editosome's individual catalytic steps of RNA editing. These include (A) RNA ligation by ligase, (B) uridine removal by exoUase, and (C) uridine addition by TUTase. (D) Compounds were also evaluated for their ability to disrupt RNA-protein interactions using EMSA. (E) The endonuclease activity assay served as a control to confirm specificity, as this assay is not encompassed by the RIDE assay. RNA substrates and corresponding fluorophores for each assay are depicted, and diagrams representing the RNA hybrids highlight the targeted enzymatic activities. In these assays, suramin served as a positive control (indicative of 100% inhibition), while DMSO were the negative control (indicative of no inhibition).*

All flavonoids (EGCG, isoquercitrin, and hyperoside) inhibited the ligase, exoUase, and TUTase activities but did not affect the endonuclease or RNA-protein interactions (Figure 14.5). This lack of effect might be due to the higher protein concentration present in these two assays.

Cefixime inhibited ligase, endonuclease, and TUTase but did not affect exoUase activity (Figure 14.5). It also hindered RNA-protein interaction in the gel shift assay, suggesting it may interact with an RNA-binding protein involved in all the assays except for exoUase activity.

Natamycin inhibited all the enzymatic activities (ligase, TUTase, exoUase, and endonuclease). It moderately affected the gel shift assay, indicating its mode of inhibition likely involves preventing the protein from binding to the substrate.

14.3.5 Exploring target specificity in RNA ligase inhibition

Each activity assay is designed to be independent of the other steps, meaning that only the activity of a specific enzyme (e.g., ligase) is required for the assay. If a compound inhibits multiple enzymatic activities, it likely targets a common element rather than inhibiting different enzymes separately. To ascertain whether the hits that hindered the ligation step of RNA editing, facilitated by the editosome, could also impact the recombinant enzyme governing the same process, we examined these compounds against recombinant RNA editing ligase (Figure 14.6). This approach helped determine if the compounds could bind directly to the ligase while disrupting the activity of the editosome through interference with RNA-protein interactions, particularly binding to mRNA-binding proteins such as Mitochondrial RNA binding Proteins (MRPs). Mordant Black 25 (MrB), a previously identified ligase inhibitor, served as the positive control (Moshiri, Mehta et al. 2015).

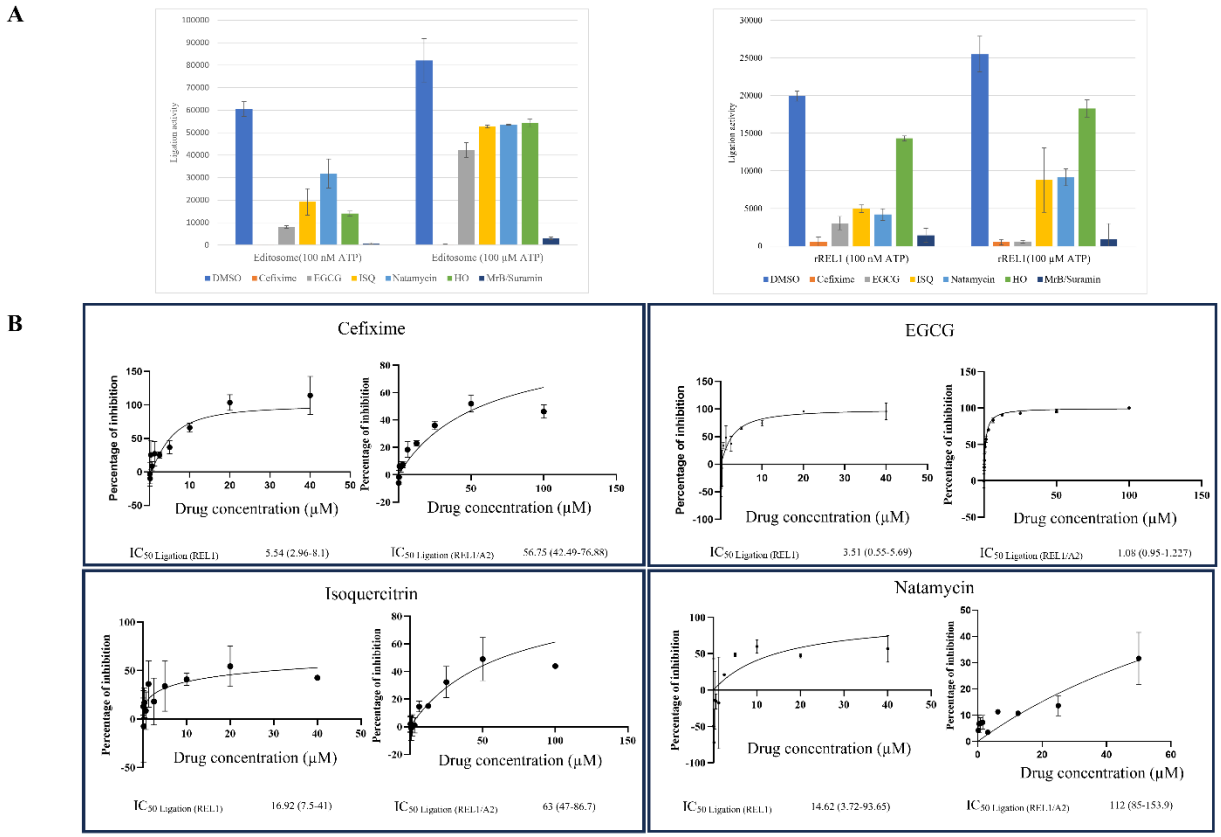


Figure 14.6. Evaluating the efficacy of hits on ligation activity. *(A) The inhibitory effects of all identified hits were assessed in our FRET-based ligation assay using two protein sources: recombinant ligase (right) and the editosome complex (left), with assays conducted at two different ATP concentrations (100 nM and 100 µM). This analysis showed differential inhibitory patterns among the compounds with varying ATP concentrations. (B) The IC_{50}s were calculated for the compounds against recombinant ligase (rKREL1) and rKREL1/KREPA2 complex. Data represent means and standard deviations, based on duplicate experimental replicates.*

The results show that all hits except hyperoside inhibited the recombinant RNA editing ligase 1 at a low ATP concentration (100 nM) (Figure 14.6A), indicating that cefixime, EGCG, natamycin, and isoquercitrin bind to the ligase and hinder the ligation process *in vitro*. This discovery opens up new possibilities for creating targeted inhibitors.

In a preliminary analysis, we tested the drugs at 40 µM with two ATP concentrations (100 nM and 100 µM) in the ligation assay to test the effect of high ATP concentrations on the

compounds' ability to inhibit editing (Figure 14.6A). Remarkably, the inhibition by hyperoside decreased from approximately 77% to 35% when tested against the editosome. The increase in ATP concentration also impacted EGCG and isoquercitrin to some degree, reducing their inhibitory effects on the editosome from 88% and 70% to 50% and 37%, respectively. However, it only slightly affected isoquercitrin's inhibition of the recombinant protein, reducing it from 81% to 68%, and had no discernible effect on EGCG.

Natamycin's efficacy against rKREL1 decreased slightly from 85% to 66%, and its mediocre inhibitory effect remained largely unaffected against the editosome (47% to 36%). Cefixime fully inhibited the ligation assay with both the editosome and the rKREL1 at both ATP concentrations.

To evaluate the efficacy of the hits against the ligase, we determined the IC_{50} of cefixime, EGCG, isoquercitrin, and natamycin in the ligation assay using either rKREL1 or the rKREL1-KREPA2 complex (Figure 14.6B). Utilizing KREPA2 would provide insight into whether the presence of an interacting partner affects the inhibitors' efficacy. KREPA2 enhances ligation activity compared to recombinant REL1 alone (Moses, Mehta et al. 2023), potentially impacting the inhibitors' efficacy. Except for EGCG, we observed less inhibition when using rKREL1/KREPA2 as the protein source. Hyperoside did not affect either rKREL1 or rKREL1/KREPA2, indicating that its target lies elsewhere in the editosome.

