Identification of Compounds inhibiting a Leishmania RNA Editing Reaction

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Abstract

Several species of Leishmania are human pathogens that afflict more than 12 million people worldwide, and the current treatment options are limited. An RNA editing reaction that is both essential and specific to the parasites is an attractive target for new drug development. The editing reaction involves the post-transcriptional modification of specific mitochondrial mRNAs through the precise deletion or insertion of uridylates. Many aspects of the editing mechanism are still unclear, and the lack of specific inhibitors to probe the reaction has hindered the field. Although high-throughput screening of chemical libraries is a powerful strategy often used to identify inhibitors, the available in vitro editing assays do not have the necessary sensitivity and format for this approach to be feasible.

A novel editing assay was developed in this thesis that overcame previous limitations as it can both detect edited product in the low femtomole range and is ideal for high-throughput format. The reporter for the assay consists of an RNA editing substrate linked to a streptavidin-binding domain that is initially held within an inactive conformation. An in vitro selection strategy optimized the linkage so that the streptavidin-binding domain is only activated by an editing-induced conformational change. The reporter RNA is labeled with a ruthenium complex, and an electrochemiluminescent signal results from the ruthenium label when the reporter is bound to the bottom of a streptavidin-coated microtiter plate where it can be stimulated by a carbon electrode. Chemical probing, mutagenesis and binding affinity measurements were used to characterize the reporter. This highly sensitive assay was optimized and validated for use in high-throughput screening, and a pilot screen of a 1280 compound library identified compounds that are the first specific inhibitors of the editing reaction. Some of the identified inhibitors will have value as probes of the editing reaction and have already provided insights into possible regulatory mechanisms. The identification of novel drugs through screens of large chemical libraries is now possible with the new assay.

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Abbreviation

HTS: high-throughput screening

ECL: electrochemiluminescent

Chapter 1
Introduction

1. Leishmaniasis

Leishmaniasis is an ancient disease caused by several different species within the Leishmania genera of the kinetoplastid protozoa. The disease is a severe public health burden. It is most predominant in the tropical and subtropical regions of the world. Eighty-eight countries are affected, of which 72 are developing countries and 13 are among the least developed [1]. It is estimated that 12 million people are suffering from leishmaniasis and the population at risk is about 350 million worldwide [2]. The annual incidence is approximately 2 million [1], with 2.4 million disability adjusted life years lost and 59,000 deaths [3].

Leishmaniasis consists of three major clinical syndromes: cutaneous leishmaniasis, muco-cutaneous leishmaniasis and visceral leishmaniasis. In cutaneous leishmaniasis, patients present with skin ulcers or nodules on the exposed parts of the body, such as the face, arms and legs. Patients can have up to 200 skin lesions, which leave permanent disfiguring scars. Lesions can also cause great pain and result in serious disability. In the muco-cutaneous forms of leishmaniasis, patients suffer from progressively destructive ulcerations of the mucous membranes of the nose, mouth, throat cavities and surrounding tissues; these disabling lesions are not self-healing. In visceral leishmaniasis, patients present with irregular bouts of fever, fatigue, weakness, loss of appetite, substantial weight loss, anemia, swelling of the spleen and liver. Visceral leishmaniasis is fatal if left untreated, mainly as a result of damage to the spleen and liver.

Over 20 leishmania species have been reported to be responsible for human infections. Major causes of visceral leishmaniasis are *L. chagasi* in Latin America, *L. donovani* in Africa, India and Asia, and *L. infantum* in the Mediterranean. *L. major, L. tropica, L. aethiopica, L. mexicana, L. braziliensis* and related species typically are causative pathogens for cutaneous leishmaniasis. *L. braziliensis* is responsible for

mucocutaneous leishmaniasis. Most species also infect animals such as canines and rodents, which serve as reservoirs and present additional obstacles for disease control [4, 5]. In addition to the Leishmania parasites, several other species of the kinetoplastid order are also causative agents of devastating human diseases, including *Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense* for human African trypanosomiasis (HAT) and *Trypanosoma cruzi* for Chagas' disease.

Leishmaniasis is transmitted by female sand flies (phlobotomine), and the Leishmania pathogens have digenic life cycles in which the parasites exist in two major forms: amastigotes in mammals and promastigotes in sand flies. A female sand fly ingests the amastigote-infected macrophages when it takes a blood meal from an infected mammal. The parasites transform into the promastigote form in the gut of the insect and multiply. After approximately one week, infectious promastigotes migrate to the proboscis and are injected into the skin when the infected sand fly takes another blood meal from a mammal. Promastigotes then attach to one or more macrophage receptors and are subsequently phagocytosed by the macrophage, where they are enveloped in a vacuole that fuses with lysosomes. Promastigotes transform into amastigotes, multiply and eventually break out of the vacuole to infect other macrophages [5, 6].

2. Current treatment and prevention

Although leishmaniasis is an ancient disease, effective treatments are still limited.

Pentavalent antimonial compounds (notably sodium stibogluconate and meglumine antimoniate) have been used as the first-line treatment for leishmaniasis in many areas for more than 70 years. These compounds induce several adverse effects including headache, myalgia, nausea, transaminase elevation and sometimes life-threatening side effects such as cardiac arrhythmia and acute pancreatitis. Also because of the prolonged usage, drug resistance has emerged dramatically in some areas. Up to 65% treatment failure has been reported in Bihar, India. This makes antimonial drugs

almost useless in drug-resistant regions, which are usually also the most leishmaniasis-predominant ones [7].

Conventional amphotericin B has replaced antimonial drugs as the first-line medicine in areas where the antimonial drugs have poor response. However, the drug has several adverse effects including fever, chills, malaise, nausea, vomiting and life-threatening hypokalemia, nephrotoxicity and first-dose anaphylaxis [8]. A new liposomal form of amphotericin B exhibits much less toxicity and is widely used in Europe and the United States. However, the cost is not affordable for the majority of leishmaniasis patients [9].

Miltefosine was initially developed as an anti-cancer drug, but shows great potential for treating leishmaniasis. It is also the only anti-leishmaniasis agent that can be administered orally, which is a large advantage for patients in poor rural areas [10, 11]. Miltefosine was first registered in India in 2002, and it has since been used throughout India and in several other countries; studies indicate that it is relatively well tolerated and has good efficacy [12, 13]. However, the current cost of this drug is too high for most leishmaniasis patients [14-16]. In addition, the drug is teratogenic, thus women of child-bearing potential have to use effective contraception during and up to 3 months after treatment. Another concern is that because miltefosine requires a long treatment course (28 days) and has a long half-life (~150 hours), parasite resistance is likely to be induced [17]. Sub-optimal compliance of the patient could lead to widespread resistance. As a result, direct monitoring of patient compliance is required. The above complications further increase the total cost of miltefosine therapy and reduce its convenience [15, 18, 19].

An intramuscular form of paromomycin may be the most promising anti-leishmaniasis drug so far, as indicated by recent studies [20-22]. Paromomycin, also known as aminosidine, is an aminoglycoside antibiotic that has been used for the parental treatment of bacterial infections worldwide since 1959. Because it is poorly absorbed

from the gut, oral paromomycin is recommended for clinical treatment of intestinal amebiasis and tapeworm infections. It has also been used as topical treatment for cutaneous leishmaniasis. Recent studies suggested that intramuscular injection of paromomycin could have good activity for the treatment of visceral leishmaniasis [20]. Large scaled Phase III trials in Indian showed well tolerance and great efficacy of this agent [21, 22]. In East Africa, paromomycin is under evaluation in monotherapy and combination therapy with traditional first line drugs, pentavalent antimonials, and the results are encouraging [23]. In addition, paramomycin also has the great advantage of low cost at US$7.5 per patient [16].

Besides these major anti-leishmaniasis medicines, several new synthesized compounds or microorganism and plant derivatives are currently being studied for their effectiveness or safety (reviewed in [24] and [8]). However, since there are still millions of suffering patients, the lack of effective, low-toxic and affordable drugs still points out a definite need for new drug development.

3. An RNA editing reaction

Many mitochondria mRNAs of kinetoplastid protozoa, including the causes of human leishmaniasis, African trypanosomiasis and Chagas' disease, undergo a unique post-transcription modification reaction named RNA editing. It was first discovered in 1986 [25] that the mRNA sequence for cytochrome oxidase II contained several uridylates that were not encoded by the corresponding DNA sequence. Later, this phenomenon was also found in many other kinetoplastid mitochondrial mRNAs (reviewed in refs [26-28]). In the editing reaction, uridylates are inserted into or deleted from nascent mRNA transcripts at specific positions in a precise number (Fig. 1.1). Some transcripts are edited by the addition of a few Us in a single region, others by the addition of hundreds and removal of tens of Us throughout the molecule. It was also found that alternative editing may occur to some mRNAs [29-31]. Although the rationale of the U-insertional/ deletional editing reaction is not fully understood, it is required to form

functional open reading frames. In leishmania species, 12 of the 19 mitochondria mRNAs are edited.

The specificity of editing is determined by a group of small RNAs termed guide RNAs (gRNAs). A gRNA pairs with its cognate pre-mRNA immediately downstream of an editing site (Fig. 1.2). Unpaired purine nucleotides on the gRNA act as guiding nucleotides for the incorporation of uridylates, and unpaired pre-mRNA uridylates define sites of deletion [32]. The gRNA also contains an oligo U tail, which was proposed to stabilize the gRNA/mRNA hybrid and facilitate the recognition of pre-edited mRNA by the multi-protein complex that catalyzes the editing reaction [33].

Several different enzyme activities are required to catalyze the editing reactions (Fig. 1.2). An endonuclease first cleaves the pre-mRNA at the editing site producing a 5' fragment with a 3' hyroxyl and a 3' fragment with a 5' monophosphate (Fig. 1.2, Step 1). U-additions and U-deletions are catalyzed by a terminal uridylyltransferase (TUTase) activity (Fig. 1.2, step 2) or an exonuclease activity respectively. The 5' and 3' fragments are then religated through ligase activity resulting in the completion of one round of the editing reaction, which extends the complimentary region between the gRNA and mRNA by at least one base pair (Fig. 1.2, step 3). A new round of editing may be reiterated at subsequent sites.

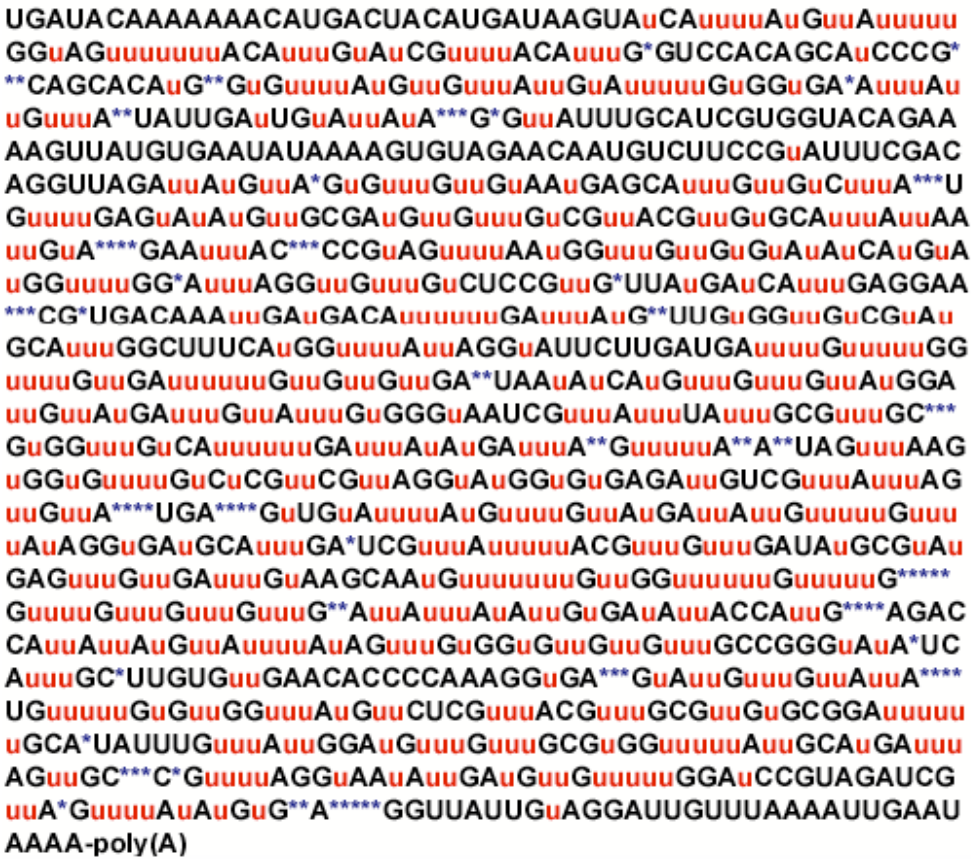

Figure 1.1. RNA editing within kinetoplasitd parasites. The sequence of edited ND7 mRNA from *Trypanosoma. brucei* [31]. Red lower-case Us are not encoded in the genome but inserted into the transcript during the editing reaction. Blue asterisks indicate encoded Us that are deleted from the transcript during editing.

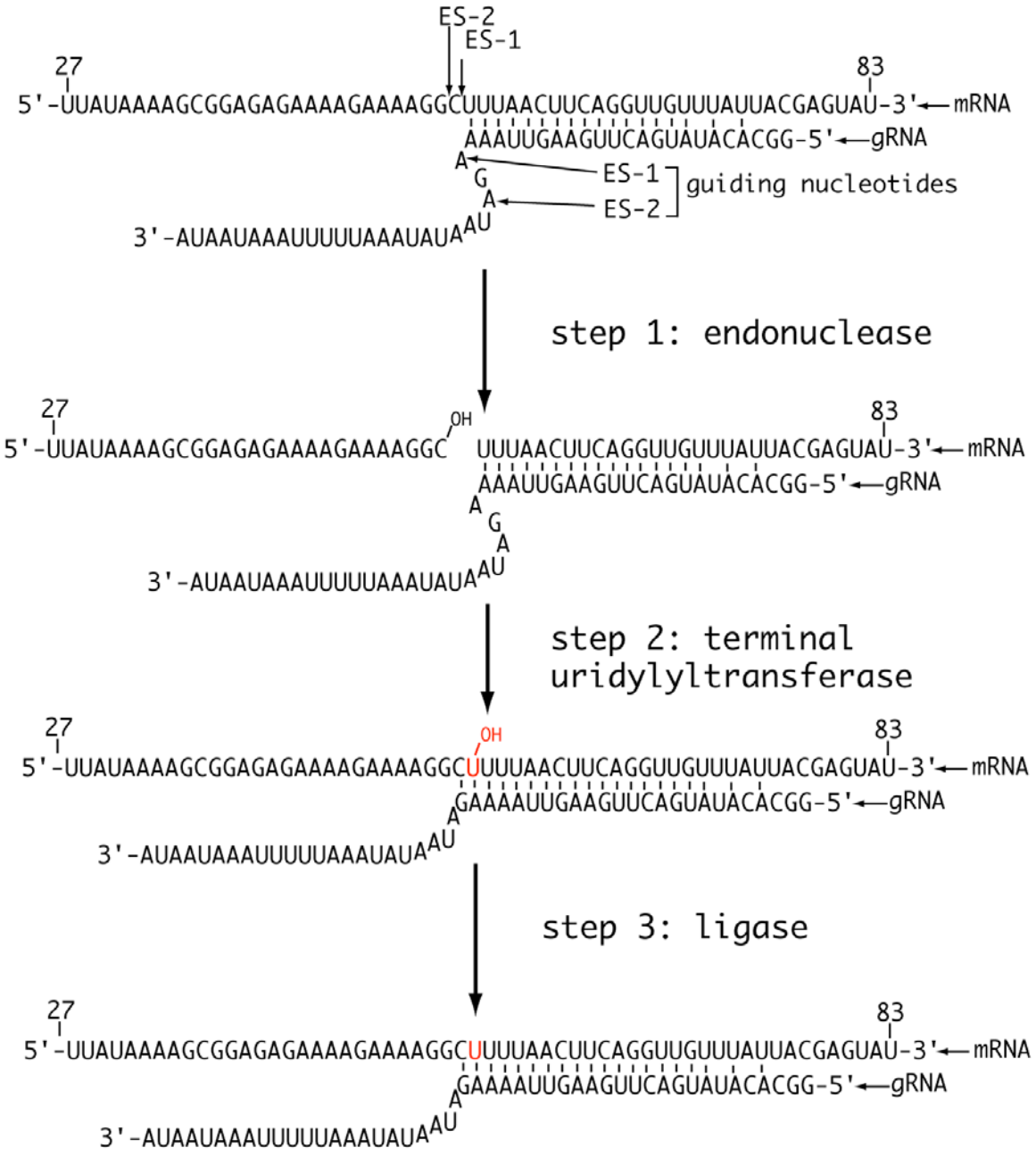

Figure 1.2. Major steps of one complete cycle of the insertional editing reaction. A gRNA forms base pairs with a pre-mRNA downstream of the editing site. Unpaired gRNA purines direct the U insertions. The pre-mRNA undergoes endonuclease cleavage (Step 1), U addition by terminal uridylyltransferase (Step 2) and religation by ligase (Step

3) to complete one editing cycle. Sequences are from parts of the *Leishmania tarentolae* cytochrome b mRNA and gRNA [34].