Cefixime, isoquercitrin, and natamycin showed significantly reduced inhibition against rKREL1/KREPA2 compared to rKREL1, possibly due to the enhanced ligation activity of rKREL1/KREPA2 or conformational changes in KREL1 induced by KREPA2. The IC_{50}s of Natamycin and isoquercitrin were even higher than their IC_{50}s in the RIDE assay, suggesting an alternative mode of action within the editosome context. Cefixime's IC50 was ten times higher against rKREL1/KREPA2 than rKREL1, yet it exerts its effect in the same range of concentrations observed in the RIDE assay mediated by the editosome.

This study showed evidence that the identified compounds inhibit RNA ligase activity and potentially disrupt RNA-editosome interactions *in vitro*. However, it is crucial to acknowledge the limitations of our assay methods, which are primarily biochemical and do not provide direct

proof of mechanism within a cellular context. The observed effects might also be mediated through mechanisms not explored in this study, including potential off-target interactions. Future studies employing knock-in/knock-out techniques and *in vivo* models will be essential to confirm these findings and elucidate the precise mechanisms by which these compounds exert their antiparasitic effects.

14.3.6 Molecular docking studies

Molecular docking studies identified the binding location and interaction mode of EGCG with KREL1 through blind docking using AutoDock Vina (Figure 14.7). EGCG was found to bind within the ATP binding pocket of the full-length KREL1, which includes both the catalytic N-terminal and C-terminal domains involved in RNA recognition and ligase auto-adenylylation (Figures 14.7A-C) (Moses, Mehta et al. 2023). EGCG exhibited a binding affinity of -9 kcal/mol, compared to -8.6 kcal/mol for docked ATP. Figure 14.7D illustrates the superimposition of EGCG and ATP, showing their similar binding characteristics. Both molecules bind in analogous poses; EGCG's benzopyran group and ATP's adenine base are located deepest within the ATP-binding cleft, while the remainder of their structures extend outward from the binding pocket. According to predictions, EGCG's benzopyran is expected to occupy the positions analogous to ATP's adenine and ribose sugar (Figure 14.7D), illustrating mimicry in their binding configuration. Interactions include Phe209, Val286, and Lys87 engaging with the benzopyran of EGCG and the adenine base of ATP, respectively. Additionally, the hydroxyl group attached to carbon 7 (C7) of EGCG's benzopyran forms a hydrogen bond with Glu86, and ATP's adenine base hydrogen bonds with Tyr58. ATP forms a pi-sigma bond with Ile61, while EGCG's galloyl group hydrogen bonds with the same residue (Figures 14.7C and 12.7E). EGCG also forms a pi-anion interaction with the highly conserved residue Lys307, crucial for the first step of adenylylation reaction (Deng, Schnaufer et al. 2004), whereas ATP is hydrogen bonded to Lys307 through its α-phosphate group. EGCG also forms two hydrogen bonds with catalytic Lys87, involving the oxygen atoms from EGCG's ring C and the galloyl group attachment. However, ATP engages Lys87 only via a pi-alkyl bond. EGCG Rings B and D (the galloyl group) efficiently fill the space occupied by ATP's phosphate chain (Figure 14.7D), binding

tightly within the ligase. A hydroxyl group on EGCG's Ring B forms a hydrogen bond with the conserved His89 (crucial for strand joining (Ho and Shuman 2002, Yin, Ho et al. 2003, Deng, Schnaufer et al. 2004)). Additionally, EGCG's Ring B forms a pi-anion interaction with Glu159, a motif III conserved residue, whereas Glu159 binds the ATP ribose sugar and phosphate groups of ATP via hydrogen and charge interactions. Amino acids that interact with both EGCG and ATP in the full-length protein are outlined in black (Figure 14.7C).

We also observed an unfavorable donor-donor interaction between Arg309 and an oxygen atom on EGCG's galloyl group, suggesting a potential for induced fit effect due to the protein's static nature in our simulation. This observation supports the hypothesis that EGCG's inhibitory effect on KREL1 at low micromolar concentration may be enhanced by protein conformational adjustments. While ATP forms more interactions within the pocket, EGCG's similar docking pose and critical residue engagement highlight its potential as a basis for developing potent KREL1 inhibitors.

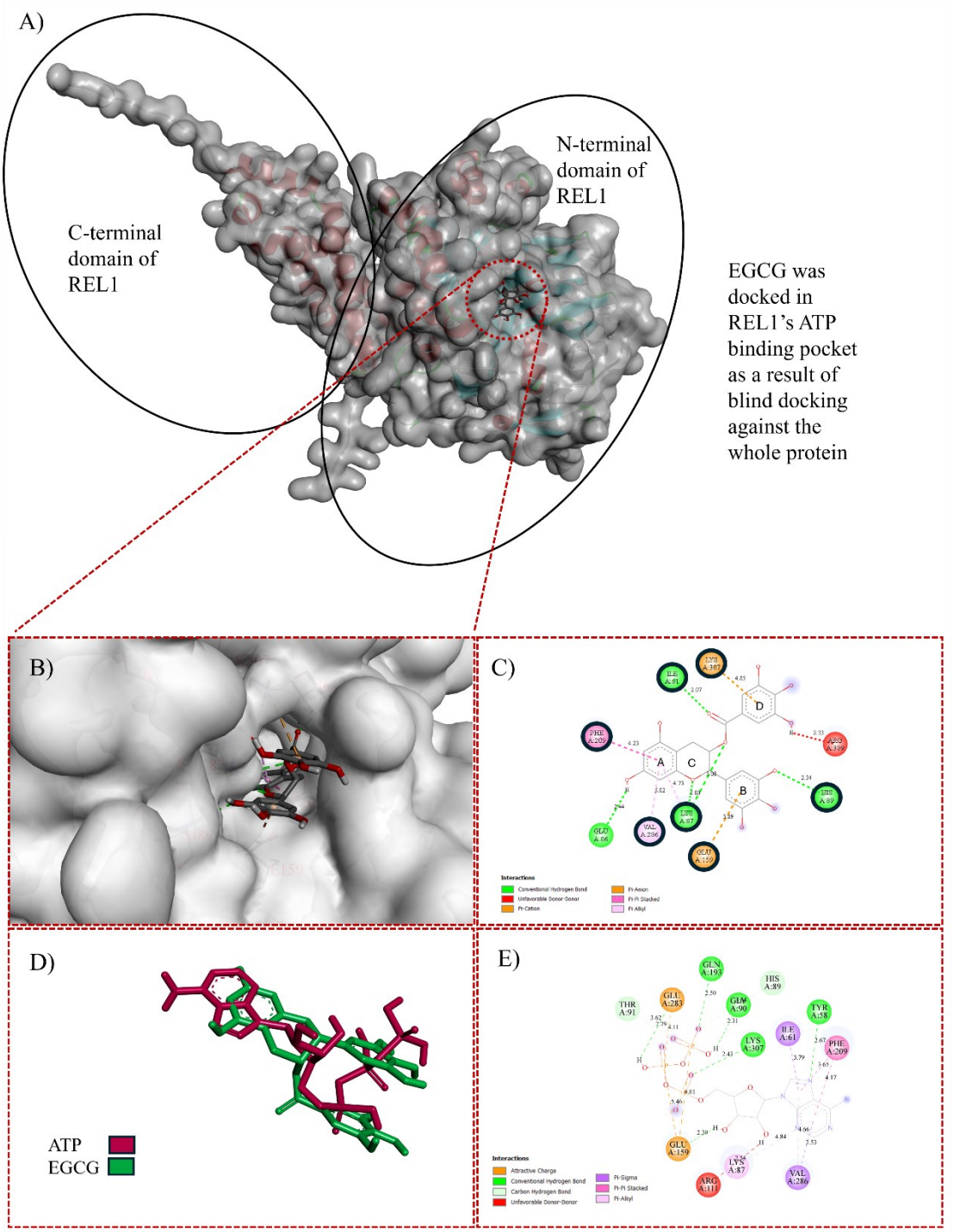


Figure 14.7. Molecular docking studies of EGCG against the full-length AlphaFold model of KREL1. *(A) Structure of KREL1 and its two domains (B) 3D view of the EGCG docked in the ATP binding pocket (C) 2D ligand interaction diagram of EGCG in the binding site. Amino acids that were shown to interact with the docked ATP are outlined in black (D) EGCG superimposed on the ATP (both docked against the full-length KREL1) (E) 2D ligand interaction diagram of ATP docked against the full-length KREL1.*

14.3.7 Expanding the ligase inhibitor pool

To expand the repertoire of ligase inhibitors, we acquired an additional set of 36 compounds, which includes flavonoids, several suramin analogs (previously known as KREL1 inhibitors), and nucleotide analogs. We screened these compounds at a concentration of 50 μM against rKREL1, the rKREL1/KREPA2 complex, and the REL1-TAP tagged purified editosome for ligation activity (Figure 14.8A). We selected this concentration because the IC_{50} values of flavonoids (excluding EGCG) fell within the moderate micromolar range. Screening compounds with closely related structures at 50 μM ensures we capture all potential inhibitors. If a compound fails to exhibit any inhibitory effect at this concentration, it confirms its lack of activity and is deemed unworthy of further pursuit. ABMA, an ATP analog, also demonstrated a 50% inhibitory effect within a similar range, justifying the same rationale for nucleotide analogs (Rostamighadi, Mehta et al. 2023).

Among this set, we found nine compounds that inhibit ligation activity, targeting either all three protein sources [rKREL1, rKREL1/KREPA2 and the editosome], two protein sources [rKREL and editosome], or exclusively the editosome. The high hit rate can be attributed to the selection of these compounds as analogs of the hits identified in this study (flavonoids) and from previous studies (Suramin) (Zimmermann, Hall et al. 2016, Rostamighadi, Kamelshahroudi et al. 2024). We identified two compounds (nucleotide analogs), MRS2289 and MRS2295, that solely inhibited ligation within the editosome, warranting further investigation to elucidate their mode of action. The synthesis of MRS2285 (compound 7 from (Kim, Brown et al. 2001)), MRS2289 (compound 17 from (Kim, Barak et al. 2001)), and MRS2295 (compound 15 from (Kim, Barak et al. 2001)) was previously described. Furthermore, we observed that suramin analogs impacted the activity of all protein sources like suramin, as shown previously (Liang and Connell 2010, Zimmermann, Hall et al. 2016, Rostamighadi, Kamelshahroudi et al. 2024).

We used an endonuclease assay as a counter-screen to determine whether a compound specifically affects the “ligation” step among the enzymatic activities of the editosome. As shown in Figure 14.8B, five compounds also inhibited the endonuclease activity, indicating that their target protein within the editosome influences other editing activities. MRS2884, which is a

polyamidoamine (PAMAM) dendrimer conjugate (Das, Zhou et al. 2009), exhibited aggregation within the well, representing its mode of action. However, two flavonoids (MRS8266 and MRS8267) did not affect endonuclease activity, suggesting their target within the editosome could be ligase or its interacting partners. Additionally, MRS8267 was inactive against the rKREL1/KREPA2, possibly due to the conformational change or the enhanced activity of the complex compared to the recombinant protein alone.

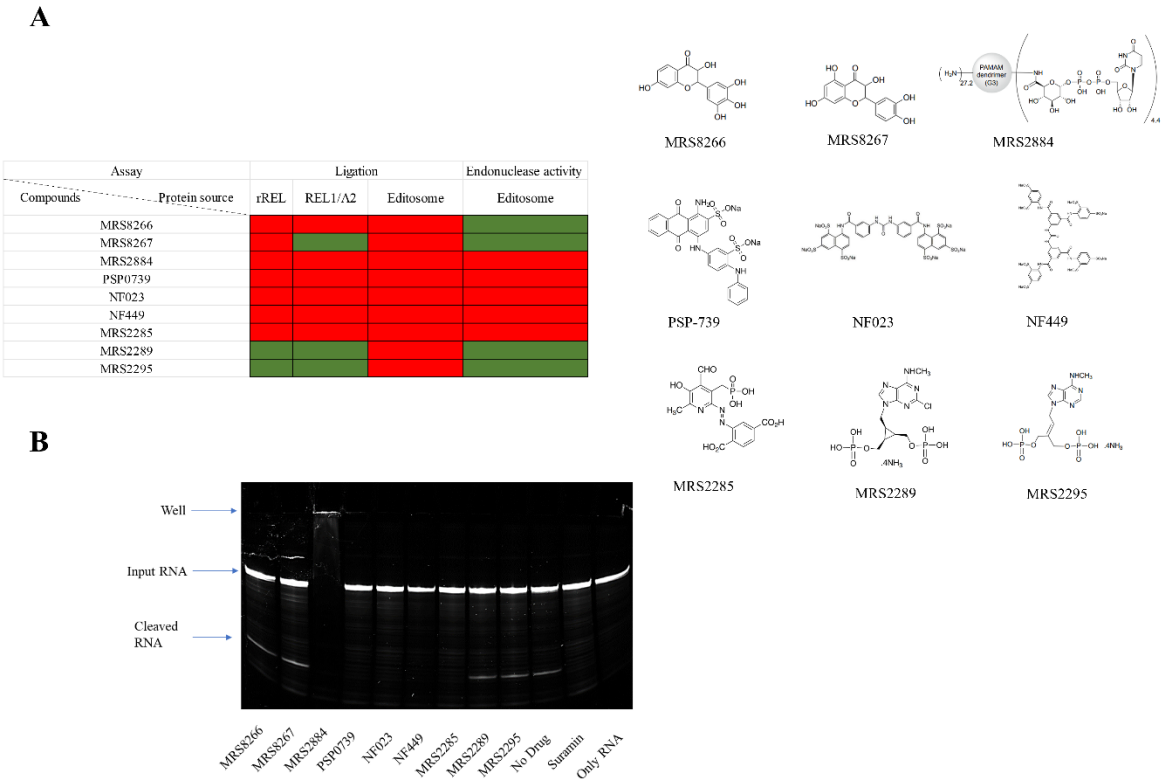


Figure 14.8. Expansion of the hit compounds. *(A) A selected set of 36 available analogs, including flavonoids, suramin (a previously identified editosome inhibitor (Liang and Connell 2010, Zimmermann, Hall et al. 2016, Rostamighadi, Kamelshahroudi et al. 2024)), and nucleotide analogs were tested at 50 μM in a ligation assay. Three protein sources were used: recombinant ligase rKREL1, rKREL1/KREPA2 complex, and the editosome. Compounds achieving greater than eighty percent inhibition were considered significant, and their chemical structures are depicted. (B) To confirm the specificity of these compounds for ligase activity within the editosome, their effect on the editosome's endonuclease activity were also evaluated. The color coding in the illustrations—red for inhibition and green for no inhibition—indicates the outcome of these assessments.*

14.4 Discussion

Repurposing drugs is an effective strategy for developing treatments for trypanosomatid diseases. To advance this research, we used a novel multiplex assay to screen the NCC library for inhibitors targeting the uridine insertion/deletion RNA editing, which is unique to kinetoplastids and absent in humans. We initially identified ten effective drug candidates against the editosome, the protein complex crucial for this editing process. Our promising findings include two compound series—three flavonoids, four anthracyclines, and three individual compounds.