The enzymatic cleavage-ligation mechanism of U-insertion/deletion editing was first proposed as early as 1990 [32] (Fig. 1.2); however, it took several years to experimentally support this model and to disprove an alternative transesterification model [35, 36] (Fig. 1.3). The transesterification model was based on the detection of chimeras in which gRNAs are covalently linked through their U tails to 3' fragments of pre-edited mRNAs. It was proposed that the 3' terminal U of the gRNA reacts with the mRNA at the editing site through a transesterification reaction, and a gRNA-mRNA chimera linked at the editing site would be formed as an intermediate of the reaction. A second transesterification reaction was proposed to occur with the 3' hydroxyl group of the 5' mRNA fragment attacking the phosphodiester bond of the gRNA-mRNA chimera at the editing site. As a result, one or more Us would be either transferred from the oligo U tail of gRNA to the mRNA in the insertional editing, or from the mRNA to the oligo U tail in the deletional editing [35, 36]. The development of several *in vitro* editing systems that were able to exam intermediate steps of the editing process made it possible to test the enzyme-cascade and transesterification models [37-41]. The time course of the reaction indicated that the chimeras formed after correctly edited product and accumulated over time. This indicated the chimeras are more consistent with being dead-end side-product rather than reaction intermediates [37, 39]. It was also shown that neither the oligo U tail nor the 3' hydroxyl group of the gRNA is required for the editing reaction, which is inconsistent with the transesterification model [37, 41, 42].

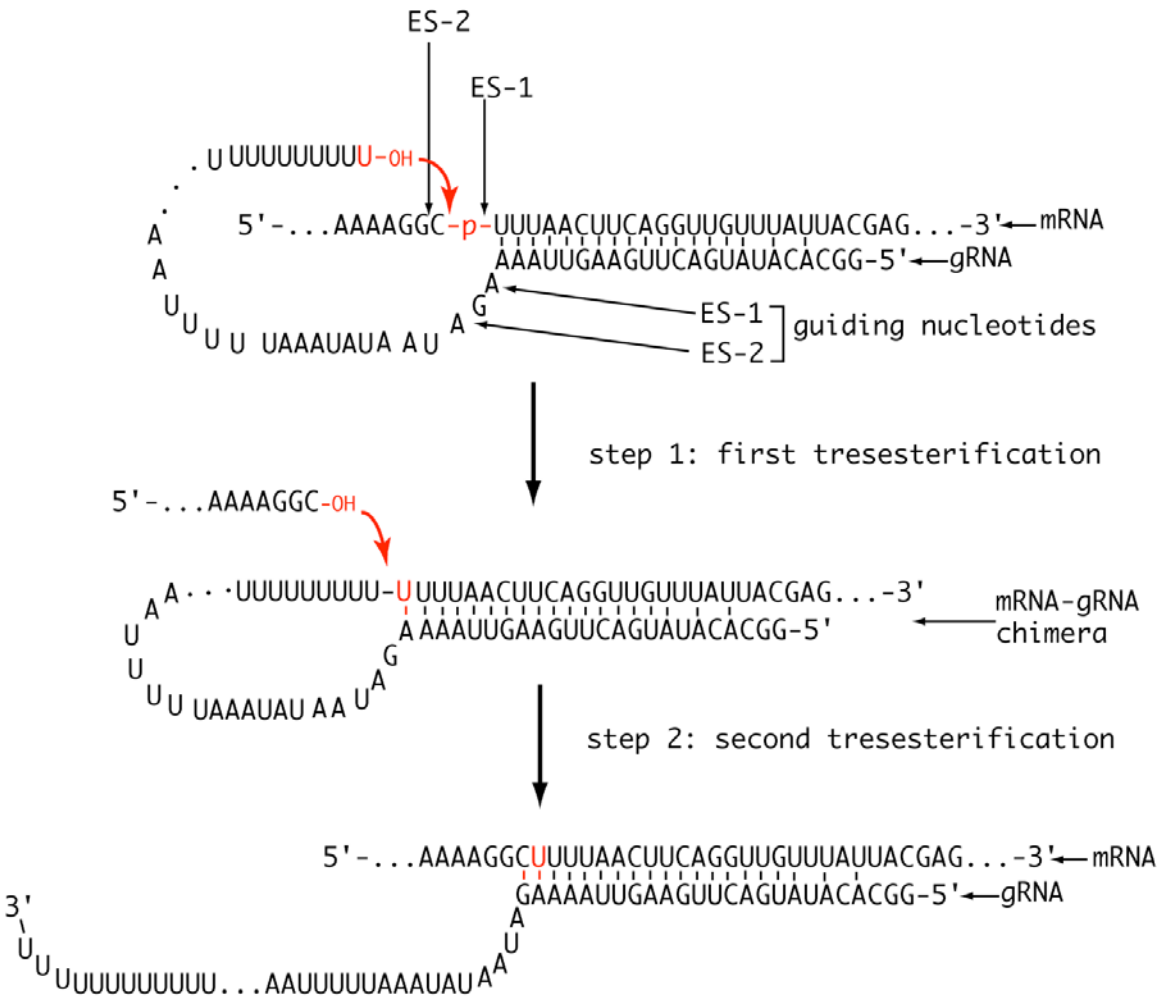

Figure 1.3. The alternative transesterification model. A gRNA-mRNA chimera results from the 3' terminal U of the gRNA reacting with the mRNA at the editing site through a transesterification reaction. A second transesterification reaction involves the 3' hydroxyl group of the 5' mRNA fragment attacking the phosphodiester bond of the gRNA-mRNA chimera at the editing site. In this model, the inserted Us would arise from the U tail of the gRNA.

The editing reaction requires the editing complex to recognize and act at specific sites, which is important to the parasites because editing at incorrect sites could disrupt open reading frames and be lethal. The mechanism of editing specificity is not fully understood. The base-pairing between gRNA and mRNA immediately downstream of editing sites partly contributes to the editing specificity. Other sequence and structural features, however, must also play a role in addition to gRNA-mRNA base-pairing since other double strand RNA structures, such as tRNAs, are not recognized as editing substrates. Besides downstream base-paring and guiding purines at the editing sites, a weak upstream helix and a bias in sequences flanking editing sites are also observed [43-46]. It is possible, nevertheless, that other essential features of editing substrate have not been identified.

4. Editing complexes

The major enzymatic activities outlined in Fig. 1.2 exist as part of multi-protein complexes that sediment at ~ 20S in glycerol gradients. gRNAs, however, do not co-purify with these complexes, but associate with larger (~40S) less defined complexes which also contain other proteins or complexes. The significance of the 40S complexes, however, is still unclear. Although not all components of the editing complexes have been fully characterized, many have been purified from *Trypanosoma brucei* and *Leishmania tarentolae* by various methods. The orthologs of these proteins have been identified in *Leishmania major* and *Trypanosoma cruzi* [47], for which the genomes have been sequenced. As many of the editing proteins were identified independently by several different groups, the nomenclature has been confusing. Most labs, though, have agreed on the naming of the proteins with defined functions.

Most of the editing complex proteins exist in pairs or sets with motif/sequence similarities [48], and the proteins that have been characterized are summarized below:

Three **endonucleases** KREN1, KREN2 and KREN3 have been identified, and all are essential for the parasite's survival [49-52]. While KREN1 cleaves pre-edited mRNA at deletion sites, KREN2 cleaves pre-mRNA at insertion sites but not those of COII pre-mRNA. In contrast, KREN3 specifically cleaves pre-COII at insertion sites. COII is the only mRNA that contains a gRNA sequence in cis, whereas all other pre-mRNAs use trans-acting gRNAs. However, editing recognition by KREN3 does not depend on structural features resulting from a cis guiding sequence, as KREN3 can cleave an artificial COII substrate with a trans-guide sequence. It is unclear either how KREN1 and KREN2 discriminate different editing sites.

KREX1 is a U-specific **exonuclease (exoUase)** that removes unpaired Us at deletion sites *in vitro*. It was found that the down-regulation of KREX1 in T. brucei affected cell growth and the stability of the 20S editing complex [53]. However, a recent study showed that the repression of KREX1 did not affect the sedimentation of the 20S editing complex but did caused KREN1 loss from the complex [54]. The reason for the difference in the effects of KREX1 loss on the 20S complex is unclear. Another protein KREX2 is related to KREX1. KREX2 of T. brucei exhibits 3'-5' exonuclease activity, but its down-regulation has little effect on parasite viability and complex stability. The Leishmania KREX2 does not have the exonuclease motif [55]. Although not essential, T. brucei KREX2 may still play a role in RNA editing by interacting with other components [54].

The **terminal uridylyl transferase (TUTase)** KRET2 is an integral 20S editing complex protein that adds Us at insertion sites [56, 57]. KRET1 is also a TUTase but is not a component of the 20S complex. Instead, it appears to indirectly associate with the 20S complex through interactions with RNA. KRET1 adds U's to the 3' end of the gRNA. Down-regulation of either TUTases leads to a decrease of edited mRNA *in vivo* [57, 58].

Two **editing ligases** KREL1 and KREL2 catalyze the *in vitro* rejoining of the mRNA fragments after U addition or removal [59-61]. However, KREL1 but not KREL2 is essential for *in vivo* editing and cell survival, which suggests that KREL1 is able to compensate for KREL2 *in vivo* but KREL2 cannot compensate for KREL1.

Other proteins, including KREPA1-4, KREPA6, KREPB4 and KREPB5, are important for maintaining the integrality of the editing complex, as they interact with other components of the complex.

All proteins discussed above except KRET1 are components of the 20S editing complexes. Three compositionally and functionally distinct 20S editing complexes have been identified in T brucei [50, 62]. Each complex is defined by a unique endonuclease (KREN1, KREN2 or KREN3) and a few associated proteins that are mutually exclusive among the complexes. The three complexes share a common set of proteins, including two heterotrimer sub-complexes. One heterotrimer sub-complex consists of KRET2/KREPA1/KREL2 and catalyzes *in vitro* U deletion/ligation with a pre-cleaved substrate RNA. The other common sub-complex contains KREX2/KREPA2/KREL1 and catalyzes *in vitro* U insertion/ligation with a pre-cleaved substrate [63]. Thus all three 20S complexes can catalyze the U addition, U removal and ligation steps of editing. However, as expected from the specificity of the endonucleases, the KREN1-containing complex only cleaves pre-edited mRNA at deletion sites but not insertion sites, the KREN2 complex only cleaves at insertion but not deletion sites [62], and the KREN3 complex is specific for COII pre-edited mRNA cleavage [50].

The organization of the proteins within the editing complexes is not well understood, but various experimental methods have provided some insight [62, 64, 65]. A recent study using electron tomography and single-partial reconstruction revealed a slender triangle model of the 20S core complex with about 30A resolution, and the REL1 ligase was localized on this model by labeling with REL1 –specific IgG [66]. Although the resolution of the structure needs to be improved, the editing complex is likely to be

dynamic and have several alternative organizations. This is because 1) the core editing complex has to interact with gRNA, mRNA and other regulatory proteins and complexes; 2) several different gRNAs have to anneal and dissociate from some mRNAs during editing, and 3) the U insertion and deletion activities are catalyzed by distinct 20S complexes. Dynamic structures are also commonly observed in other RNA processing complexes.

5. Complexes that interact with the 20S editing complex

While the 20S editing complexes directly catalyze editing and are able to perform the complete editing reaction *in vitro*, other proteins and complexes have been identified for roles that are associated with editing. The **KRET1 complex** catalyzes the addition of the 3' oligo(U) tails to gRNAs. In L. tarentolae, most KRET1 is present in a complex of ~500kDa containing three or four KRET1 molecules, and recombinant KRET1 forms active oligomers *in vitro*. The KRET1 complex can associate with the 20S editing complex via an RNase-sensitive link [58].

The **MRP complex** contains two related proteins MRP1 and MRP2, which form a heterotetramer. It has been shown that MRP1 binds to RNA nonspecifically but catalyzes complementary RNA annealing *in vitro* [67]. In addition, immunoprecipitates of MRP1 and MRP2 contain gRNAs [68, 69]. The above results are suggestive that the MRP complex may facilitate base-pairing between gRNAs and their cognate pre-mRNAs.

The **kinetoplastid poly(A) polymerase (KPAP) complex** adds short (~20-25 nt) poly A tails to the mitochondria mRNAs. It has been suggested that the short A-tail decreases the stability of the pre-edited RNA, however, it is converted by the editing reaction into a signal that is required and sufficient for stabilizing the partially and fully edited RNAs [70-72]. Upon completion of the editing process, the short A-tail is extended as an (A/U) heteropolymer tail. In addition, KPAP complex was also found to

interact with the 20S editing complex, and the interaction was not affected by RNase [72]. Above results indicate that KAPA complex is associated both compositionally and functionally with the editing process.

The GRBC (gRNA binding complex) contains a core heterotetramer of two related proteins GRBC1 and GRBC2. This complex was found to directly bind gRNAs and interact with RET1, MRP1/2, the KPAP complex and 20S core complex. The deletion of GRBC1 or GRBC2 resulted in loss of gRNAs and the inhibition of editing [73].

The protein **MERS1** also associates with GRBC. Knock-down of MERS1 led to a decrease of edited mRNA, however, the level of pre-mRNAs was not significantly changed. The pre-edited mRNAs are relatively stable in mitochondria and typically accumulate if not processed by editing. Therefore it was proposed that rather than affecting the editing process, MERS1 plays a role in the stability of the edited mRNA [73].

6. Identification of drugs that target the editing reaction

Numerous experiments have shown that either the knock-out or knock-down of the genes encoding the editing proteins affects cell survival of the blood-stream form of the parasites [49-52, 61, 74-77]. In addition, the U insertional/deletional editing reaction is unique to the kinetoplastid species, and there are not any human proteins with significant homology to the core editing complex proteins. As a result, the editing complex is an attractive target for drug development to treat kinetoplastid diseases. As already indicated, these diseases include leishmaniasis, human African trypanosomiasis and Chagas disease; they afflict millions of people in the tropic and subtropic developing regions and have been long neglected.

Despite the advance in knowledge of the editing reaction and editing complexes, no compound has been identified that specifically inhibits the editing reaction. An in silico screening strategy was previously used to identify novel drug-like compounds that can be docked to the known crystal structure of KREL1 [78]. Although this approach is promising, it is limited by differences in structure or drug accessibility that result from the study of individual proteins outside the context of the intact multi-protein complexes. The approach also does not fully exploit the large drug-binding landscape that would potentially be presented by the intact complexes.

High-throughput screening (HTS) of chemical libraries is an alternative strategy to identify novel drugs inhibiting the editing reaction, but it also has limitations. These are primarily related to the *in vitro* editing assays not having the sensitivity and format necessary for HTS to be practical and economically feasible [37, 39, 41, 46, 79]. All current *in vitro* editing assays are gel-electrophoresis based, and most *in vitro* substrates are edited with poor efficiency. Therefore, these assays are not applicable for HTS.

7. A previously identified editing substrate

An editing substrate RNA with features favoring the insertional editing reaction has earlier been described [46] (Fig. 1.4). The substrate contains a gRNA sequence (red) in cis, which directs two uridylate insertions at the editing site. It becomes edited *in vitro* at least 10^3 times more efficiently than any natural editing substrates, and the two helixes flanking the editing site can greatly affect the editing efficiency. The substrate RNA is required to be circularized prior to the editing assay so that higher mobility products will not result from the addition of Us to the 3' end by a terminal uridyyl transferase (TUTase) activity that is present within the editing extract [46, 80].

The editing substrate RNA can be used to monitor the efficiency of an editing reaction. Because two Us are inserted at the editing site during editing, the edited RNA

will be two nucleotides larger than the pre-edited form. The edited and pre-edited RNAs can be resolved by gel electrophoresis, and the ratio between the two forms can be used to quantify the editing efficiency. However, this assay can only monitor limited amount of reaction because of the high labor intensity that is involved, thus it still does not meet the high-throughput requirement.

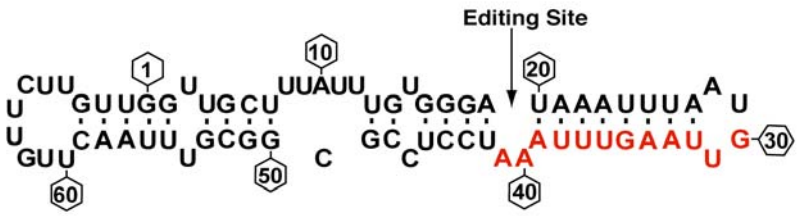

Figure 1.4. An *in vitro* editing substrate. The sequence highlighted in red is the cis–acting gRNA. The two guiding As opposite the editing site direct two uridylate insertions at the editing site.