Among the primary hits, the anthracyclines family—including daunorubicin, idarubicin, doxorubicin, epirubicin—and mitoxantrone were eliminated from further studies due to their interference with the ribozyme activity and the detection system. This aligns with previous research indicating that anthracycline derivatives are characterized by distinctive red fluorescence emission associated with their conjugate systems (Beale, Block et al. 2010, Catitti, De Fabritiis et al. 2022), and their ability to intercalate with DNA(Chaires, Fox et al. 1987, Wang, Ughetto et al. 1987, Moore, Hunter et al. 1989, Chaires 1990) or form adducts with it (Coldwell, Cutts et al. 2008). Also, a recent study proposed a model illustrating doxorubicin docked within the major groove of the 32 bp U-helix, establishing polar contacts with G-U base pairs (Acquah and Mooers 2023). This aligns with our findings indicating that doxorubicin inhibits the active ribozyme from cleaving the substrate, as demonstrated in the polyacrylamide gel data (Figure 14.3B). Mitoxantrone, a classic DNA intercalator that also forms DNA adducts, similarly inhibited the FRET cleavage of the active ribozyme (Parker, Buley et al. 2004, Bhattacharyya, Basu et al. 2014). Although these inhibitors were not pursued in this study, some could serve as promising starting points for targeting the RNA structure to inhibit editing, as proposed in another study (Acquah and Mooers 2023).

After confirming the initial hits (cefixime, natamycin, EGCG, isoquercitrin, and hyperoside), the IC_{50} values of these compounds in the multiplex assay were determined. However, except for EGCG, which showed sub-micromolar activity, all other hits showed low to moderate micromolar efficacy. Moreover, their antiparasitic activity also fell within the moderate

micromolar range. Considering that the *in vitro* data shows that the IC_{50} of enzyme inhibition concentration is in the same range as the cell-based activity (except for EGCG), we speculate that RNA editing could potentially be the target for the compounds exhibiting antiparasitic effects, which warrants further investigation. In the case of EGCG, the enzyme inhibition occurs at a concentration lower than that required for efficacy against the parasite, suggesting either poor compound uptake by the parasite or by the mitochondrion where the editosome resides.

Additionally, we analyzed the mode of action of the identified hits against the editosome, revealing their inhibition of multiple enzymatic activities and RNA-protein interaction, as demonstrated in EMSA. This suggests that the compounds may target either the integrity of the complex or its RNA binding capability. Many previously discovered compounds showed this mode of action, including MrB, aurintricarboxylic acid (ATA), PPNDS, NF449 (Moshiri, Mehta et al. 2015), and several compounds from our recent HTS (Rostamighadi, Kamelshahroudi et al. 2024). Even though the inhibitors target non-catalytic regions of the editosome, having these inhibitors allows us the opportunity to refine them further, potentially leading to the development of more effective lead compounds. Flavonoids, in particular, have been proposed as promising candidates for further optimization in treating kinetoplastid diseases (Tasdemir, Kaiser et al. 2006, Baldim, de Alcântara et al. 2017, Boniface and Elizabeth 2019, Ebiloma, Ichoron et al. 2020, Boniface, Ferreira et al. 2023). However, it is worth noting that flavonoids are known for their promiscuous activities, yet they continue to be pursued as potential drug leads (Baell and Walters 2014, Ingólfsson, Thakur et al. 2014). For instance, EGCG was identified in three other studies screening the NIH clinical collection, reporting various activities, including the inhibition of HIV-integrase, modulation of nonviral gene delivery to adipose-derived hMSCs, and inhibition of amyloid-β (Abrahams, Mosebi et al. 2018, Kozisek, Hamann et al. 2020, Johnson, Wang et al. 2022).

Given that ligation activity was targeted by the identified hits, assessing these compounds against the recombinant protein could confirm their ability to inhibit the isolated enzyme. Among the five confirmed hits, we found that hyperoside did not inhibit the recombinant enzyme to the same extent as the editosome, unlike the other hits. This underscores a critical point that

inhibiting a specific enzymatic activity within a complex does not necessarily mean that the enzyme responsible for that activity is being targeted. Also, a compound that inhibits a recombinant enzyme may not necessarily target the same protein in a complex containing that enzyme. Moreover, increasing the ATP concentration by a thousand-fold (up to 2X drug concentration in the reaction) resulted in over a thirty percent reduction in the inhibition of flavonoids (EGCG, isoquercitrin, and hyperoside) (Figure 14.5) when assessed against the editosome, but not when evaluated against the recombinant ligase. This suggests that these flavonoids competed with ATP in the presence of the editosome. Flavonoids have been shown to inhibit kinases by competitively mimicking kinase substrates, such as triphosphate (ATP and GTP) (Srivastava 1985, Middleton, Kandaswami et al. 2000, Silva, Biluca et al. 2021).

Cefixime, another confirmed hit, maintained its inhibitory effect against both the editosome and the recombinant ligase even with increased ATP concentrations. As a cephalosporin antibiotic, cefixime can establish covalent bonds through its beta-lactam group with its intended targets (Beadle, Nicholas et al. 2001, Nicola, Tomberg et al. 2010). However, since it did not affect the viability of *T. brucei*, it likely could not reach its target inside the mitochondria. Further studies focusing on formulation or modifications to functional groups are required to enhance its cell permeability.

Natamycin has been suggested to induce mitochondrial membrane depolarization, elevate intracellular Ca^{2+} levels, and cause significant changes to the plasma membrane in *Leishmania* cells. Consequently, these disruptions lead to depletion of cellular ATP levels and the generation of reactive oxygen species (ROS), ultimately culminating in apoptosis-like and necrotic cell death (Awasthi and Mitra 2018). We propose that natamycin may also influence RNA editing within the mitochondria, where the edited products (such as those involved in the electron transport chain) are crucial for ATP generation. However, further investigation is needed to validate the editosome as the *in vivo* target of natamycin.

We also aimed to increase the pool of ligase inhibitors available for future studies. Therefore, we screened a small group of compounds, including flavonoids, suramin analogs, and nucleotide analogs. Our rationale was based on three initial hits that were flavonoids that

inhibited ligation. Additionally, another study previously identified myricetin as an inhibitor of rKREL1/KREPA2 (Zimmermann, Hall et al. 2016). These flavonoids share a common structural scaffold, comprising a 15-carbon skeleton organized into three rings (designated as A, B, and C) with different substituents attached (Jackson, Knisley et al. 2011, Dias, Pinto et al. 2021). We identified two additional flavonoid compounds (dihydrorobinetin and (+)-dihydroquercetin) that inhibited ligation. The potential mechanisms underlying the bioactivity of flavonoids in cells may involve the formation of protein-flavonoid adducts or flavonoid-triggered protein oxidation (Joyner 2021). Nucleophilic residues in proteins might form covalent bonds with flavonoid quinones, or flavonoids could oxidize specific amino acids such as cysteine, methionine, or tyrosine (Joyner 2021). Notably, even minor structural modifications can significantly affect the inhibitory activity among flavonoids (Proença, Freitas et al. 2017, Proença, Freitas et al. 2019). For instance, we found that dihydrorobinetin and (+)-dihydroquercetin inhibited ligation, whereas apigenin and naringenin did not. Dihydrorobinetin and (+)-dihydroquercetin are structurally similar dihydroflavonols, differing from the flavonoids apigenin and naringenin, which have a 3-hydroxy-4-keto function in the B-ring absent in the dihydroflavonols.