8. Aptamers and *in vitro* selection

The editing substrate described above was identified through an *in vitro* selection-amplification strategy, which is also referred to as SELEX (Systematic Evolution of Ligands by Exponential Enrichment) [81, 82]. SELEX utilizes the same principle as natural evolution: heritable phenotypes that can best survive a particular selective pressure are enriched from a large population of sequence variants. The great advantage of the *in vitro* selection approach is that large numbers of sequence variants ($\approx 10^{15}$) can be explored in order to obtain a DNA or RNA molecule with specific characteristics. Although current knowledge has advanced in predicting DNA/RNA structures, it is still difficult to rationally design nucleic acid sequences that interact with specific targets. Mutagenesis is one means to investigate sequence variability when only a few nucleotides are involved. However, this approach would not be feasible when larger numbers of sequence variants need to be explored.

The SELEX procedure involves several cycles of enrichment and amplification of a molecule with the desired properties from a large population of sequence variants (Fig. 1.5). A population of DNA or RNA containing large tracts of random sequence is initially generated. Molecules within this population that meet the selection criterion are then partitioned from the bulk population by various methods; affinity chromatography is a common approach to enrich for various properties but many other strategies have also been exploited. Because of the large number of variants in the starting population and the selection efficiency usually being less than 100%, the partitioned DNA/RNA has to be amplified for subsequent enrichment. Multiple cycles of selection and amplification can result in a population of molecules that meet the intended criteria.

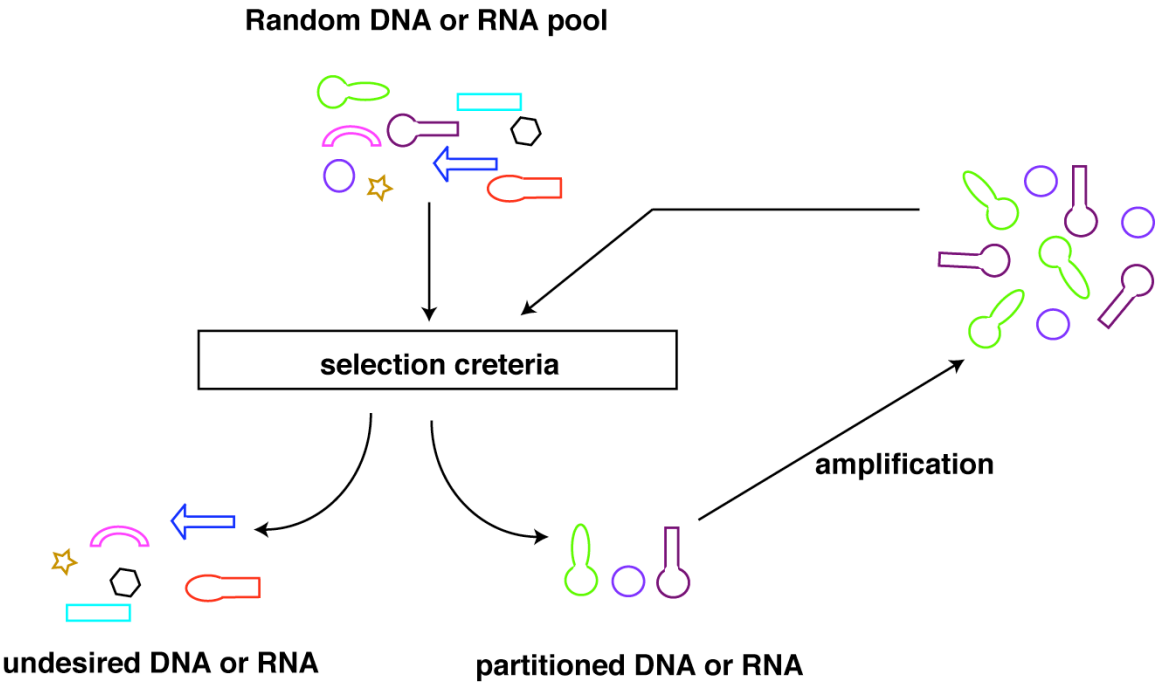

Figure 1.5. Overview of the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) procedure. A randomized DNA or RNA pool is generated, and molecules that survive the selection pressure are separated from the bulk pool. The partitioned molecules are subsequently amplified using standard molecular biology techniques. Multiple cycles of selection and amplification can result in a population of molecules that meet the selection criteria.

The *in vitro* selection method has been widely used to obtain aptamers, which are nucleic acid molecules that bind to a specific target with relatively high affinity [81, 82]. The targets for aptamers include small molecules, proteins, nucleic acids, and even cells, tissues and organisms [83-86]. In addition to target-binding aptamers, DNA/RNA molecules with other characteristic have also be selected using the same strategy but with different selection criteria. Such molecules include ribozymes, which are nucleic acids with catalytic activities [87]. *In vitro* selection-amplification was also one of the major strategies used to develop the reporter for the high-throughput editing assay described in this thesis.

9. The principle behind the assay development

In order to monitor the editing reaction in a high-throughput assay, the editing reporter is required to convert the sequence change resulting from editing into a detectable signal change. While it is difficult to realize this convention directly, it is possible that the sequence change of an RNA molecule could affect its conformation. In numerous examples, such as aptamers, ribozymes, and riboswitches, the conformation of an RNA determines its function. Therefore, the principle for developing the editing reporter was to search for an RNA in which the editing reaction induced a conformational change that could in turn generate a signal change (Fig. 1.6).

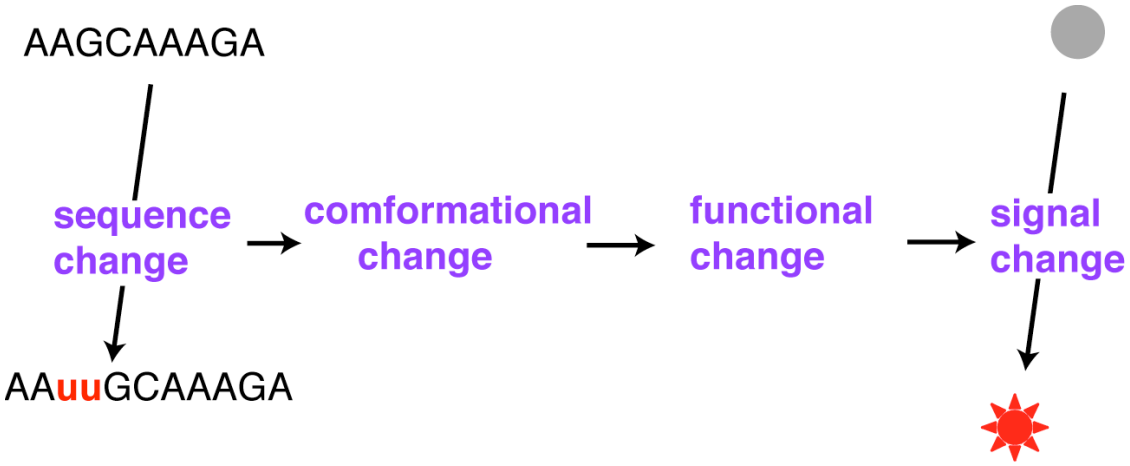

Figure 1.6. The principle behind the development of the high-throughput editing assay. The sequence change resulting from the editing reaction will cause a conformational change within the reporter RNA; the conformational change of the reporter RNA will in turn cause a functional change, which is coupled to a signal change.

Three different approaches were developed based on the above principle. In each approach, the proposed reporter consisted of an editing domain and a functional domain. The functional domain is held in an inactive conformation when the reporter RNA is in the pre-edited form. The sequence change caused by the *in vitro* editing reaction alters the conformation of the reporter and activates the functional domain. In the first approach, the activated functional domain was designed to bind to and stimulate the fluorescence of a dye (Chapter 2). In the second approach, the functional domain was designed to inhibit an enzyme cascade reaction (Chapter 2). In the third approach, the functional domain was designed to bind to streptavidin and generate an electrochemiluminescent (ECL) signal (Chapter 3). *In vitro* selection strategies were used to obtain these reporters for the different strategies. Reporters selected for the third approach were found to have properties that make high-throughput editing assays feasible. The editing assay with the selected reporter was optimized and validated for use in HTS (Chapter 4). A pilot screen of a 1,280 compound library was conducted, and five compounds were identified that inhibit the *in vitro* editing reaction. The pilot screen demonstrated the feasibility of using the new assay in screens of large chemical libraries.

Chapter 2

Fluorescent and Enzymatic Cascade Strategies for Developing a High-Throughput Editing Assay

INTRODUCTION

In this chapter, a purification method was developed to prepare the mitochondrial editing extract required for high-throughput drug screens. In addition, two different approaches for obtaining an RNA reporter suitable for a high-throughput editing assay are described. In the first approach, the design of the reporter RNA was based on an *in vitro* editing substrate [46] and a malachite green (MG) aptamer that increases the fluorescence of MG when bound to it [88]. Parts of the MG aptamer and the editing substrate were linked through a double helical connecting region. The linkage was optimized so that a conformational change induced by *in vitro* editing would increase the affinity of the MG aptamer for MG, resulting in an increased fluorescence. An *in vitro* selection strategy was used to enrich the desired RNAs from a randomized RNA population. Several RNAs were selected that exhibited an increased binding affinity for MG that was dependent upon editing. However, the resultant fluorescent signals were not significant enough for these RNAs to be able to function as reporters in the proposed assay. In order to improve upon the sensitivity, a second approach was attempted using a reporter RNA containing an aptamer that inhibits a coagulation cascade [89]. This strategy involved linkage of the editing substrate to the cascade-inhibiting aptamer such that inhibition would only occur upon editing. The approach, however, was not successful primarily because of the poor reproducibility of the cascade reaction. Although neither approach was successful, the knowledge gained laid the foundation for the successful strategy described within the next chapter.

RESULTS

1. Preparation of the editing extract

The major constraint on the assay development is related to the apparent low abundance of the editing complex within the mitochondria and the resultant practical and economical limitations in obtaining enough material for HTS. This constraint defines the

sensitivity required of the assay, and several aspects of the approach were designed to optimize it. First, a species and strain of Leishmania was used that can be grown to high densities in media that does not require serum or other expensive supplements. It can be grown to a density over ten-fold higher than that of any *Trypanosoma brucei* strains used to study the editing reaction. Second, a scalable purification strategy was developed to maximize the yield of the editing activity and at the same time minimize activities that may inhibit the assay. Details of this purification are provided in the Materials and Methods section. Approximately 100,000 units of editing activity are obtained from a 25 L culture, where one unit of editing activity is defined as the quantity of extract resulting in 1 fmol of correctly edited product within 1 h under previously defined conditions [46]. For the drug screens to be economically and practically feasible, it is necessary for the assay to be sensitive enough to detect the edited product resulting from approximately 50 units. This would make it possible to carry out approximately 40,000 reactions with the extract harvested from a 500 L bioreactor culture.

2. Development of an MG aptamer as a reporter for the editing reaction

2.1. Background- an aptamer that can induce fluorescence of MG

An aptamer previously selected to bind to MG also stimulates the fluorescence of MG and other related triphenylmethane dyes [88, 90] (Fig. 2.1 A and B). MG itself is not significantly fluorescent, as most of the absorbed excitation energy is released instead through vibrational and rotational motion. However, upon binding to the aptamer, the MG is held in a rigid conformation that results in more of the absorbed excitation energy being released through fluorescence; a ~2360-fold increase in fluorescence is observed. The MG aptamer contains an internal loop and two stems; mutations at these sites inhibit the aptamer's binding to MG.

The MG aptamer was previously exploited as an *in vitro* sensor for ATP [91]. The ATP sensor consisted of the MG aptamer and a previously reported ATP aptamer. The

two aptamers were linked through their double helical regions, which served as a "communication module". When the ATP aptamer bound an ATP molecule, the resultant conformational change affected that of the MG aptamer via the communication module, resulting in an increased binding affinity for MG and consequently an increased fluorescence.

2.2. An editing reporter based on the MG aptamer

An RNA reporter for a high-throughput editing assay was designed analogous to the ATP sensor described above. The reporter has three major domains (Fig. 2.1 C): an MG binding domain (green) containing the internal loop of the MG aptamer, an editing domain (black and red) that is part of the previously selected editing substrate, and a helix (blue) that connects the MG domain and the editing domain. Two guiding As in the editing domain (red) were designed to direct the insertion of two uridylates at the editing site (indicated by arrow) during *in vitro* editing. When the reporter RNA is in the pre-edited form, the connecting helix would be destabilized without the two uridylates at the editing site; this helix was previously demonstrated to be critical for MG binding. As a result, the pre-edited form has no or only a weak affinity for MG and does not stimulate a significant fluorescent signal. In contrast, when the reporter RNA is edited, the insertion of two uridylates would restore the connecting helix, resulting in increased MG binding and fluorescence. Inhibition of the editing reaction by a drug would be indicated in the HTS by a diminished fluorescent signal.

2.3. The *in vitro* selection of the editing reporter

There are three criteria required of the editing reporter for the high-throughput assay:

1. It is required to have no or very low affinity for the MG dye in its unedited form.
2. It is required to be edited efficiently by the *in vitro* editing reaction.
3. It is required to have a relatively high affinity for the MG dye in its edited form.

The above requirements place a lot of sequence restrictions on the proposed reporter, especially the connecting helix. Because the connecting helix is both one stem of the MG binding domain and immediately upstream of the editing site, it would be expected to affect the dye binding ability as well as the editing efficiency of the reporter [46, 90, 92]. A previous study indicated that the outer helix of the MG binding domain also affects the fluorescent signal of the bound MG dye (Fig. 2.1 C) [91]. Therefore, simply connecting the previously selected editing substrate with the MG aptamer would not generate a reporter that best meets the stated criteria, as sequence that is known to fit one requirement may not be compatible with the other two. In order to identify the optimal sequence, six base pairs within both helices (Fig. 2.1 D) were randomized resulting in a pool of potentially 4^{12} or ~10^{7} sequence variants. An *in vitro* selection approach was developed to identify the optimal RNA sequence from the randomized pool.

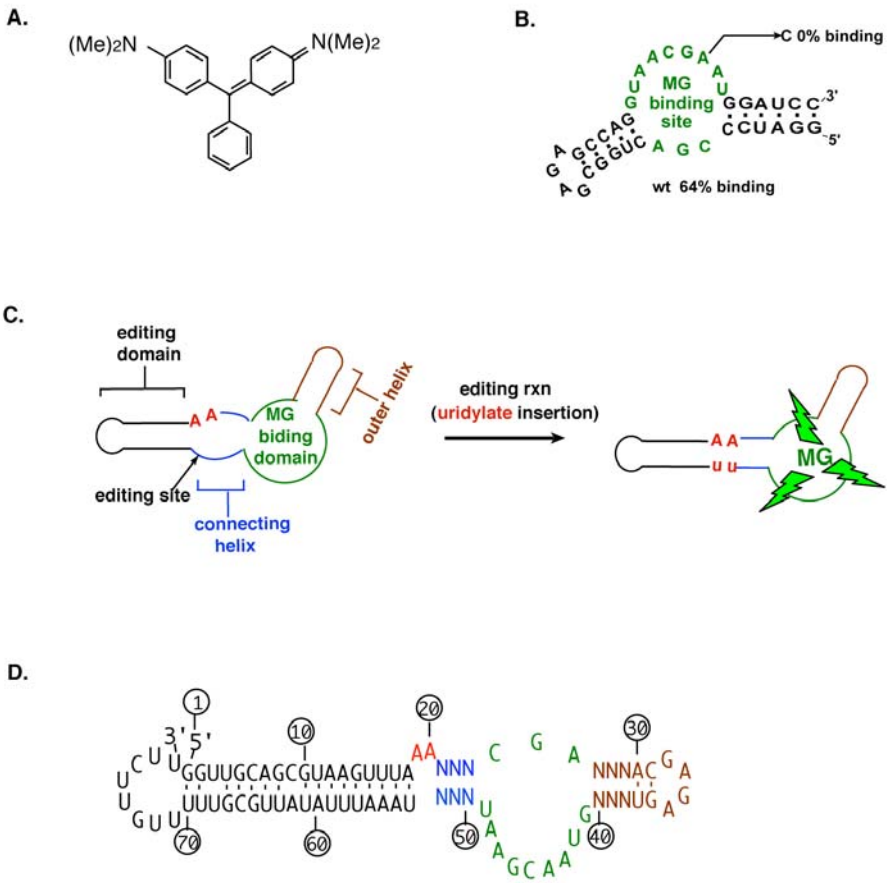

Figure 2.1. Development of an MG aptamer as a reporter for the editing reaction. (A) The structure of MG. (B) The secondary structure of the MG aptamer. The internal loop and MG binding site are indicated in green. An A-C mutation decreases the binding affinity of the RNA for MG [90]. (C) The design of an MG-aptamer based editing reporter. The reporter contains an MG binding domain (green), an editing domain (black), a connecting helix (blue), and an outer helix (brown). The two guiding As are highlighted in red. In the absence of two Us at the editing site (indicated by the arrow), the connecting helix is disturbed, leading to no or very low MG affinity of the MG binding domain. As a result, no significant fluorescence is generated. U insertions catalyzed by the editing complex would restore the connecting helix, resulting in increased MG binding and fluorescence. (D) The design of the RNA used for the selection of a MG-based reporter. Six base pairs flanking the MG binding domain were randomized. Each pair position is numbered. Colors are as indicated above.