Suramin, known to inhibit editosome activities as well as rKREL1/KREPA2 (Liang and Connell 2010, Zimmermann, Hall et al. 2016, Rostamighadi, Kamelshahroudi et al. 2024), led us to explore its analogs, leading to the discovery of NF449 and NF023, which are known to inhibit editing activities (Liang and Connell 2010, Moshiri, Mehta et al. 2015). We also identified PSP0739, which shares functional groups with suramin and inhibited ligation. PSP0739 and suramin were found to inhibit the $P2Y_{12}$ receptor, which uses ADP as its primary physiological agonist (Boyer, Zohn et al. 1994, Hoffmann, Ziegler et al. 2008, Baqi, Atzler et al. 2009, Baqi and Müller 2010). These three compounds (PSP0739, NF449, and NF023) act similarly to suramin, inhibiting the recombinant ligase, rKREL1/KREPA2, and ligation by the editosome. They also inhibited the endonuclease activity by inhibiting RNA-protein interactions (EMSA data not shown).

As the ligase hydrolyzes ATP, we tested a group of nucleotide analogs and identified three additional hits that can be further utilized in SAR studies for lead compound development.

14.5 Conclusion

Employing a drug repurposing approach to identify novel uridine insertion/deletion RNA editing inhibitors, we screened the NCC library using our recently developed RNA editing insertion/deletion RIDE assay. This effort yielded five promising compounds (cefixime, natamycin, EGCG, isoquercitrin, and hyperoside), most of which exhibited significant antiparasitic activity. These compounds demonstrated inhibition across multiple enzymatic steps of editing performed by the editosome. Notably, cefixime and natamycin were found to inhibit RNA-protein interactions, as evidenced by EMSA analysis. Both cefixime and EGCG exhibited low micromolar efficacy against recombinant RNA editing ligase 1. Furthermore, we broadened the repertoire of ligase inhibitors by screening a selection of flavonoids, nucleotide analogs, and suramin analogs. Our findings highlight the discovery of novel RNA editing inhibitors that hold promise for further optimization toward lead drug development, especially considering their established safety profiles in humans. Such developments could involve structural modifications to reduce toxicity or strategies to improve target specificity, ensuring that any repurposed drug maintains efficacy while minimizing adverse effects in clinical settings.

14.6 Materials and Methods

14.6.1 RNA preparation

RNA substrates used in the multiplex assay, 5′Ins, 3′Ins, 5′Del, 3′Del2, gHHRc, and FRET substrates, were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) as previously described (Rostamighadi, Mehta et al. 2023). The gHHRc competitor, a DNA sequence, was also prepared by IDT. Fluorescent-labeled RNA sequences used in the mode of action assays (5′Del-/C3/, 5′Ins-/Cy5/, PreA6-/FAM/) were chemically synthesized and labeled by IDT. All sequences have been detailed previously (Rostamighadi, Mehta et al. 2023, Rostamighadi, Kamelshahroudi et al. 2024).

14.6.2 Compound libraries

The NIH clinical collection library (NCC), consisting of 707 compounds, was obtained from the National Center for Advancing Translational Sciences (NCATS), NIH. The compounds were dissolved in DMSO at a concentration of 10 mM, distributed across nine 96-well plates. Daughter plates were prepared with drugs at 200 µM for screening purposes. Fresh powders of the final hits were also ordered from Sigma, and the stocks were prepared.

14.6.3 Ribozyme Insertion/Deletion Editing (RIDE) assay

This assay, which encompasses both uridine insertion and deletion editing, was performed as described before with a minor modification (Rostamighadi, Mehta et al. 2023). First, appropriate RNA hybrids for deletion ([5′ Del (2 pmol), 3′ Del2 (2 pmol), gHHRc (4 pmol)] and insertion editing [5′ Ins (2 pmol), 3′ Ins (2 pmol), gHHRc (4 pmol)] for insertion editing) were annealed in separate microtubes. This was achieved by denaturing at 70 °C in a water bath for five minutes, then gradually cooling them to 27 °C at 1°C per minute. The RNA hybrids were then mixed and added to the master mix. The final reaction contained 25 mM HEPES (pH 7.9), 10 mM Mg (OAc)2, 50 mM KCl, 1 mM EDTA, 0.1 µM ATP, 5 mM CaCl2, 0.1% Triton X-100, 1 µL of the purified editosome (calmodulin eluate from KREL1-TAP tag purification (Stuart, Panigrahi et al. 2004, Rostamighadi, Mehta et al. 2023, Rostamighadi, Kamelshahroudi et al. 2024) from *T. brucei*, approximately at 40 ng/mL), 10 µM UTP and a compound of interest (DMSO, a small molecule, or positive control like suramin), in a 20 µL final volume. The assay was set up in 96-well plates, securely covered securely, and incubated for four hours at 28°C. To separate the edited ribozyme from the gRNA, 40 pmol of DNA guide competitor, fully complementary to the gRNA, was added. This mixture was then incubated at 85°C for 10 min and afterwards at 25°C for 5 min. Then 20 pmol of each of FRET1 and FRET2 substrates were added, and FAM and Cy5 signals were kinetically monitored at 37°C every minute for thirty minutes using an RT-qPCR machine.

14.6.4 Electrophoretic mobility shift assay (EMSA)

To study the effect of compounds on the RNA-protein interaction, we used a fluorescent-based gel shift assay as described before (Rostamighadi, Kamelshahroudi et al. 2024). Briely, gA6[14] gRNA labeled with 6-carboxyfluorescein by Integrated DNA Technologies (IDT, Coralville, IA) served as the probe. For each reaction, 10 pmol of RNA was preheated at 95 °C for 5 min in a dry block incubator and then cooled to room temperature (RT). The probe was then incubated with 5 µL of 10-fold concentrated KREL1 TAP-tagged editosome complex (2.5 nmol per reaction) before addition to the mixture. Each EMSA reaction contained 20 mM Tris-HCl (pH 7.9), 150 mM KCl, 5 mM MgCl2, 100 µg/ml BSA, 10 % glycerol, 1 mM DTT, 20 units of RNasin ribonuclease inhibitor (Promega, Madison, WI), 10 pmol of the RNA probe and the protein-drug mixture, totaling 20 µL in volume. The mixture was incubated at RT for 30 min to allow RNA binding by the editosome. It was then mixed 1:1 with 30% glycerol and loaded on a 4% (w/v) native polyacrylamide gel. The gel was run for 15-20 minutes at 150 V in 0.5X TBE buffer (Tris, boric acid, EDTA) at 4 °C and analyzed using ChemiDoc mp imaging system (Bio-Rad, Hercules, CA) to visualize the fluorescent-labeled RNA bands.

14.6.5 Counter-screen assay

In this assay, genuine editosome inhibitors were differentiated from compounds that interfered with the FRET assay, such as those inhibiting the ribozyme activity or hampering fluorescent signals. This assay follows the same condition as the multiplex assay, with one minor modification: each reaction contained only one pmol of active HHR (A6Rbz) and no editosome. The reaction was incubated at 27 °C for 30 min. Subsequently, 20 pmol of FRET substrate was added, and the fluorescent signal was kinetically read to assess the ribozyme's activity in the presence of compounds. In parallel, after adding FRET substrates and a further incubation at 37 °C for 30 min, the reaction was mixed with the 2X TBE-urea loading buffer, heated to 95 °C, and then loaded on a 20% polyacrylamide, 7M urea denaturing gel. The gel was run for one hour at 18 W and directly visualized using a ChemiDoc mp imaging system (Bio-Rad, Hercules, CA).

14.6.6 Viability assay

We used the Alamar blue assay to test the effects of compounds on the viability of *T. brucei* PRA-382 parasites (Lister 427 VSG 221 bloodstream-form) in a dose-response manner using 96-well plates, as previously described (Räz, Iten et al. 1997, Sykes and Avery 2009, Rostamighadi, Kamelshahroudi et al. 2024). It is important to note that this assay does not distinguish between trypanostatic and trypanocidal effects. Brielfy, parasites were seeded at a density of 2000 cells/ml in each well and incubated at 37 °C for 24 h. The next day, compounds were added to each well at their desired concentrations, with the final assay volume set at 200 µL. The plate was then incubated at 37 °C for an additional 48 h to allow the compounds to exert their effects. Subsequently, we added 20 µL of Alamar blue reagent (from Life Technologies, Carlsbad, CA) to each well, followed by further incubation at 37 °C for four hours. Fluorescence was measured at 590 nm using a BioTek Synergy H4 Hybrid Microplate Reader (Agilent Technologies, CA, USA).

14.6.7 Mode of action assays

Each enzymatic step of uridine insertion and deletion editing (endonuclease, TUTas, exoUase, and ligase) was replicated and monitored by a unique fluorescent gel-based *in vitro* assay, as previously described (Rostamighadi, Kamelshahroudi et al. 2024). The assays were adaptations of former radioactivity-based editing assays, performed similarly to the multiplex assay but with variations in the RNA substrates used, gel analysis, and some assay components (Igo, Palazzo et al. 2000, Igo, Weston et al. 2002, Wang, Salavati et al. 2002, Moshiri, Mehta et al. 2015, Rostamighadi, Mehta et al. 2023). Trimolecular RNA hybrids were prepared by heating the RNA substrates at 70 °C for 5 min and allowing them to cool to RT. The sequences used in assays were as follows: [5′ Ins-/Cy5/, 3′ Ins, gHHR] for TUTase, [5′ Del-/Cy3/, 3′ Del, gHHRc] for ExoUase, [5′ Ins-/Cy5/, 3′ Del, gHHRc] for ligase and [PreA6-/Cy5/, gA6] for endonuclease activity assays. These RNAs were then mixed with the same master mix as used in the multiplex assay, with the following modifications that 100 nM ATP and 100 µM UTP were added only to the ligase and TUTase activity assays. ATP was not added to the endonuclease, TUTase, and exoUase assays to prevent inadvertent ligation by ligase. Each assay included 50 fmol of the

KREL1 TAP-tagged purified editosome protein, except for the endonuclease assay, which used 250 fmol. After the reactions, a loading dye (7 M Urea in TBE buffer) was mixed in 1:1 ratio with the reaction mixture, then loaded onto a 20 % polyacrylamide with 7 M urea and run for 2 h at 18 W. Gel analysis was performed using the ChemiDoc mp system (Bio-rad) to detect fluorophore-labeled RNA bands.

In precleaved assays like the FRET-based ligation assay, RNA substrates [5′ Ins, 3′ Del, gHHRc] were used under the multiplex assay conditions along with the desired ATP concentrations and protein sources, including recombinant KREL1 (Moses, Mehta et al. 2023) (rKREL1), or recombinant $KREL1_{51\text{-}459}$/$KREPA2_{56\text{-}176}$ (rKREL1/KREPA2) commercially expressed in BL21(DE3) *Escherichia coli* and purified by GenScript (Piscataway, NJ, USA), or the KREL1-TAP-tagged purified editosome from *T. brucei* (Rostamighadi, Mehta et al. 2023)). Other precleaved assays, including precleaved insertion and precleaved deletion assays, followed the same format as the multiplex assay but with specific precursor RNA substrates added for insertion or deletion accordingly.

14.6.8 Molecular docking studies

To investigate the potential interactions of EGCG with KREL1, we performed blind docking studies using the full-length AlphaFold model of KREL1. Docking was carried with the AutoDock Vina package of PyRx 0.8, as described before (Dallakyan and Olson 2015, Eberhardt, Santos-Martins et al. 2021, Rathod, Shinde et al. 2023) (https://pyrx.sourceforge.io/). The protein model, obtained from the AlphaFold2 database (AF-P86927-F1), was prepared using BIOVIA Discovery Studio, uploaded in PyRx 0.8 and converted to the PDBQT format. The 3D SDF format file of the ligand, EGCG, was downloaded from PubChem, subjected to energy minimization (EM), and converted to PDBQT format using the OpenBabel plugin of PyRx(O'Boyle, Banck et al. 2011). Within AutoDock Vina, we selected both the ligand and target protein and defined a grid box to cover the entire protein with dimensions of *X*: 104.9 Å, *Y*: 126.1551 Å, and *Z*: 123.3374 Å, and center coordinates *X*: 0.4318, *Y*: 12.7782, and *Z*: 29.7122. The optimal pose, which showed the lowest binding affinity and zero RMSD, was chosen for further analysis using BIOVIA Discovery Studio. We used the docking pose of ATP

within the full-length KREL1 as a control to compare against the blind docking results for EGCG.

15. Concluding Remarks and Future Directions

Kinetoplastid pathogens cause debilitating parasitic diseases in humans and livestock. The current drugs are not suitable and have many disadvantages, highlighting the pressing need for new drug development (De Rycker, Wyllie et al. 2023).

Among many conserved pathways within these early diverged protozoans, mitochondrial RNA editing has been the topic of research for a long time (Aphasizheva, Alfonzo et al. 2020). This enigmatic post-transcriptional modification entails the insertion and/or deletion of uridine residues in mitochondrial transcripts. A large protein complex known as the RNA editing holoenzyme is responsible for mediating this pathway. This multi-protein complex is formed by an RNA-mediated association of three different complexes, namely, RECC, RESC, and REH2C. RECC performs the catalytic steps of uridine insertion/deletion, while RESC and REH2C have roles in coordinating pre- and post-editing processes and controlling editing fidelity, respectively. RECC exists in three different isoforms and contains multiple enzymes (including endonucleases, TUTase, exoUase and ligases) and structural and RNA binding factors. By using "editosome" interchangeably with RECC in this work, we focus on the central catalytic activities necessary for RNA editing, highlighting the RECC's critical role within the broader RNA editing complex.

The essentiality of all catalytic core activities of the editosome for the parasite viability and the vast drug-binding landscape of the high molecular mass editosome complex make it a prime drug target for pan-kinetoplastid drug development (Salavati, Moshiri et al. 2012). Moreover, identifying small molecules interfering with different steps of RNA editing helps us demystify the less-known dynamic assembly/disassembly of RNA editing machinery components by slowing the dynamics of protein complexes and providing snapshots of the complex's lifetime (Disney 2008). This chemical genetic approach has been successful in the case of other large protein complexes such as spliceosome and ribosome (Moazed and Noller 1989, Ermolenko, Spiegel et al. 2007, Jurica 2008, O'Brien, Matlin et al. 2008, Yusupova and Yusupov 2017). These precedents validate the approach and provide a framework for navigating the complexities inherent in targeting such large and multifaceted biological systems. The successes seen with

these other complexes suggest that, with continued refinement, similar methods could reveal novel inhibitors for the editosome.

Additionally, the pursuit of developing a drug that targets a conserved pathway to combat various kinetoplastid parasites appears promising. This approach has been explored with other targets in kinetoplasts, including the kinetochore, proteasome, and topoisomerase II (Khare, Nagle et al. 2016, Xie, Dick et al. 2019, Saldivia, Fang et al. 2020, Rao, Gould et al. 2023). However, it is important to acknowledge that developing a universally effective pan-kinetoplastid drug may not be practical or desirable (Khare, Nagle et al. 2016). Such a drug would need to achieve high concentrations in diverse tissues and subcellular compartments, given that different parasites infect various cell types and reside in distinct organs. This necessity could potentially elevate the risk of toxicity. Alternatively, it may be necessary to optimize the analogues of novel scaffolds to achieve desirable pharmacological profiles tailored to each specific kinetoplastid infection (Khare, Nagle et al. 2016). Tailoring drug analogs for specific infections would allow for more precise targeting of the parasites, reducing the risk of off-target effects and increasing the therapeutic index. This precision medicine approach is increasingly recognized as crucial, especially given the diverse pathology of kinetoplastid diseases, which can manifest in a variety of ways depending on the species and stage of infection.