The *in vitro* selection approach contained three major steps corresponding to the three criteria for the reporter (Fig. 2.2): the first step was to remove RNAs that bind to MG in the absence of editing, the second step was to obtain RNAs that are good editing substrates, and the third step was to enrich RNAs that bind to MG with high affinity in their edited form.

The first step of the selection was designed to obtain pre-edited RNAs that do not significantly interact with MG (Fig. 2.2 A). This is important in order to minimize the background fluorescence in the absence of editing. An affinity column was synthesized by coupling MG isothiocyanate with adipic acid dihydrazide agarose. The quality of the column was tested by using radiolabeled positive and negative control RNAs; the wild type MG aptamer was used as a positive control and an A27-C mutation previously demonstrated to inhibit binding was used as a negative control [90] (Fig. 2.1 B). The column profile indicated that 64% of the positive control and 0% of the negative control RNA bound to the column respectively, which was consistent with reported results for both of these RNAs [90].

The MG column was used to remove those RNAs that have affinity for MG in their pre-edited form (Fig. 2.2 A). The pre-edited randomized population was transcribed from the corresponding DNA template with T7 RNA polymerase as described within the Methods section. P^{32}-labeled nucleotide triphosphates were included within the transcription in order to radiolabel the RNA, making it possible to monitor during the selection (Fig. 2.3 A). The RNA pool was first heated at 65°C and incubated at 37°C to facilitate correct folding. The RNA was applied to the column followed by washing with 15 column volumes of selection buffer (0.1 M KCl, 5 mM MgCl, 10 mM Na-Hepes, pH 7.4). To remove those RNAs that have high MG binding affinity even in the absence of editing, only the column flow-through (column volume 1-6) was collected. It was still possible, though, that some pre-edited RNAs with affinity for the MG dye could be included within the flow-through fractions because of incorrect folding of the RNA. In order to reduce this complication, the collected fractions were ethanol precipitated,

refolded and passed through a second column. The flow-through fractions were then used for the next step of the selection.

The second step of the selection (Fig. 2.2 B) was performed to ensure that the selected RNAs are good substrates for the editing reaction. The non-binding RNAs obtained from the first step were incubated with the editing extract at 27°C for 1 hr. As two uridylates would be inserted during editing, the edited RNAs could be resolved from the non-edited population by denaturing polyacrylamide gel electrophoresis. A gel slice was excised at the location corresponding to 2 U-insertions, and the RNA was eluted. Since the terminal uridylate transferase activity within the editing extract can add uridylates at the 3' hydroxyl terminus of RNA, the resultant 3' extended RNAs could co-migrate with the edited RNAs. In order to inhibit this complication, the 3' end of the RNAs had been blocked prior to the editing reaction through ligation to the 5' end by treatment with RNA ligase; this results in a circular RNA. The edited RNA eluted from the gel was amplified by RT-PCR. A T7 promoter present on one of the PCR primers allowed the DNA product to be transcribed into RNA for the next step.

The third step of selection was for RNAs that have high affinity for MG when in the edited form. Radiolabeled RNA selected from the previous step was loaded onto the MG affinity column described above (Fig. 2.2 C). After extensively washing with 15 column volumes of selection buffer, specifically bound RNA was eluted with five column volumes of an elution solution containing 1mM MG. The eluted RNAs were amplified by RT-PCR and T7 RNA polymerase mediated transcription. This selection step was repeated four times in order to obtain a significant enrichment of the specifically bound RNAs. Fig. 2.3 B shows the increase in population of specific MG binders from the 2^{nd} through to the 4^{th} round of selection.

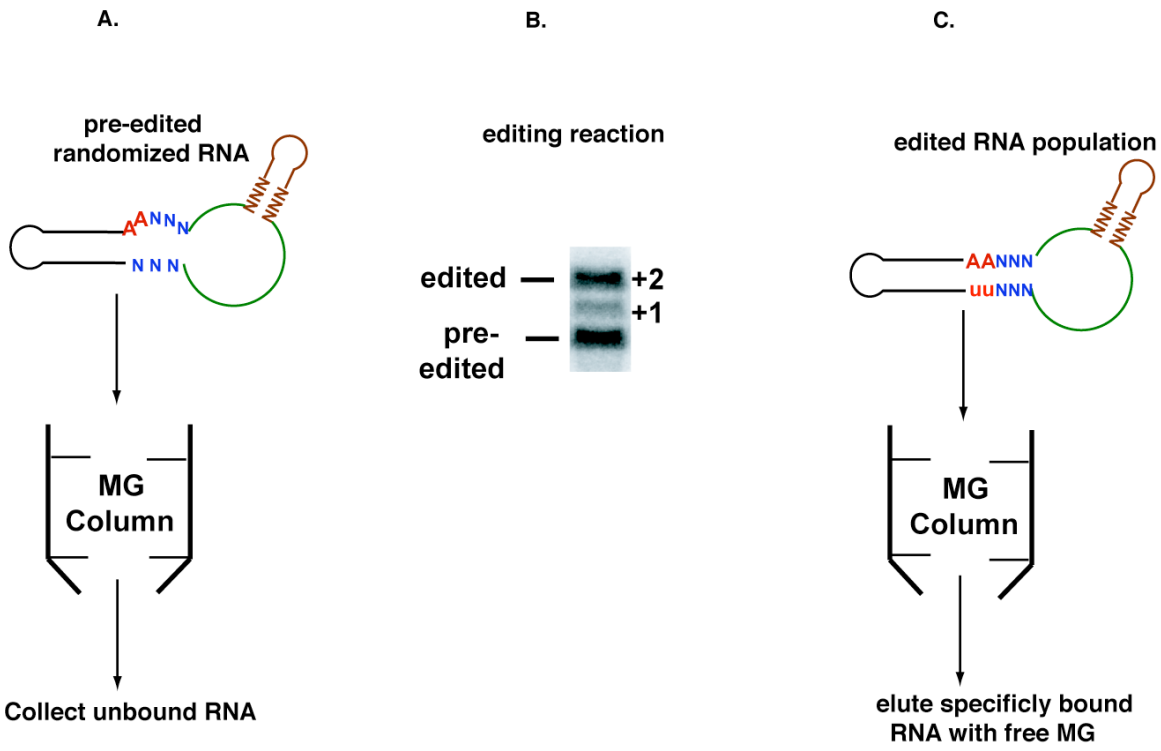

Figure 2.2. The selection of a reporter for the editing reaction. (A) The elimination from the population of pre-edited RNAs with affinity for MG. The randomized pre-edited RNA population was loaded onto an MG affinity column. Non-binders were collected and used as substrates for the editing reaction. (B) The selection of RNAs that are good editing substrates. After the editing reaction, RNAs were resolved by gel electrophoresis. Edited RNAs corresponding to the size expected for 2 U-insertions were excised and eluted from the gel. (C) The selection of edited MG-binding RNAs. Edited RNAs were loaded onto a fresh MG affinity column. Specific MG binders were eluted with buffer containing free MG.

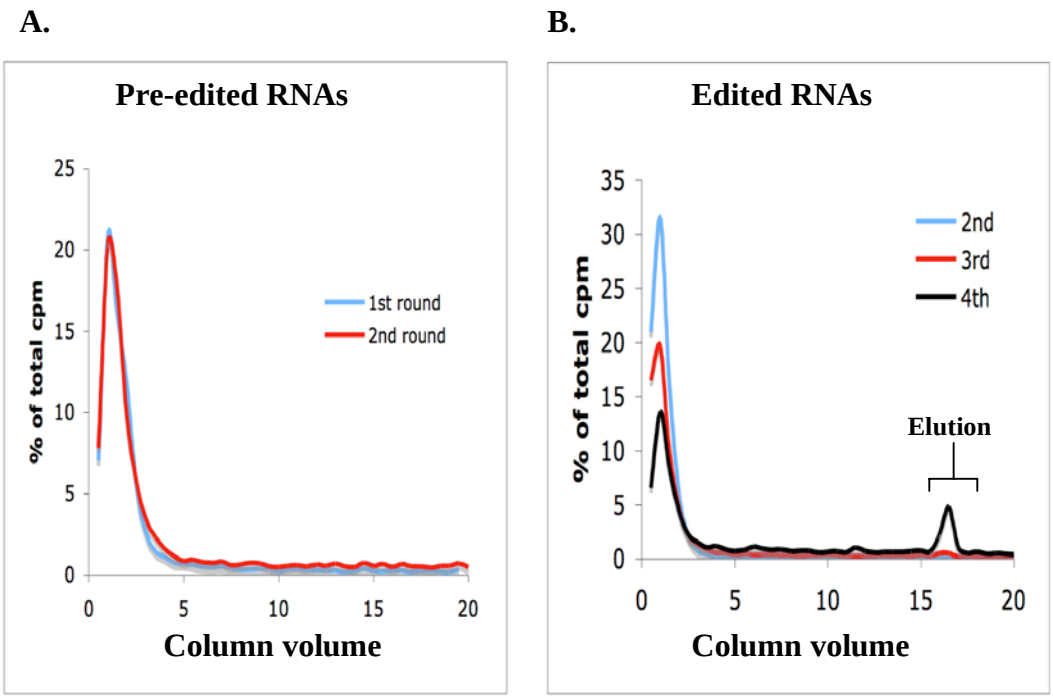

Figure 2.3. The MG column selection steps. (A) Removal of pre-edited MG-binding RNAs. The randomized unedited RNA population was passed through an MG column twice. Fractions that contained non-binding RNAs (column volume 1-6) were collected. (B) Selection of edited MG-binding RNAs. After editing, the RNAs were passed through a fresh MG column. The MG column was washed with 15 column volumes of selection buffer, and specific MG-binding RNAs were eluted with 5 column volumes of an elution solution containing 1 mM MG (labeled elution). Collected RNAs were amplified and reselected on the MG column a total of four times. The population of RNAs specifically eluted from the column is detectable in the last round of selection.

RNAs from the 4th round were cloned and sequenced. The sequence of 77 unique clones was obtained. Twenty-four clones contain correctly edited sequences, whereas 16 remained unedited, which could have resulted from incomplete enrichment during the second step of the selection. The remaining clones appeared to be either incorrectly edited or artifacts of PCR or cloning. The 24 correctly edited clones are presented in Fig. 2.4, and the most frequently observed base pairs selected at the two helical regions of the reporter are summarized in Fig. 2.5.

2.4. Summary of the results for the selection of the MG-editing reporter

Eight selected clones, # 007, #019, #023, #118, #121, #128, #133,and #134, were first tested for their ability to stimulate MG fluorescence. The edited form of each RNA was synthesized, and it was heated and allowed to refold before being incubated with MG. The induced fluorescent signal of each reaction is indicated in Table 2.1. The fluorescent signals from four edited RNAs, # 007, #019, #128 and #133 were the highest. As a result, the corresponding pre-edited RNAs of these four were synthesized and tested alongside the edited forms. As shown in Table 2.2, there is a 4-8 fold difference in fluorescence between reactions containing the edited and the corresponding pre-edited RNA. This result suggests that RNAs with editing-dependent affinity for MG had been successfully selected.

Figure 2.4. The sequence of correctly edited RNAs obtained from the MG selection. Blue and brown nucleotides indicate positions that were randomized at the start of the selection within the connecting helix and outer helix, respectively.

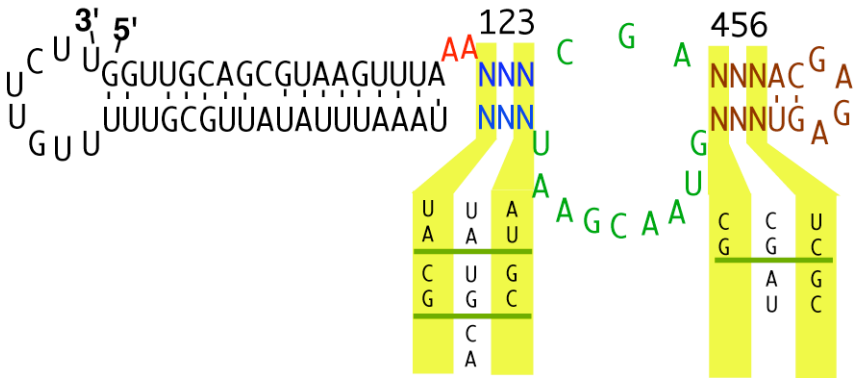

Figure 2.5. Summary of the selection results. The most frequently observed base pairs selected from the randomized region of the reporter RNA are indicated.

Table 2.1. Fluorescence of MG in the presence of selected RNA.

RNA[a]	FU
007	12.36±0.08
019	6.4± 0.6
023	0.7± 0.5
118	1.49± 0.02
121	0.7± 0.3
128	5± 1
133	6± 1
134	3± 1
Background	-0.1± 0.3

[a] MG (0.16 μM) was mixed with the edited form of the indicated RNA (0.125 μM) in an 80 μl volume. Fluorescent units (FU) were measured in duplicate.

Table 2.2. Fluorescence of MG in the presence of edited or pre-edited reporter.

RNA[a]	7	19	128	133
Edited Form (FU)	17.0 ± 0.7	9 ± 2	9.1 ± 0.8	7.6 ± 0.3
Pre-edited Form (FU)	2.6 ± 0.3	1.4 ± 0.2	2.2 ± 0.2	0.88 ± 0.04

[a] MG (0.16 μM) was mixed with the edited or pre-edited form of the indicated RNA (0.125 μM) in a 80 μl volume. Fluorescent units (FU) were measured in triplicate.

The fluorescent measurements recorded in Table 2.1. and Table 2.2. required that there be 10 pmol of edited RNA within the 80 μl volume of each microtiter plate well. However, as already indicated it would not be economically and practically feasible to generate this quantity of edited RNA during the high-throughput assays. Experiments were performed to reduce both the RNA concentration and the total reaction volume, but this could not be done without a significant loss in the fluorescent signal; the fluorescence was close to the background noise when the desired amount of RNA was used (data not shown). In an attempt to improve upon the sensitivity, three different fluorescent plate readers were used during the reaction optimization, but the signal-to-background ratio was comparable among all three. This suggested that a more sensitive assay was required.

Another deficiency of the selected RNAs was their editing efficiency. Radiolabeled pre-edited RNAs were analyzed by gel electrophoresis after the *in vitro* editing reaction. None of the selected RNAs were edited efficiently and some not at all (data not shown). This result indicated that the selection step requiring the RNA to be a good editing substrate also needed to be improved.

3. Use of an enzyme cascade as a reporter for the editing reaction

3.1. Background: exploitation of a coagulation cascade to increase the sensitivity of the reporter

A coagulation cascade was exploited as an attempt to obtain a more sensitive reporter for the high-throughput assay [89]. The coagulation cascade is derived from the blood coagulation reaction. As illustrated in Fig. 2.6 A, the cascade contains several protease components, fibrinogens, and a suspension of microspheres. The reaction is triggered by a protease, RVV-X, that activates factor X, and the resultant fibrins form a clot that cause the microspheres to precipitate. The solution is cloudy before the initiation of the reaction but is transparent after the clot precipitates the spheres (Fig. 2.6 B). The reaction is monitored by spectrophotometry at 405nm for 4 hrs at 5 min intervals, and the

time point resulting in a 50% reduction in absorbance ($t_{1/2}$) is used as the metric for RVV-X activity. An aptamer was previously selected to bind and inhibit RVV-X [89]. It was reported that even low femtomole levels of the RVV-X aptamer significantly increased the $t_{1/2}$ of the reaction. A VEGF sensor had previously been developed using the RVV-X aptamer.

A reporter for the editing reaction was designed based on the RVV-X aptamer, and the principle of this design is analogous the MG-aptamer approach: a conformational change resulting from the editing reaction will activate the reporter's binding affinity for RVV-X, thus an editing-dependent inhibition of the cascade reaction would be obtained. In the proposed HTS, compounds that inhibit the editing reaction would be detected by an increased $t_{1/2}$ for the clotting reaction.

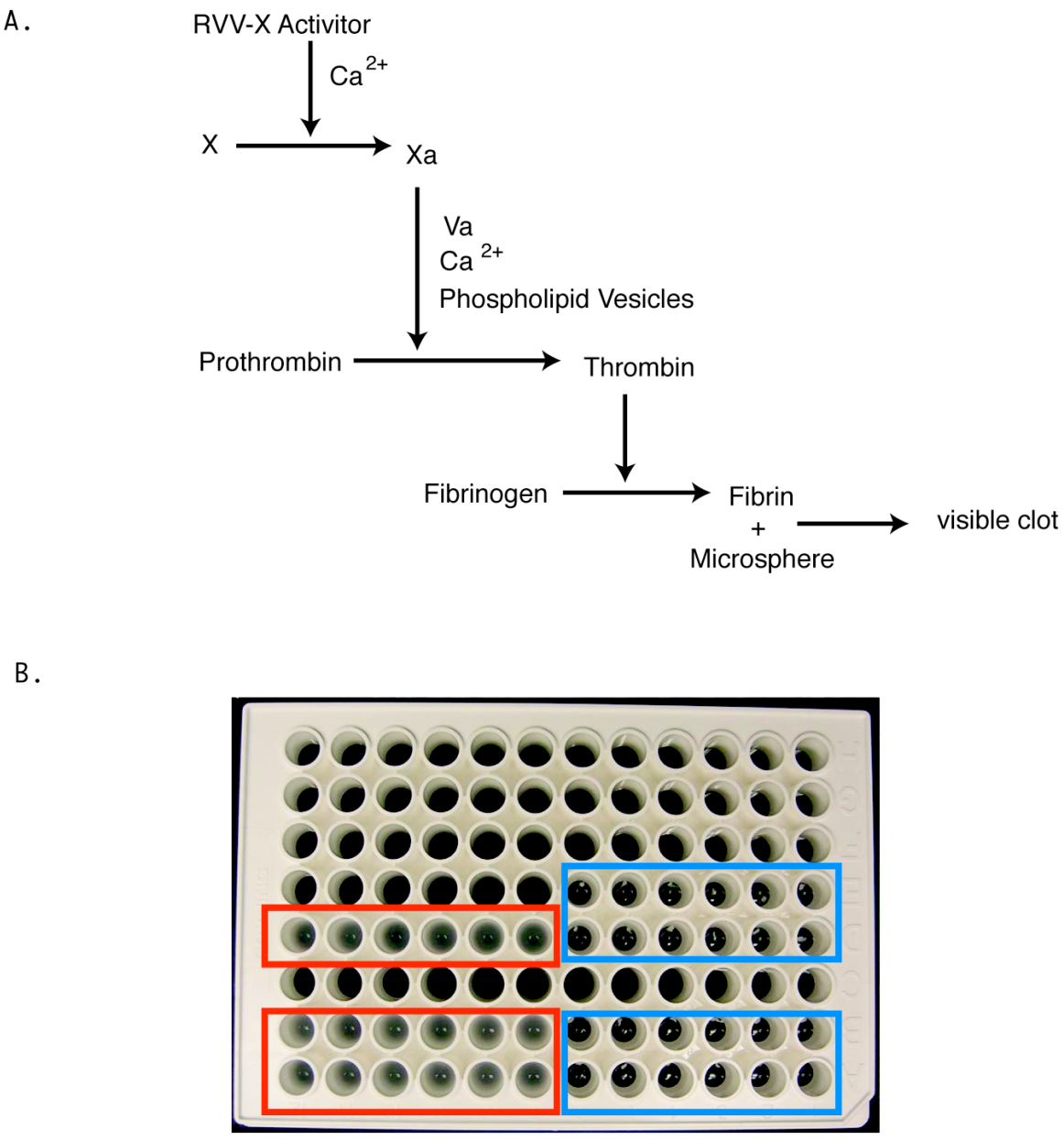

Figure 2.6. The coagulation cascade assay exploited as a reporter for the editing reaction. (A) The major steps of the coagulation cascade reaction. (B) Visual presentation of a clear bottom 96-well plate containing the coagulation cascade reagent. Reactions with or without the RVV-X initiator are boxed by blue or red, respectively. Coagulation results in precipitation of the spheres and a less turbid solution.

3.2. The coagulation assay

Coagulation cascade reactions in the presence and absence of the RVV-X aptamer were performed (data not shown), but the results suggested that several properties of the cascade were not well suited for high-throughput assay development. First, the $t_{1/2}$ of the coagulation reaction is used as the metric for the aptamer's inhibitory effect, and this requires recording the absorbance of the reaction for 2-4 hrs at a 5 min interval, which would be cumbersome for a high-throughput screen. Second, the reported RVV-X aptamer is poorly characterized. The reported inhibitory effect could not be reproduced using the published minimized aptamer (RNA 37s in refs [89]), but a longer RNA did have an inhibitory effect (RNA 9c in refs [89]). However, gel shift analysis also indicated that the interaction between the aptamer and RVV-X has a large non-specific component (data not shown). These complications would result in too many obstacles in the design of the RVV-X binding module of the reporter. Third, the cascade reaction is not stable. It was initially observed that microspheres precipitated in the absence of RVV-X. This was likely due to non-specific stickiness between microspheres and other protein components in the solution. Several methods were tried to prevent this effect, and it was found that the reaction could be stabilized by using hydroxylate polystyrene beads instead of the originally reported polystyrene beads. However, the minus RVV-X control precipitated occasionally for no obvious reason even with these modifications.

In summary, the coagulation cascade and the RVV-X aptamer are not well suited for developing a high-throughput editing assay.

DISCUSSION

The practical and economical limitations of preparing the mitochondrial editing extract for the HTS of large chemical libraries defined the sensitivity required of the editing assay. Two approaches were attempted in this chapter to obtain a suitable reporter for the editing reaction that had this sensitivity. In the first approach, reporter RNAs were

required to bind to MG and induce fluorescence only in response to editing. Several RNAs were obtained using an *in vitro* selection strategy, and the fluorescence of MG induced by the edited and pre-edited forms were significantly different. This result indicates that the selection strategy is feasible. However, appreciable fluorescent signals could not be generated when RNA was used at the lower concentrations required for HTS. In addition, the selected RNAs were not edited *in vitro* efficiently enough to be reporters for the proposed HTS. In the second approach, a coagulation cascade was exploited in order to develop a more sensitive assay. However, it was found that this approach is not well suited for high throughput editing assays because of the poor reproducibility and potential complications of the cascade reaction. Although neither approach was successful, valuable knowledge was gained in improving the strategy:

1. A system with high sensitivity is required, as suggested by the MG approach.

The selected RNA-MG generated an appreciable fluorescent signal when 10 pmoles of the RNAs were used within an 80 μl assay volume. Although this concentration is actually much lower than what was used in the previously reported applications of the MG-aptamer [91], it is too high for high-throughput editing assays. Even with the streamlined purification method, it is only practical and economically feasible to obtain enough extract provided the assay is able to reliably detect femtomole quantities of edited product. Attempts to reduce both the concentration of RNA and the assay volume were unsuccessful as the ratio of the signal to instrument background became close to one as the amount of RNA was reduced to one picomole. The fluorescent signal of the reaction is defined by the following equation:

Fluorescence = excitation coefficient × concentration × quantum yield × excitation intensity × path length × emission collection efficiency

On the basis of equation 1, a reduction in the concentration of the RNA is predicted to decrease the signal, as was observed. Reducing the volume of the reaction could affect the path length and may account for the decreased signal that resulted with the attempts to use lower volumes. In addition, at least two other factors contributed to the MG-RNA

not generating appreciable fluorescent signals at lower concentrations. First, the stoichiometry of dye binding to the RNA is only 1:1. If this could be increased, the effective concentration of the fluorophore in the above equation would also be increased. Second, RNA-bound MG is not a strong fluorophore. Although the fluorescent activity of MG is increased by 2360-fold upon aptamer binding, the bound MG only has a quantum yield (QY) of 18.7% [88]. A different fluorophore has been reported with similar aptamer-dependent fluorescent activity but a higher QY of 32.4% [88]. This fluorophore, however, is not commercially available, and because no other molecules have been reported with the aptamer-induced fluorescent activity, this assay approach was not further pursued. However, if fluorescent detection will be used in different assay strategies, it would be beneficial to couple multiple fluorophores to one reporter as well as to choose fluorophores with higher QY.

2. The selection pressure should be increased for reporters that are good substrates for the editing reaction.

In the MG approach, sequencing of the selected RNAs indicated that 16 of the 40 valid clones were in the pre-edited form, and none of the five tested selected RNAs was a good substrate for the editing reaction. This indicates that the selection step for the enrichment of RNAs that are good editing substrates (step 2) was not stringent enough. The low stringency probably resulted from there being only one round of editing selection (step 2). In order to increase editing selection stringency, a primer that overlaps the editing site is required to regenerate the pre-edited RNA so that multiple rounds of editing selection could be performed. In the MG approach, however, positions immediately upstream of the editing site were randomized for the selection (Fig. 2.1 D), which made it impossible to use an overlapping primer to regenerate pre-edited RNA. To enrich RNAs with good editing efficiency, nucleotides upstream of the editing site need to be fixed in future attempts to allow more rounds of editing selection.

3. A stable signal amplification system is needed, as suggested by the cascade approach.

The cascade approach was chosen because it includes a series of signal amplification steps, which could potentially provide a higher sensitivity than the MG dye-based assay. However, several intrinsic deficiencies undermined the value of this approach. First, the cascade reaction requires shaking and reading with 5-10 min interval for the entire process. This would not be well suited for large scale high-throughput screens. Second, the coagulation assay contains a number of protein and protease components, which could render the assay vulnerable to a high background and the reproducibility of the assay was observed to be poor. The large number of components in the coagulation assay could also increase the number of false positive and negative results in the drug screen for compounds that interfere with the editing assay. Third, because the reported RVV-X aptamer was not well characterized, designing of editing reporters based on it would be difficult. Other signal amplification methods should be exploited, as they are likely to increase the detection sensitivity. However, such a method must be stable. In the next chapter, a chemiluminescent signal amplification method will be utilized that has the required sensitivity and stability.

MATERIALS AND METHODS

1. Preparation of the editing extract

A 25 L culture of the *Leishmania tarentolae* UC strain was grown to a density of ~1.5 × 10^8 cells/mL, and the cells were lysed as previously described [93]. A mitochondrial fraction was enriched through differential centrifugation, and taken up in 200 mL of resuspension buffer (0.5 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 25 mM HEPES at pH 7.5, 10 μg/mL leupeptin, 10 mM MgCl2, and 1 mg/mL Pefabloc). The material that was soluble in 1.0% TX-100 was loaded onto a 15-mL SP-Sepharose column. After the column was washed with 10 volumes of resuspension buffer containing 100 mM KCl, the editing activity was eluted with 200 mM KCl in resuspension buffer. Column flow rate was 1ml/min. The fractions containing editing activity were pooled,

and the KCl concentration was reduced to 150 mM by dilution with resuspension buffer. The extract was loaded onto a 2 mL Q-Sepharose column and after washing with 10 column volumes of resuspension buffer containing 150 mM KCl, the column was eluted with 225 mM KCl in resuspension buffer; the column flow rate was 3 ml/min. Fractions were assayed for editing activity as indicated below. Approximately 100,000 units of editing activity are obtained from the fractionation, where 1 unit of editing activity is defined as the quantity of extract resulting in 1 fmol of correctly edited product within 1 h under previously defined conditions [46]. Although the editing activity would be enriched further by other purification strategies [94-97], this streamlined fractionation is relatively free of RNases and other complicating activities, results in relatively high yields, and can be readily scaled to produce sufficient material for high-throughput screens.

2. The *in vitro* editing reactions

For a 50 μl *in vitro* editing reaction, 1 pmol of pre-edited RNA was denatured for 5 min at 65°C in 10 μL of denaturing buffer containing 0.2 mM EDTA and 25 mM Tris (pH 8.0, 27°C). The RNA was then mixed with 25 μL of a 2× editing buffer (2 mM ATP, 2 mM UTP, 2 mM dithiothreitol, 10 μg/mL leupeptin, 12 mM $MgCl_2$, and 1 mg/ml Pefabloc SC) and incubated for 10 min at room temperature. The editing reaction was initiated through the addition of 15 μl of the appropriate column fraction. After 1 h incubation at 27°C, 2 μl of 250 mM EDTA and 1 μl 10% SDS were added to stop the reactions. RNA was precipitated with ethanol after phenol/ CH_3Cl extraction. Edited RNA was resolved from the unedited by gel-electrophoresis.

3. MG affinity column preparation

To synthesize the MG agarose column, 300 mg of MG isothiocyanate was initially dissolved in 300 μl of dimethylformamide (DMF). The dissolved MG was then reacted at room temperature overnight with 10 ml of adipic acid dihydrazide agarose that had been equilibrated in 0.1 M $NaHCO_3$ (pH 8.3). Unreacted MG was removed by

extensive washing with DMF and water. A 0.6 mm diameter column was loaded with 600 μl of MG agarose. The column was treated with *E. coli* tRNA and washed with 5 column volumes of selection buffer (0.1 M KCl, 5 mM $MgCl_2$, 10 mM Na-Hepes, pH 7.4) prior to use.

4. The selection for MG-binding reporters

Step one: The pre-edited randomized RNA population (Fig. 2.1 D) was transcribed from the corresponding DNA template. The transcription reactions contained 4 mM ATP, 4 mM CTP, 4 mM UTP, 1 mM GTP, 5 mM GMP, 0.3 mCi/μl ^{32}P-ATP, 5 mM dithiothreitol, 22 mM $MgCl_2$, 2 mM spermidine, 40 mM Tris (pH 8.2, 20°C), 0.01% Triton X-100 and 0.5 units/μl of T7 RNA polymerase (Invitrogen). RNA was gel-purified, and 300 pmol RNA was used in a 120 μl circularization reaction containing 60 units of T4 RNA ligase (New England Biolab), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 0.25 mg/ml BSA, 15% DMSO (v/v%), 10 mM Tris (pH 8.7, 22°C). The circularized RNA migrates slower than linear RNA on a polyacrylamide gel and was excised and purified. Fifty picomoles of pre-edited circular RNA was heated in at 65°C in 95 μl heating buffer (0.1 M KCl, 10 mM Na-Hepes, pH 7.4) for 5 min. The RNA was incubated at room temperature for 10 minutes after the addition of 5 μl of 100 mM $MgCl_2$ to facilitate folding. The RNA was applied to the column followed by washing with 15 column volumes of selection buffer (0.1 M KCl, 5 mM MgCl, 10 mM Na-Hepes, pH 7.4). Fractions, approximately the size of one column volume, were collected, and 5% of each fraction was quantified by scintillation counting. Fractions 1-6 contained over 70% of the flow-through RNA. These fractions were precipitated and applied to another MG column. Binding and washing were as described. Collected RNA was precipitated for the next step.

Step two: Two picomole of RNA collected from step one was incubated with approximately 700 units of editing extract in a 100 μl *in vitro* editing reaction as was described in Methods Section B. The edited RNA was resolved from the non-reacted

RNA on a 9% polyacrylamide 8 M urea gel. Slower migrating RNA, consistent with 2 U insertions, was excised and eluted. RNA was then amplified by RT-PCR, and radiolabeled RNA was transcribed for subsequent enrichment.

Step three: Fifty picomoles of RNA was loaded onto an MG column as described above for step one. After washing the column with 15 column volumes of selection buffer, specifically bound RNAs were eluted with 5 column volumes of elution solution (0.5 mM MG oxalate, 0.1 M KCl, 5 mM MgCl, 10 mM Na-Hepes, pH 7.4). Eluted fractions were collected and quantified via scintillation counting. Collected RNA was amplified by RT-PCR followed by T7 RNA polymerase mediated transcription. This step was repeated four times until a significant percentage of RNA was observed in the elution fractions.

5. Fluorescence measurements

RNA was heated and incubated as described for the first step of selection. MG was added at the end of the incubation. Fluorescent signals were taken using several different fluorescent plate readers: FLEXstation (bucher biotec), SpectraMAX (bucher biotec), and FLUOstar (BMG labtech). Experiments were performed at the excitation wavelength of 610 nm and emission of 640 nm.

6. Coagulation cascade assay

The following reagents for the coagulation cascade assay were purchased from Haematologic Technologies: Fibrinogen, Prothrombin, Factor Va, Factor X, snake venom protease RVV-X, and phospholipid vesicles (PCPS). Polystyrene microspheres (10% solids, 0.77−1.0 μm in diameter) were from Bangs Laboratories. Hydroxylate microspheres (2.5% solids, 0.8 μm) were from Polysciences.

The coagulation cascade contained 600 nM PCPS, 230 nM Fibrinogen, 170 nM Prothrombin, 870 fM Factor Va, 580 pM Factor X, and 0.025% of microspheres in 50 mM imidazole-HCl and 3 mM $CaCl_2$ buffer, pH 7.8. One hundred microliters of the reaction mix were dispensed into each well of a clear bottom 96-well plate, and 20 µl RVV-X or aptamer-RVV-X was added to initiate the reaction. Reactions were performed at 37°C with shaking for 2 hr. The reaction was monitored by taking the absorption at 405 nm at 5-10 min intervals.

To test the inhibitory effect of reported RNA aptamers (RNA 37s and RNA 9c in [89]), each RNA was heated at 65°C for 5 min in 16 µl of 50 mM imidazole-HCl, pH 7.8. Two microliters of 50 mM imidazole-HCl and 27 mM $CaCl_2$ buffer, pH 7.8 were added, and the mix was incubated at room temperature for 5 min to facilitate RNA folding. Two microliters of RVV-X were then added and incubated at room temperature for 20 min to facilitate aptamer-RVV-X binding. This was then mixed with the other components of the coagulation reaction and monitored as described above.