Ultimately, identifying RNA editing inhibitors will serve as foundational starting points for novel drug discovery and significantly enhance our understanding of the RNA editing machinery. To achieve this goal, we developed multiple ribozyme-based precleaved *in vitro* RNA editing assays that bypassed the rate-limiting endonuclease cleavage activity (Igo, Palazzo et al. 2000, Igo, Weston et al. 2002, Carnes and Stuart 2007). These assays require much less ATP and UTP compared to the previous full-round RNA editing assay (Moshiri and Salavati 2010), facilitating the identification of competitive inhibitors, as demonstrated by ABMA as a proof of concept. This reduction in nucleotide requirements, along with bypassing endonuclease activity and optimized protein concentration not only make the assays more cost-effective but also more efficient, enabling the screening of a broader range of compounds within a shorter

timeframe. Such advancements are critical for accelerating the pace of drug discovery, particularly in HTS scenarios.

Upon optimizing assay conditions, we discovered that the delayed addition of ATP to the reaction, along with the inclusion of a cytidine residue in the gRNA sequence (Cruz-Reyes, Zhelonkina et al. 2001, Wang, Salavati et al. 2002), markedly enhances the editing efficiency. The z-factor calculated for the precleaved assays were all >0.7, demonstrating their suitability for screening a large library of compounds. We subsequently modified the pre-mRNA ribozyme sequence and introduced the RIDE assay, a sophisticated technique capable of simultaneously monitoring both uridine insertion and deletion editing within a single reaction. This advancement substantially enhances the scope of targets that can be observed and screened for potential inhibitors in a unified assay. By enabling the simultaneous observation of multiple editing events, the RIDE assay provides a more comprehensive picture of how compounds interact with the editosome. This holistic view is invaluable for identifying inhibitors that may have multifaceted effects on the editing process, thus providing a richer understanding of how to disrupt the function of this critical protein complex.

The development of these advanced assays not only allows for more detailed investigations into RNA editing but also enables the targeting of specific complexes involved in this process. By using an upgraded version of the RIDE assay that requires sequential editing, including both uridine insertions and deletions, we can target additional complexes involved in editing, such as REH2C and RESC. This approach could uncover novel therapeutic targets by revealing how these complexes contribute to the dynamics and functionality of the editosome. Disrupting the interactions between RESC and REH2C, for example, could impair the parasite's ability to produce functional mitochondrial transcripts, thus offering a new strategy for developing drugs that exploit vulnerabilities in the parasite's RNA editing machinery.

Additionally, we developed fluorescent gel-based RNA editing assays to visualize the activity of each enzyme involved in RNA editing and characterize the mode of action of RNA editing inhibitors. Although these assays were developed using TAP-tagged purified editosomes, they can be further adapted to reconstituted catalytic editosomes with recombinant proteins, as

previously demonstrated (Kang, Rogers et al. 2005, Kang, Gao et al. 2006), making them suitable for a gray box screening approach (targeting protein complexes that have been reconstituted *in vitro*) (Wong 2011, Gestwicki 2022). This adaptability is crucial because it enables researchers to identify compounds that specifically target enzymes involved in RNA editing, rather than disrupting the entire complex or binding to structural proteins. While this approach may not directly aid in understanding the dynamic assembly/disassembly of the complex, it is valuable for discovering potential therapeutic compounds that inhibit key enzymatic activities within the RNA editing machinery.

In Chapter IV, we report the first published large-scale screening of compounds conducted to identify mitochondrial RNA editing inhibitors (Rostamighadi, Kamelshahroudi et al. 2024). We first miniaturized the FRET-based full-round deletion editing assay, which had been previously developed and used in a pilot-scale screen in our lab (Moshiri and Salavati 2010, Moshiri, Mehta et al. 2015). This assay was employed as the primary screening tool. The miniaturization process not only reduced costs and improved assay efficiency but also enabled the screening of a large number of compounds, thus increasing the likelihood of identifying potent inhibitors.

The protein source used for the screen was the REL1-TAP tagged editosome, which contains fewer impurities than the glycerol gradient fractions. TAP-tagging is a fairly standard and reliable method for protein purification, enabling the isolation of protein complexes with high specificity and purity. This enhanced purity improves the chances of discovering enzyme-specific inhibitors while decreasing the likelihood of identifying non-specific inhibitors.

Regarding the composition of the purified material, the TAP tag method was specifically used to purify complexes associated with REL1, a core component of the editosome. Western blot analysis confirmed the presence of RECC subunits. Although the study primarily focused on these core components, we did not extensively test for the presence of other complexes like RESC, REH2C, or auxiliary factors. However, the use of TAP-tagged REL1 is designed to selectively purify proteins that are part of or closely associated with the editosome, minimizing the inclusion of unrelated proteins or complexes.

As for the consistency of purifications, using a standardized TAP-tag approach helps maintain consistency across different preparations, reducing variability. Any inconsistencies in purification would likely manifest as variations in assay performance or inhibitor specificity. The reliance on a TAP-tagged purification strategy provides confidence that the preparations are sufficiently pure for the intended screening assays. Therefore, we believe that the use of the REL1-TAP tagged editosome minimizes potential weaknesses in the assays related to inconsistent preparations.

Upon successful execution of the HTS, seven primary hits were found to inhibit RNA editing *in vitro* in the low micromolar range. This hit list was selected for progression based on chemical clustering, potency, and ligand efficiency. With defined clusters in place, rapid rudimentary SAR data was generated over a total of 31 analogs, defining the essential elements in the structure associated with activity against RNA editing *in vitro* along with their trypanocidal and leishmanicidal efficacy in cell-based viability assays. These SAR studies are critical, as they provide insights into which chemical modifications enhance or diminish biological activity, guiding the optimization of lead compounds into more potent and selective inhibitors.

Furthermore, secondary assays enabled us to characterize the mode of action of each inhibitor against the editosome. We identified various categories of inhibitors, encompassing enzyme-specific inhibitors, editosome inhibitors with broad-spectrum impacts, and subcomplex-specific inhibitors. Such mechanism of inhibition study is vital for understanding the breadth of the effects these compounds could have, informing decisions about which hits to prioritize for further development based on their specific mechanisms of action and potential therapeutic index. Interestingly, eight compounds demonstrated antiparasitic activity against *T. brucei* and *L. donovani* in the low micromolar range, making them excellent candidates as starting points for drug discovery campaigns.

Finally, we used a drug-centric drug repositioning approach in Chapter V to perform a pilot-scale screen using the novel RIDE assay developed in Chapter III. We tested a library of FDA-approved drugs and late-stage development candidates, resulting in the identification of ten primary hits. These included two series of compounds—anthracyclines and flavonoids—as well

as three singletons. This approach leverages existing safety and pharmacokinetic data and accelerates the drug development timeline, which is particularly advantageous in addressing urgent public health needs posed by kinetoplastid diseases.

Further analysis showed that five hits, including members of the anthracyclines family and one singleton, namely mitoxantrone, exhibited non-specific inhibition by interfering with the reporter system, either through inhibiting ribozyme activity or quenching the fluorescence. The remaining five final hits were tested in dose-response assays and exhibited sub-micromolar to moderate micromolar efficiency against editosome. The identification of these hits underscores the potential of repurposing known drugs for new therapeutic applications, providing a valuable shortcut in the drug discovery process.