Chapter 3

An ECL Aptamer Switch for a High-Throughput Editing Assay

INTRODUCTION

The initial attempts to develop a high-throughput editing assay indicated that both a sensitive and stable system is required. In this chapter, reporter RNAs were designed to bind to immobilized streptavidin in response to editing. A modified version of the *in vitro* selection strategy developed in chapter 2 together with a PCR-mutagenesis strategy were used to obtain this reporter. Several different labeling methods to detect the streptavidin-immobilized reporter were explored, and an ECL detection system was found to be the most sensitive. Femtomole amounts of edited product can be detected with this assay. A mechanism is proposed for how the streptavidin-binding of the reporter is "switched on" by editing, and experiments were performed to test it.

RESULTS

1. The detection method of the assay.

The editing assay outlined in Fig. 3.1 A was designed to maximize the detection sensitivity and also to be compatible with high-throughput format. The reporter consists of an RNA editing substrate linked to a streptavidin-binding domain that is initially held within an inactive conformation. The reporter RNA is incubated with editing extract within streptavidin-coated microtiter plates. The insertion of three Us at the editing site induces a conformational change that activates the streptavidin-binding domain. As a result, the edited reporter becomes bound to the streptavidin at the well bottom, and the pre-edited RNA is removed by washing. The choice of a streptavidin-binding domain as the functional domain was for two main reasons: first, streptavidin-coated microtiter plates are widely available; second, work by others had identified a streptavidin-binding aptamer [98] with relatively good binding affinity that potentially could be exploited. A major remaining challenge was finding a sensitive enough method to detect the immobilized RNA.

Two fluorescent methods were initially tested for their suitability to detect the streptavidin-bound RNA. The previously reported streptavidin-binding aptamer was used for these studies [98]. The aptamer was incubated in a streptavidin-coated microtitre plate after which the non-bound RNA was removed by washing. In the first detection method, the bound RNA was stained with Ribo Green dye (Fig. 3.1 B, left panel). Ribo Green binds specifically to nucleotide bases, and the bound dye exhibits a QY of 0.65, which is more than 1000-fold higher than the QY of the unbound dye. In the second method, the streptavidin aptamer was covalently labeled with a fluorescent dye Alexa Fluor 488 prior to the binding reaction (Fig. 3.1 B, middle panel). The QY of Alexa Fluor 488 is 0.92, and about 4 dye molecules were integrated into one aptamer under the optimized reaction condition. Both methods are relatively simple and with low cost. Both methods are also more sensitive than the MG approach described in chapter 2, with a detection limit about 100 fmol aptamer in an 80 μl volume. However, this still would not be sensitive enough for the intended HTS.

An ECL labeling/detection method was subsequently tested and found to have the required sensitivity (Fig. 3.1 B, right panel). In this method, a special mictrotiter plate was used in which the well bottoms are coated with streptavidin and have electrodes running underneath them. The streptavidin aptamer was labeled with a ruthenium complex. The binding of the aptamer to the immobilized streptavidin brought the ruthenium complex within close proximity to the plate bottom. When a current was applied to the electrodes underneath the well bottom, electrons were transferred to the ruthenium label, and luminescent signals were generated. This method has a much higher sensitivity than the fluorescence based method, with 1 fmole streptavidin aptamer being easily detected. As a result, the chemiluminescent method was chosen as the detection method.

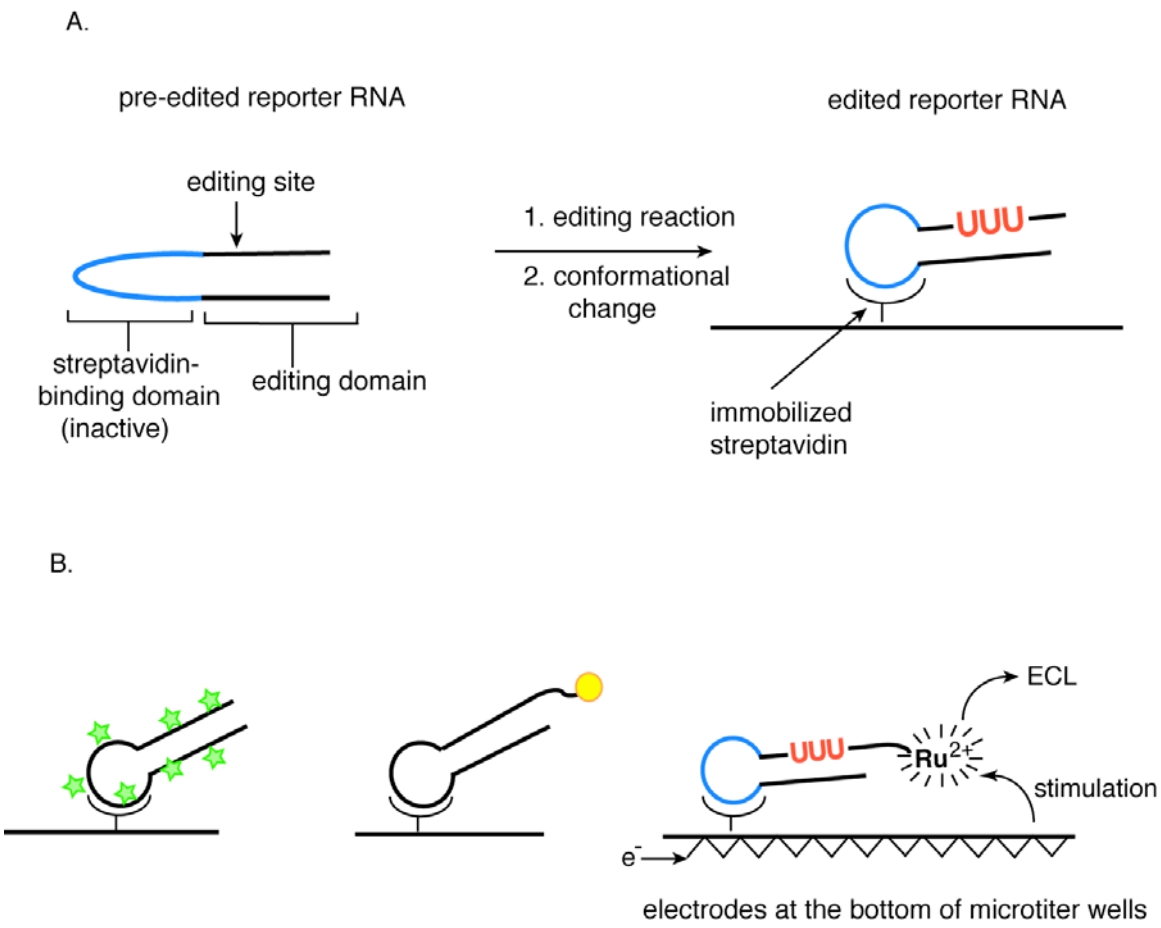

Figure 3.1. Development of a high-throughput assay for an editing reaction. (A) An editing-induced streptavidin-binding RNA. In response to the insertion of three Us (red) by the *in vitro* editing reaction, the reporter RNA undergoes a conformational change that activates the streptavidin-binding aptamer (blue). (B) Three methods of detecting the reporter RNA following the removal of the unbound RNA by washing. Left panel: the bound RNA was stained with Ribo Green dye. Middle panel: the reporter RNA is covalently labeled with a fluorescent dye Alexa Fluor 488 prior to the binding reaction. Right panel: the reporter RNA is labeled with a ruthenium complex; upon immobilization of the RNA at the bottom of a streptavidin-coated microtiter plate and electrical stimulation, the ruthenium complex will generate an ECL signal.

2. The design of the reporter.

The major challenge in developing the assay was to obtain a reporter RNA with the appropriate properties. In order for a significant fraction of the edited RNA to become bound to the streptavidin-coated plate, the edited RNA is required to have a relatively good affinity for streptavidin ($K_d \leq 1x10^{-7}$ M). At the same time, the RNA in its pre-edited state is required to have minimal affinity since any binding will lead to a high background signal and the deterioration of assay sensitivity. The reporter is further required to be an efficient substrate for the editing reaction. In other words, the reporter's streptavidin binding function needs to be inactive until switched-on by the editing reaction. Satisfying all three criteria is complicated as sequence that significantly influences editing efficiency could also have constraints related to streptavidin-binding and the switching mechanism.

Four different *in vitro* selection strategies were used in parallel in order to obtain a reporter RNA with the desired properties. The four RNA constructs with the indicated randomized regions were used for the selections (Fig. 3.2). All four constructs contain part of an aptamer (underlined) that had been selected earlier in the lab to be an efficient substrate for insertional editing [46]. The editing aptamer was previously demonstrated to have many of the attributes associated with natural mRNA substrates; these include a dependence on guiding nucleotides, similar sequence and secondary structural constraints and the co-purification of the editing activities. In construct C1 and C2, the editing aptamer part was linked to the binding site of a previously described S1 streptavidin aptamer (blue, [98]). Twenty-one positions of C1 were randomized, which included part of the editing domain, the non-consensus sequence of the S1 aptamer domain, and the region connecting the editing and the streptavidin domains. Construct C2 contained a longer connecting region and a total of 40 randomized positions. Construct C3 and C4 did not have the S1 streptavidin aptamer domain, but instead contained a long randomized region; 70 positions were randomized in C3 and 90 nt in C4.

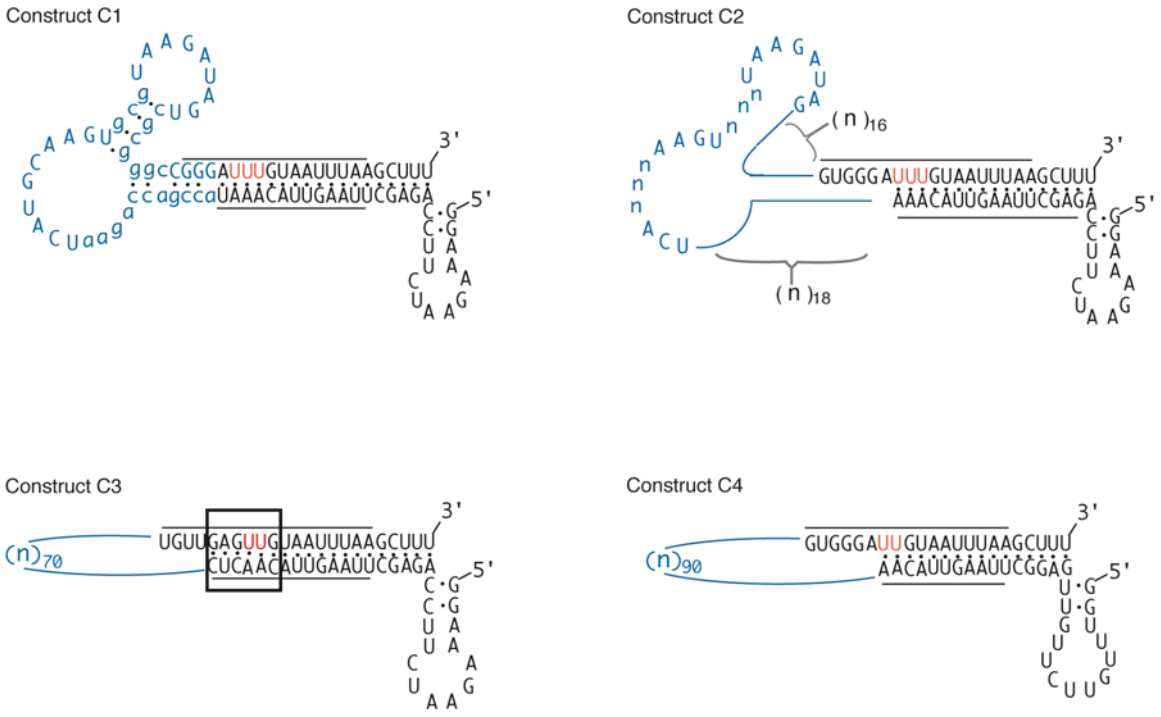

Figure 3.2. Four different RNA constructs for the *in vitro* selection. All four constructs contain part of a previously selected editing aptamer (underlined). Construct C1: the editing apatmer was linked to the binding site of a previously described S1 streptavidin aptamer (blue, Srisawat & Engelke, 2001); 21 positions (shown in lower case) were randomized. Construct C2: the editing apatmer was linked to the S1 streptavidin aptamer (blue) via 40 randomized positions (indicated by "n"). Construct C3: the editing aptamer was linked to a randomized 70 nt region (blue). Editing creates a receptor for the GNRA type of tetraloop (boxed). Construct C4: the editing aptamer was linked to a randomized 90 nt region (blue).

The rationale for using four different constructs in the selections was to increase the probability of obtaining an RNA with the desired properties. The first major uncertainty going into the *in vitro* selection was whether the S1 streptavidin-binding aptamer would be compatible with the required switching function. The advantage of containing the streptavidin-binding aptamer within the starting constructs C1 and C2 is that the only constraint put on the randomized regions during the selection would be the optimization of the linkage with the editing substrate domain. As a result, the sequence and structural permutations of the critical linkage region could be more exhaustively explored than if the streptavidin-binding domain had to be simultaneously selected from a completely randomized sequence. In contrast, it was also possible that the S1 streptavidin aptamer would be incompatible with the desired switching function of the reporter, and as a result constructs C3 and C4, in which the S1 aptamer sequence was replaced with larger random regions, were also used in parallel.

A second uncertainty was whether the desired switching function would be physically possible. The switching function will be related to a streptavidin-binding domain whose activity is in some manner dependent upon the editing reaction. In order to ensure that the helix created by the editing reaction would be compatible with a known tertiary interaction, construct C3 was engineered so that editing would create a receptor for the GNRA type of tetraloop [99]. The interaction between tetraloops and their receptors are one of the major classes of RNA tertiary interactions. Having the tetraloop receptor formed by the editing reaction provided a possible mechanism through which a streptavidin-binding domain that was dependent upon the interaction could have been selected.

A related uncertainty was the optimal number of guiding nucleotides to use in the four constructs. A large number of inserted Us increases the free energy difference between the edited and pre-edited RNAs that could be used to maximize differences in streptavidin binding. However, the efficiency of the editing reaction decreases as the

number of insertions increase, which would also decrease the reporter's efficiency. As a result, the constructs used in the selections had either 2 or 3 guiding As.

A fourth uncertainty was whether it would be better to explore a relatively small random region exhaustively or to explore a much larger random region at a much more superficial level. The number of sequence permutations that can be explored is limited to approximately 10^{14} molecules by economical and practical considerations involved with the RNA synthesis and handling. For example, the 21 randomized positions in C1 results in $4x10^{12}$ theoretical sequence variants. Assuming no other biases, 300 pmol of a 21nt randomer would result in a 99% probability of having all of the possible combinations [100]. In contrast, the 40 randomized positions of C2 results in 1 $x10^{24}$ theoretical variants which would require several thousand kilograms of RNA in order to have comparable representation. The advantage of using the 40 N construct, however, is that it is possible to select for larger structural motifs that would not be present within the 21 N RNA even though the most optimal sequence of any given motif is highly unlikely to be within the starting population.

3. *In vitro* selection and characterization of the reporter

An in vitro selection strategy was used to obtain the desired reporter RNA. Femtomole amounts of edited product can be detected with the selected reporter. A mechanism is proposed for how the streptavidin-binding of the reporter is "switched on" by editing, and chemical probing, mutagenesis and binding affinity measurements were used to test this mechanism.

The work describing the *in vitro* selection and characterization of the reporter has been published and can be found in "An electrochemiluminescent aptamer switch for a high-throughput assay of an RNA editing reaction." Liang, S and Connell, GJ (2009), RNA, 15. 1929-1938

DISCUSSION

Four different selection strategies were used in this chapter, but only the C1 selection was successful. C1 contains a previously selected streptavidin-binding domain and an editing domain; the selection pressure was exclusively on the linkage region connecting the two domains. All possible sequence permutations in the linkage region of construct C1 could be explored, unlike the three other constructs in which the random sequence was significantly greater. The C3 and C4 selections resulted in RNA pools that had two sub-populations (not shown): one subpopulation was a good substrate for the editing reaction but had low affinity for streptavidin, and the other had high affinity for streptavidin but was only poorly edited. The C3 and C4 constructs required a complete streptavidin-binding motif to be selected from the randomized regions in addition to the editing switch function. Such requirements placed significant constraints on the randomized sequence and was probably a major cause of the selection failures.

The PCR mutagenesis was another important contribution to the success of the C1 selection. After a sub-optimal population had built-up during the selection, the PCR mutagenesis introduced additional sequence variation that could be acted upon by subsequent selection pressure. Deletions were selected that occurred in regions that were fixed in the original starting population, and an extra nucleotide was inserted in the streptavidin-binding domain. These mutations were found to be critical for the properties of the selected reporter RNA.

It was also critical to maintain a fine balance among the different types of selection pressure. The desired reporter is required to have three properties: good editing efficiency, high affinity for streptavidin in the edited form and low affinity for streptavidin in the pre-edited form. Over emphasizing any single type of selection pressure resulted in an enrichment of a sub-population that was strong in one property but not in the other two. Once an undesirable sub-population became predominant during the

selection, it was not possible to restore more optimal sequence even with additional PCR mutagenesis.

Two reporter RNAs, RNA A and B, were obtained from the C1 selection; these RNAs differ at only one nucleotide position. Although the affinity of edited RNA A for streptavidin is higher than that of RNA B, the affinity of the corresponding pre-edited form is also higher. Both RNAs are edited *in vitro* with equal efficiency. In order to determine which RNA should be used as a reporter for the ECL editing assay, several common metrics for HTS, the z' factor, the coefficient of variance (CV) and signal/background ratio, were considered [107]:

$$z' = 1 - (3\sigma_{c+} + 3\sigma_{c-})/(\mu_{c+} - \mu_{c-})$$

$$CV = \sigma/\mu$$

$$\text{signal/background ratio} = \mu_{c+}/\mu_{c-}$$

In these equations, μ_{c+} and μ_{c-} are the mean values for the positive and negative control assay signals respectively, and σ_{c+} and σ_{c-} are the corresponding standard deviations for these values. For the ECL editing assay, the positive and negative controls would be the assay in the presence and absence of the essential cofactors ATP and UTP. ECL editing assays using RNA A as the reporter generated a stronger signal than that with RNA B. However, the signal/background ratio of RNA B is higher (Fig. 3.4 C). Assuming the same CV will be obtained with both RNAs, a higher signal/background ratio will lead to a higher z' factor. A z' factor ≥ 0.5 is usually accepted as the standard that has to be met by a HTS assay. Therefore, RNA B was chosen as the reporter for the HTS performed within the next chapter.

Chapter 4

Identification of Inhibitors of the RNA Editing Reaction

INTRODUCTION

The ECL editing assay developed in chapter 3 was used in an HTS for compounds that inhibit the RNA editing reaction. Many aspects of the assay needed to be optimized for it to pass a validation test that is a prerequisite for HTS. In this chapter, the reaction volume, concentration of editing extract and reporter RNA, as well as the experimental protocol were optimized for HTS. The z' factor for the optimized assay is >0.5, which is a criterion that is widely utilized to determine the suitability of an assay for HTS. A pilot screen of a 1280 compound library was performed in duplicate, and positive hits were tested for their specificity. Five compounds were identified from the screen that inhibited the editing reaction. Although some of the identified inhibitors may have potential as both probes of the editing reaction and as potential lead compounds in the development of new therapeutics, the major goal of the pilot screen was to demonstrate the feasibility of using the new assay in screens of larger libraries containing 20,000 to 50,000 different compounds. Screens of this size are usually required in order to identify an inhibitor that can survive the attrition associated with the toxicology and pharmacokinetic studies necessary for new drug development.

RESULTS

1. Optimization of reaction conditions

The ECL editing assay conditions were optimized as a prerequisite to HTS. This was necessary for three reasons. First, economical and practical constraints required that minimal quantities of the reporter RNA and editing extract be used in each reaction. Second, the linear range of the assay needed to be determined in order to maximize the sensitivity of the assay for the identification of inhibitory compounds. Third, the signal-to-background ratio and reproducibility of the assay needed to be kept high in order to decrease the likelihood of both false positives and negatives.

ECL reactions were initially performed using 9, 12 or 24 μl volumes within a 384-well microtiter plate (Fig. 4.1). The ECL assay signal did not decrease with a reduction in volume. However, the reaction volume for the assay was chosen to be 12 μl for all future studies because smaller volumes did not appear to mix efficiently within the microtiter plate wells. If a 1536-well microtitre plate could be utilized in place of the 384-well plates, it is estimated that this volume could be reduced to 4 μl without a significant deterioration in the signal/background ratio. Unfortunately, 1536-well ECL plates are not currently commercially available.

Titrating down the concentration of both the reporter RNA and the editing extract was performed with caution, as reducing the concentration of either one could potentially adversely affect the reaction kinetics. ECL editing reactions were initially performed with 1-6.5 units/μl of extract with the reporter concentration held fixed at 20 nM. The reactions were performed both in the presence and absence of the essential editing co-factor UTP (Fig. 4.2 A). The signal in the presence of UTP increased relatively linearly in the range of 1-3.5 units/μl of editing extract but started to plateau with higher amounts of extract and actually decreased when more than 5 units/μl of extract were used. This is consistent with an earlier observation indicating that higher concentrations of editing extract inhibited the binding of the edited reporter RNA to streptavidin (data not shown). Similarly, the background noise in the minus UTP reactions also decreased with increased units of extract. The titration experiment was repeated with different preparations of editing extract, and a similar pattern was observed (data not shown). The reporter RNA was then titrated from 2 to 20 nM with the editing extract held fixed at 5 units/μl. Under this condition, the reporter concentration could be reduced to 8 nM without significantly compromising the signal-to-background ratio as determined by the ratio of the ECL intensity obtained in the presence and absence of UTP (Fig. 4.2 B). The titration of the editing extract concentration was repeated with the optimized 8 nM reporter RNA concentration (Fig. 4.2 C); the optimal concentration of editing extract was finalized to be 5 units/μl, as it generated the optimal signal/background ratio and is also within the linear range of the plot.

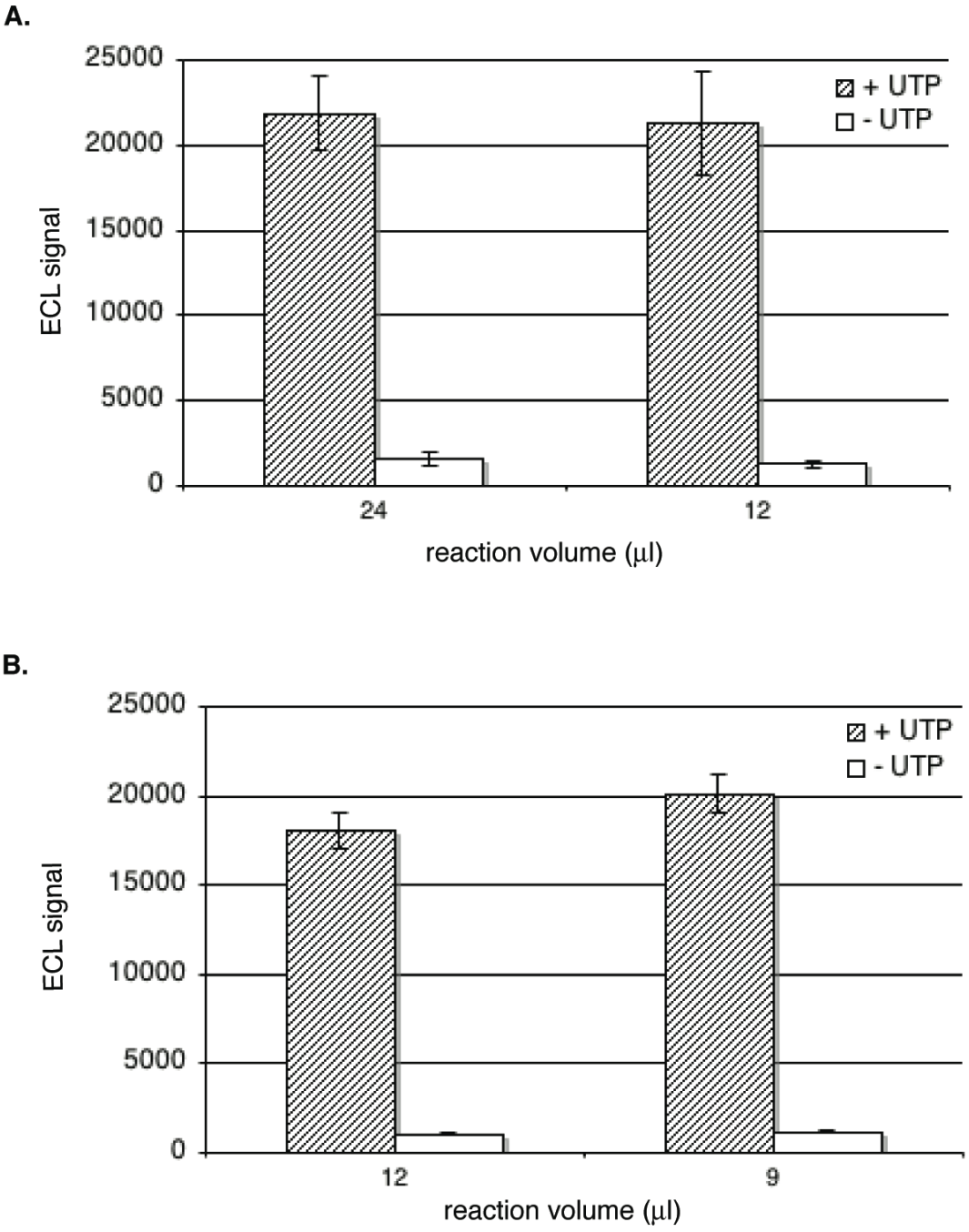

Figure 4.1 Minimization of the assay volume. The ECL reactions were performed in the presence (striped) or absence (white) of UTP, an essential co-factor for the editing reaction. (A) ECL signals with 24 µl vs. 12 µl volumes (n=4). (B) ECL signals with 12µl vs. 9µl volumes (n=4).

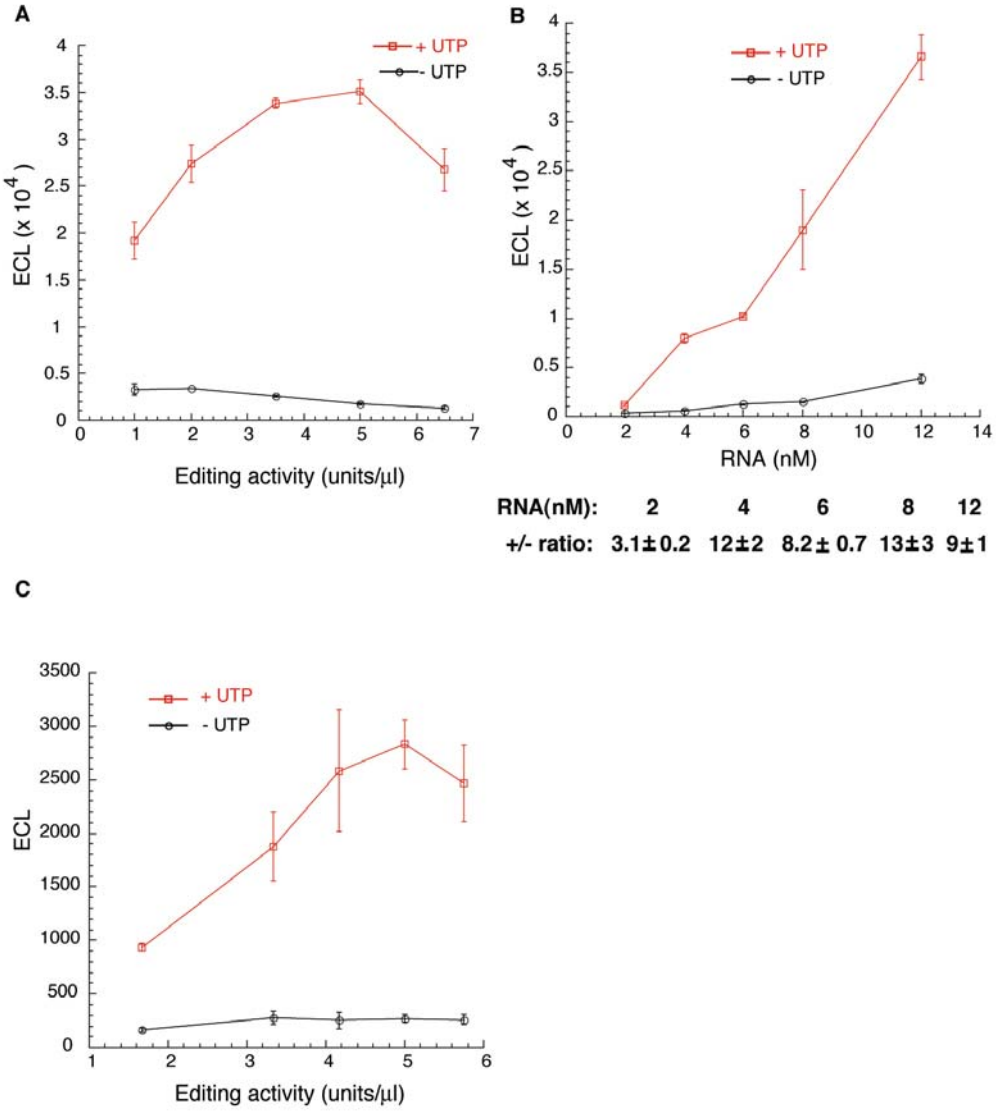

Figure 4.2 Minimization of the editing extract and reporter RNA concentrations. The ECL reactions were performed either in the presence (+UTP, red line) or absence (-UTP, black line) of UTP. (A) Reactions contained 20 nM of the pre-edited reporter and the indicated units of editing activity (n=2). (B) Reactions contained 5 units/µl of editing activity and the indicated concentration of reporter. The ratio of the ECL signal obtained from each plus UTP reaction to the signal obtained with the corresponding minus UTP reaction is indicated below the plot (+/- ratio, n=3) (C) Reactions contained 8 nM of the pre-edited reporter RNA and the indicated units of editing activity (n=3).

A series of experiments were also performed to modify the editing assay so that it would be more suitable for the HTS format. These changes include plate blocking, reaction mixing, incubation temperature and reaction time. Details are provided in Materials and Methods of this chapter.

2. Validation of the assay

Drug discovery assays need to pass a validation test in order to be used in HTS. The z' factor is a common indicator for an assay's quality[107], which is defined as:

$$z' = 1 - (3\sigma_{c+} + 3\sigma_{c-}) / (\mu_{c+} - \mu_{c-})$$

In this equation, μ_{c+} and μ_{c-} are the mean values for the positive and negative control assay signals respectively, and σ_{c+} and σ_{c-} are the corresponding standard deviations for these values. A z' factor ≥ 0.5 is usually accepted as the standard that has to be met by a HTS assay.

Assay plates in a special stripey format were used to obtain the z' factor. The layout of a 384-well stripey plate is presented in Fig 4.3. It consists of four types of 16-well columns that are replicated six times across the entire plate: the positive control column (red, +extract/+UTP) contained the complete ECL reaction; the minus UTP column (grey, +extract/-UTP) served as a negative control; the minus extract column (yellow, -extract/+UTP) was a second negative control, and the buffer only column (green, buffer only) was used to assess the background of the system. To validate the assay and confirm its reproducibility, two stripey plates were assayed on each of two different days.

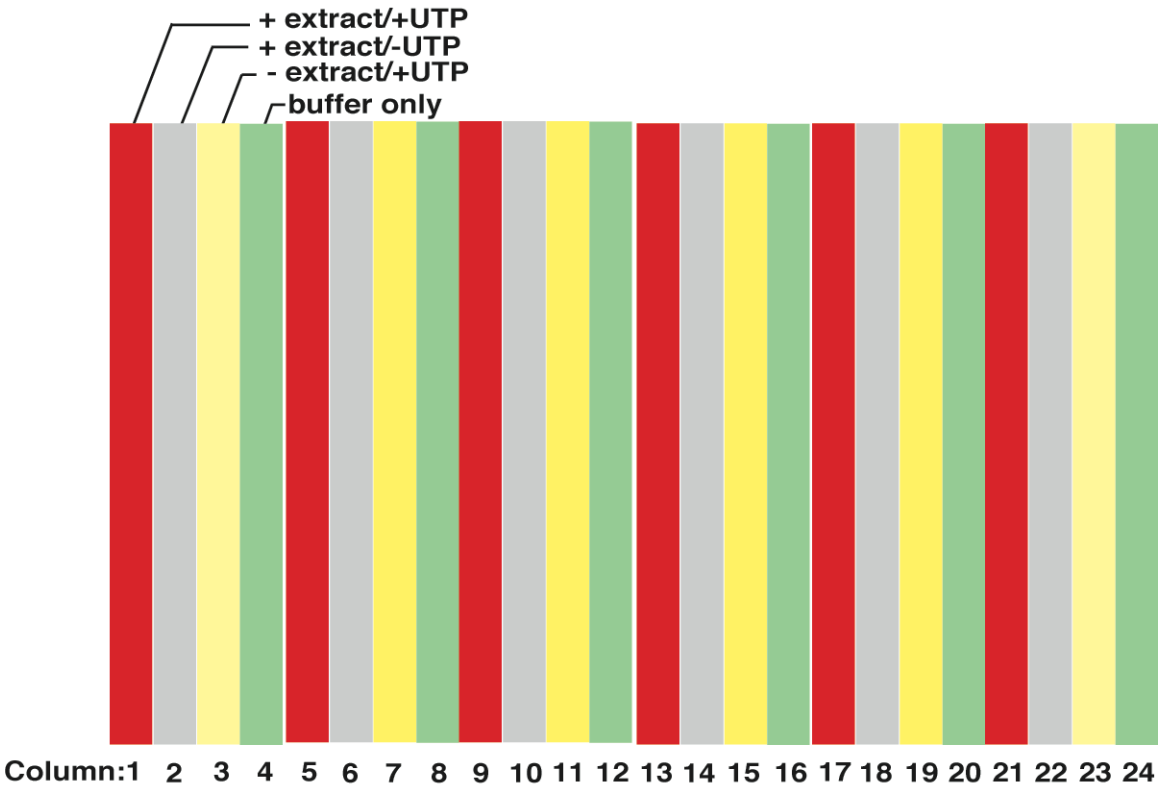

Figure 4.3 Format of a 384-well stripey plate. The stripey plate consisted of four types of 16-well columns that were replicated six times across the entire plate: the positive control column (red, +extract/+UTP), the minus UTP negative control column (grey, +extract/-UTP), the minus extract negative control column (yellow, -extract/+UTP), and the buffer only column (green, buffer only). Details of the reactions are provided within the Materials and Methods section of this chapter.