MOA studies revealed that these hits (four flavonoids plus cefixime) impede multiple steps of RNA editing by targeting either the integrity of the complex or its RNA binding capability, similar to previously identified hits such as MrB, aurintricarboxylic acid (ATA), PPNDS, NF449 (Moshiri, Mehta et al. 2015), and several compounds from our recent HTS (Rostamighadi, Kamelshahroudi et al. 2024)These findings are significant as they highlight the versatility of these compounds in disrupting RNA editing at various stages. This could potentially lead to the development of combination therapies that could target multiple points in the RNA editing pathway, thereby reducing the likelihood of resistance development. EGCG, natamycin, and HO also exhibited antiparasitic capabilities in the moderate micromolar range. Considering their safety profiles, these hits can be further pursued to develop novel drugs for kinetoplastids.

Further testing of the hits against the editosome and recombinant ligase showed that only EGCG effectively inhibits the editosome at single digit micromolar concentrations. This indicates that EGCG can bind the ligase within the context of the editosome. Docking studies further supported this by showing that EGCG shares similar binding characteristics with ATP in REL1’s catalytic site. While EGCG's ability to inhibit ligase activity suggests its potential as a specific inhibitor of the ligation process in RNA editing, a general disadvantage of using flavonoids like EGCG as lead compounds is their poor bioavailability and rapid metabolism, which can limit their

effectiveness in vivo. These issues often necessitate structural modifications or the development of delivery systems to improve their pharmacokinetic properties.

Overall, we have identified novel RNA editing inhibitors through pilot (chapter V) and large-scale (chapter IV) screening, which have shown various modes of action against editosome and parasiticidal effects. Future studies will need to prioritize these hits by examining their selectivity against closely related enzymes within the same family or those sharing similar substrates and binding domains. However, this factor must be balanced with other properties, such as physicochemical and ADME properties, when determining which compounds to advance. Balancing these factors is critical for ensuring that the lead compounds exhibit potent antiparasitic activity and possess favorable pharmacokinetic and toxicity profiles, which are essential for successful drug development.

The compounds' effects on commercially available orthologous editosome-related proteins, including recombinant T4 RNA ligase, *E. coli* RNase III endonuclease, 3′-5′ exonuclease, and terminal nucleotidyl transferase, can be assessed. The most effective ligase inhibitors can also be tested against human DNA ligase. This exercise will unveil both the merits and shortcomings of each series, enabling an informed decision regarding the most promising group of compounds to advance. Such comparative studies are essential for understanding off-target effects and optimizing selectivity, which is critical for minimizing potential side effects and enhancing the therapeutic efficacy of the inhibitors.

Furthermore, exploring RNA editing inhibitors also provides a valuable opportunity to enhance our understanding of the fundamental biology of kinetoplastid parasites. Studying how these inhibitors affect the parasite at various stages of its life cycle can reveal insights into the role of mitochondrial function in parasite survival and the adaptation mechanisms employed by these organisms to thrive in different environments. These insights could open new avenues for therapeutic intervention beyond the editosome, potentially leading to the discovery of novel drug targets.

A limitation of the current study is the relatively moderate potency of the identified hit compounds, with activities observed in the micromolar range. While inhibiting at these levels and mode of action studies might indicate the compounds' potential to disrupt the editosome's integrity, they fall short compared to more established active pharmaceuticals, which often operate in the low nM range against *in vitro* targets. It also suggests that while the identified compounds may act as disrupters, they might not bind with high specificity or affinity to discrete pockets within the protein structure of the editosome.

One of the possibilities for how these compounds exert their effects is through the inhibition of protein-protein interactions within the editosome. Inhibiting protein-protein interactions can offer advantages, such as targeting the interface regions of proteins that are crucial for complex formation, potentially leading to a more significant disruption of protein function. However, there are also disadvantages, including the often large and flat interaction surfaces of protein-protein interfaces, which make it challenging to achieve high specificity and potency with small molecules.

Therefore, future research should focus on enhancing the affinity and specificity of these compounds. This can be achieved through structure-based drug design, leveraging detailed structural information of the editosome and its interaction with small molecules to optimize these hits into more potent and selective inhibitors. By targeting specific protein-protein interaction sites or other critical functional domains within the editosome, it may be possible to develop highly effective and selective compounds.

Further optimization of the hit compounds can also involve medicinal chemistry efforts to improve their drug-like properties. By systematically modifying the chemical structure of these compounds, it is possible to enhance their binding affinity, improve their metabolic stability, and reduce off-target effects. Additionally, utilizing pharmacophore modeling to enhance the identified RNA editing inhibitors could yield more promising candidates with higher specificity and potency.

Furthermore, the potential for drug resistance development cannot be overlooked. Given the adaptability of parasitic organisms and their ability to evolve resistance mechanisms rapidly, it is crucial to consider strategies that can mitigate this risk. One such strategy could be the development of combination therapies, where multiple compounds targeting different stages of the RNA editing process are used together. This approach could reduce the likelihood of resistance development by creating multiple barriers to parasite survival.

Another important milestone in developing a new drug is determining if the compound reaches the intended target *in vivo*. Observing "specific cellular reactions" when a drug is administered is insufficient unless there is a concrete mechanistic proof of the drug's interaction with its intended target (Stefaniak and Huber 2020). As previously detailed (Gazestani, Nikpour et al. 2016, Carnes, McDermott et al. 2017, Kirby and Koslowsky 2020), RNA sequencing and qPCR, can be used to detect RNA editing *in vivo* following the treatment of cells with the hits. RNAi investigations have indicated that the loss of RNA editing significantly impacts cell viability approximately three days after RNAi induction (Schnaufer, Panigrahi et al. 2001, Carnes, Trotter et al. 2005). For the hits found from the HTS, we conducted a time-to-kill assay for each compound and determined the time it takes to eliminate all the parasites in the culture media (Rostamighadi, Kamelshahroudi et al. 2024). Consequently, we propose extracting RNA just before cell death and then sequencing the RNA and/or using it in a qPCR assay to assess the extent of editing in specific mitochondrial gene transcripts. This approach will confirm that the compound's target is indeed the editosome. Establishing such mechanistic proof of target engagement is critical, as it validates the intended mode of action and provides a robust framework for understanding how these compounds can be optimized for greater specificity and efficacy.

Alternatively, Cellular Thermal Shift Assay (CETSA®) can be used to quantify the engagement of compounds with their intended targets either within living cells or in cell lysates (Sanchez, Ronzetti et al. 2022). A compound meeting basic criteria at this stage would be escalated into further optimization processes. CETSA® provides a direct and quantitative measure of target engagement, offering a valuable tool for confirming that the compounds are interacting with their

intended targets in a physiologically relevant context. If compounds do not affect editing despite affecting cell viability, the targets of the potent compounds will be deconvoluted. Various methods have been employed for studying target engagement in trypanosomes, including overexpression libraries, metabolomics approaches, thermal proteome profiling (TPP), and RNAi library (Begolo, Erben et al. 2014, Creek and Barrett 2014, Collett, Kitson et al. 2019, Corpas-Lopez and Wyllie 2021). These complementary approaches can provide a comprehensive understanding of the molecular interactions and pathways influenced by the hits, thus enabling the identification of potential off-target effects and informing the rational design of more selective and potent drug candidates. Target engagement studies can greatly increase the chances of a lead compound's success as it advances through the drug discovery pipeline.

In conclusion, the comprehensive exploration of mitochondrial RNA editing as a drug target has provided valuable insights into the development of novel therapeutics. We have identified several promising lead compounds by leveraging advanced assays and high-throughput screening. Moving forward, detailed SAR studies, target engagement assays, and optimization of physicochemical properties will be crucial steps in transforming these hits into effective and safe drugs. The journey from hit identification to drug development is complex and multifaceted, requiring an integrated approach that combines molecular biology, chemistry, and pharmacology. However, the potential benefits of developing effective treatments for kinetoplastid diseases make this endeavor both worthwhile and urgent, promising to significantly impact global health by addressing the unmet medical needs of millions of affected individuals.

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