An unexpected signal gradient was observed with the first set of two stripey plates (Fig 4.4 A). Signals at the left end of the plate were significantly lower than that at the right end of the plate, but the signals within each column had a satisfying reproducibility. Signals of both the positive and negative control columns increased from left to right across the plate (Fig. 4.4 A). This suggested that the signal gradient is not intrinsic to the assay but is more likely associated with either the reagent delivery or plate washing conditions. If this is the case, a signal gradient in the opposite direction would be expected if the plate is rotated 180 degrees during reagent delivery and washing, and this was confirmed (Fig. 4.4 B).

A series of experiments were performed to identify the cause of the signal gradient. In the first stripey set, all liquid handling, which includes reagent delivery and plate washing, was done by a Multidrop Combi (Thermo Scientific), which is an automated delivery system. One possible cause of the left-to-right gradient was that different volumes were added in a left-to-right direction across the plate. To investigate this possibility, a fluorescein-labeled probe was used as a control to test the accuracy of the delivery equipment. No fluorescent gradient was observed with the fluorescein probe, suggesting that the delivery of the assay components was accurate. A second possibility was that the force generated by the washing step could be non-uniform across the plate; it had previously been suggested for ELISA-based assays that the aggressiveness of some automatic plate washers could cause high variance. In the ECL assay, if the flow is more forceful towards one end of the plate, it could cause more dissociation/shearing of the streptavidin-bound reporter RNA, whose affinity for the immobilized streptavidin is relatively low (10^{-7}M). Several different plate washers were used in an attempt to reduce the left-to-right variance, and the Perkin-Elmer Flexdrop automated dispenser was found to significantly reduce but not completely eliminate the left-to-right variance. Rotating the plate 180 degrees during washing did not cause a corresponding shift in the direction of the variance which suggested that another variable was contributing to the remaining variance. It was eventually discovered that the streptavidin-reporter complex was unstable in the tripropylamine-containing read buffer

used to generate the ECL signal, and that this instability was a major source of the remaining variance. To avoid differences in dissociation caused by temperal differences in the delivery of the read buffer, a Biomek FX Laboratory Automation Workstation (Beckman Counter) was used to simultaneously add 35 μl of the read buffer to each microtitre well.

Four stripey plates were performed on two seperate days with z' factors of 0.53, 0.59, 0.59, and 0.65. Two stripey plates were further performed in the presence of 0.01% DMSO, which is the solvent used to dissolve the chemicals of the library. The z' factors were 0.53 and 0.59 for these two plates, indicating that the DMSO added with the chemicals during the HTS would not adversely affect the assay. These results indicated that the ECL editing assay was acceptable for HTS.

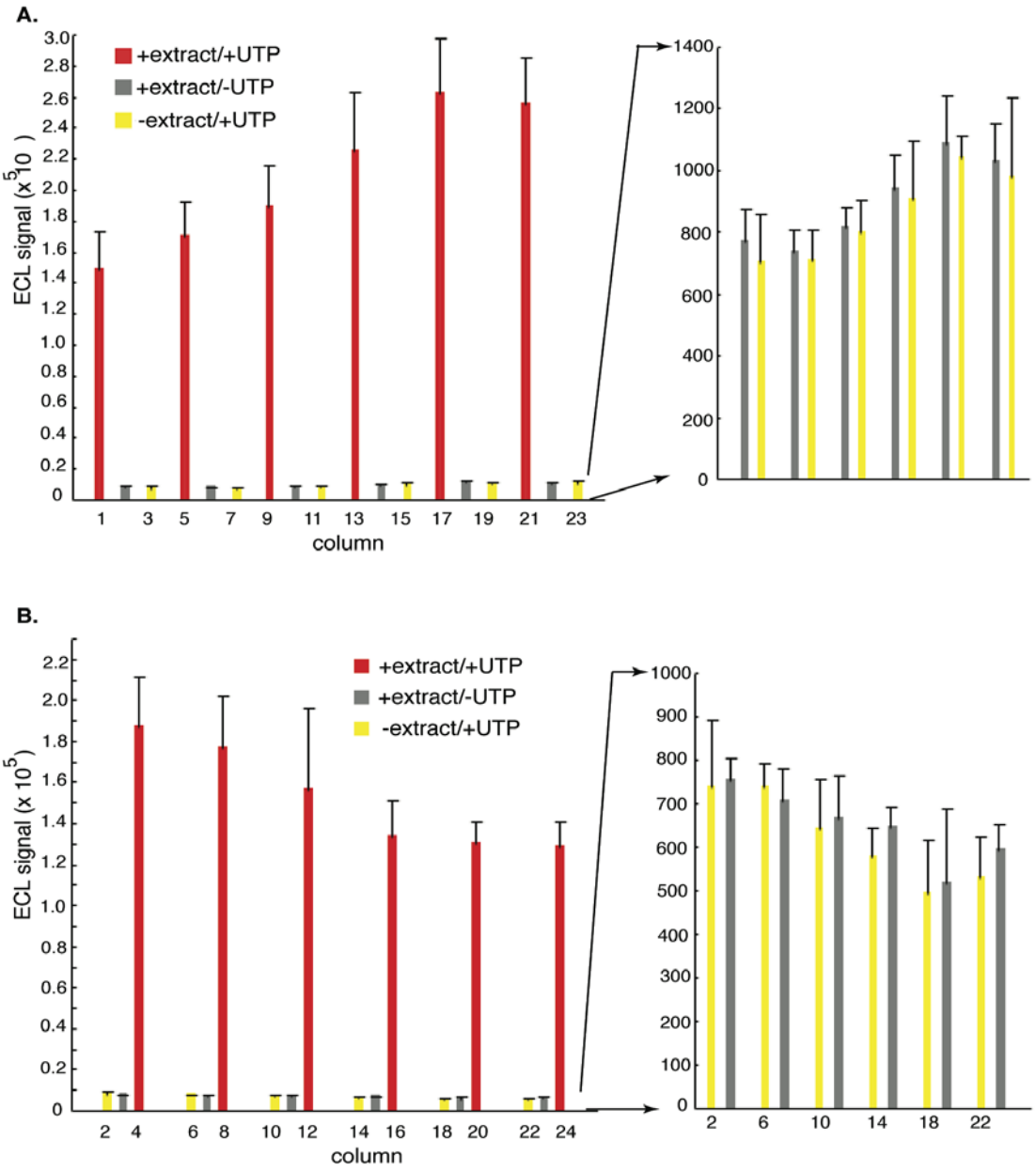

Figure 4.4 The ECL signal gradient problem detected on the stripey plates. The mean ECL values and the standard deviations were obtained from the positive control column (red, +extract/+UTP), the minus UTP negative control column (grey, +extract/-UTP), and the minus extract negative control column (yellow, -extract/+UTP). The signals of the two negative control columns are also plotted on an enlarged scale to indicate that the gradient effect also occurred independent of the editing reaction. (A) Reagent delivery and plate washing were in the direction from column 1 to column 24. (B) Reagent delivery and plate washing were in the direction from column 24 to column 1.

3. A pilot drug screen

A pilot screen was performed which further validated the application of the ECL editing assay in HTS. A library of pharmacologically active compounds from Sigma-Aldrich (LOPAC 1280) was used for the validation. This library contains 1280 well-characterized compounds that cover a broad range of cellular targets. It is one of the most commonly used libraries to validate new drug discovery assays. The library compounds, dissolved in DMSO, were transferred to individual wells and incubated for 15 minutes with a reaction mix containing editing extract and the reporter RNA. The editing reactions were then initiated through the addition of UTP. The final concentration of each test compound in the screen was 10 μM. Positive (+ UTP, no compound) and negative control (- UTP, no compound) reactions were included within both outer columns on each 384-well plate as a means to monitor the assay quality. The screening was performed in duplicate, and 28 compounds were identified that had over a 30% inhibitory effect in both replicates.

False positives are common with HTS, and compounds that interfere with streptavidin binding or the generation of ECL would cause inhibition of the assay signal. Ruthenium Red was one of the 28 compounds identified from the screen and since it consists of three coordinated ruthenium ions, it is likely interfering with the activation of the ruthenium complex of the reporter RNA. Three other selected compounds inhibited the assay signal significantly below that of the minus UTP negative control which is suggestive that they are either interfering with streptavidin binding or the generation of the ECL signal, and as a result these were not characterized further.

In order to test the remaining hits identified from the LOPAC screen, an editing assay that is independent of the ECL/streptavidin binding –linked reporter was initially used to confirm the inhibitory effect of the compounds. A radiolabeled editing substrate RNA was circularized to block the TUTase activity of the editing extract from acting at the 3' end of the substrate RNA [46, 80]. Editing extract was pre-incubated with the

radiolabeled RNA and the selected compounds or DMSO for 15 minutes prior to initiating the editing reaction through the addition of UTP. After resolution of the edited product by gel electrophoresis and PhosphorImage analysis, the efficiency of the reaction was quantified. Eleven compounds were initially tested with the radiolabeled-based assay, and seven demonstrated an inhibitory effect greater than 30% (not shown). However, the inhibition of four of the seven compounds was not reproducible among different batches of editing extract. A common cause of false positives is promiscuous inhibitors that form aggregates with enzymes nonspecifically, and this type of inhibition has been shown to be inconsistent and highly sensitive to the target enzyme concentration.

A method that has been used in other systems to overcome the non-specific aggregate based inhibition is to include 0.01% TX100 within the assay [111, 112]. When the radiolabeled editing assay of the initial panel of eleven LOPAC hits was repeated in the presence of TX-100, only three compounds remained inhibitory. The remaining panel of 13 LOPAC hits was also assayed with the radiolabeled substrate in the presence of TX-100 and three additional detergent-resistant inhibitors were identified, bringing the total to six. These include GW5074, 6-hyroxy-DL-DOPA, mitoxantrone, NF023, protoporphyrin and D-sphingosine. The 6-hydroxy-DL-DOPA is highly susceptable to oxidation, and it was found that whereas older solutions reproducibly inhibited the editing reaction, fresh solutions did not. As a result, this inhibitor was not characterized further. The inhibition of the remaining five compounds on the radiolabeled editing assay is summarized in Fig. 4.5.

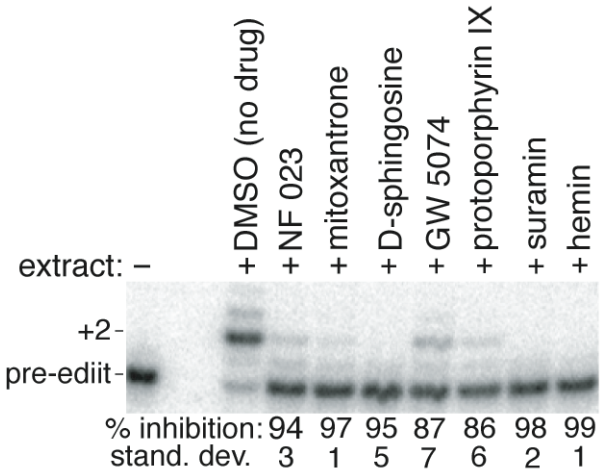

Figure 4.5 Testing the effect of the selected inhibitors on a radiolabeled-based *in vitro* editing assay. The editing reactions were incubated with the indicated compounds or DMSO for 15 minutes prior to the initiation of the reaction with UTP. The pre-edited RNA and the correctly edited product containing 2 U-insertions (+2) are indicated. The efficiency of editing was calculated for each reaction from the ratio of correctly edited product to total RNA. The percent inhibition indicated for each reaction is the ratio of its editing efficiency relative to that of the DMSO (no drug) control (n=3).

Two additional inhibitors were investigated in this study. NF023, which was identified in the LOPAC screen, is closely related to suramin which is a drug of undefined mechanism that has been used to treat *T. brucei* infections (reviewed in [113]). When tested on the editing assay, suramin was found to inhibit 98±2% (n=3) of the editing activity when used at 10 μM (Fig. 4.5). Protoporphyrin, which was also identified from the screen, is the final porphyrin precursor in the synthesis of heme. Heme was also tested in the editing assay and found to be an extremely effective inhibitor (Fig. 4.5). This potentially could be relevant to the *in vivo* regulation of editing as the heme/protoporhyrin concentration in the parasite's environment varies dramatically during the life cycle of the parasites.

The IC-50 value for the inhibition by the detergent resistant inhibitors of the RNA editing reaction and several unrelated enzymes were measured using the radiolabeled assay. The IC-50 values for the inhibition of the editing reactions were all in the range of 1-3 μM. β-galactosidase and chymotrypsin are commonly used as assays for non-specific inhibition [114], and the effect of the identified inhibitors were tested on these enzymes. No significant inhibition of the enymes was detected using mitoxantrone, NF 023, and sphingosine; the IC-50 values for these compounds are all > 1 mM. The IC-50 value for GW5074 is aproximately 200 μM with chymotrypsin and > 250 μM with β-galactosidase. The IC-50 of protophophyrin could not be determined because the drug is not soluble at concentratiuons higher than 10 μM in the β-galactosidase and chymotrypsin assay buffers. However, the activity of both enzymes was only inhibited approximately 10% by 10 μM protophophyrin.

DISCUSSION

This thesis identified the first specific inhibitors of the Leishmania RNA editing reaction. For the inhibitors of the RNA editing reaction to reach their site of action and have therapeutic value, the compounds need to be able to cross both the plasma membrane and mitochondrial membrane of the Leishmania parasites. Since the parasites

reside within the phagolysosome of macrophages of infected individuals, the inhibitors are also required to cross the plasma and phagolysosome membranes of the macrophage.

Because of the barriers to the distribution of potential anti-Leishmania drugs, highly charged inhibitors of the editing assay such as NF 023 would be unlikely to have therapeutic value as they may not be able to cross the membranes and reach their site of action at significant concentration. NF 023, however, is a closely related analog of the drug suramin, which although not effective against leishmaniasis has been widely used to treat *T. brucei* infections. The highly negatively charged suramin is able to enter *T. brucei* cells because it becomes bound to lipoprotein within human sera and *T. brucei* has high affinity lipoprotein receptors. The anti-trypanosome mechanism of action of suramin has not been identified. Although the inhibition of the editing reaction by NF 023 and suramin is suggestive of a mechanism, suramin has been shown to inhibit several other enzymes that could also be contributing to the drug's therapeutic benefit.

The remaining four identified inhibitors are more lipid-soluble and as a result have a greater likelihood of being able to reach their site of action within the mitochondria. Mitoxantrone is a topoisomerase II inhibitor that is approved as a second-line treatment for prostate cancer. The drug is a strong intercalator of DNA and could possibly be disrupting RNA structure although this has not been reported. It is a highly toxic drug, and as result probably would not have application in the treatment of leishmaniasis. However, there are several structural analogues of the drug that are under clinical trial that are reported to be less toxic and these could have more therapeutic potential. GW5074 is an inhibitor of RAF-1, which is a kinase that is part of the MAPK/ERK signal transduction pathway and as a result is likely to have major side effects if used therapeutically. Since some inhibition of both β-galactosidase and chymotrypsin were detected at 10 μM, it is also unlikely that the compound would be specific enough to have therapeutic potential. Sphingosine is an 18-carbon lipid molecule. It is an inhibitor of protein kinase C, and as a result it could also have major side effects if used therapeutically. Protoporphyrin and heme, as already indicated, are

interesting because both compounds could be natural regulators of the editing reaction. The trypanosomatid parasites undergo major biochemical and morphological changes between the insect and bloodstream stages and although editing is essential in both stages, the reaction occurs much more extensively within the insect stage than within the bloodstream form.

In order to identify novel drugs from HTS that have both the desired pharmodynamic and pharmacokinetic properties, it is usually necessary to screens libraries containing 20,000 to 50,000 chemical compounds. The ECL editing assay was validated for HTS, and the pilot drug screen demonstrated that it is ready to be used in screens of larger chemical libraries. The pilot drug screen indicated that false positives potentially could significantly complicate larger screens as much time and expense is required to identify them. Among 28 selected positive hits from the LOPAC screen, only 6 retained significant inhibitory activity in the presence of 0.01% Trtion X-100, suggesting detergent should be included in future HTS to reduce the number of non-specific hits. In fact, detergent is frequently included in primary screens to eliminate false positives [111, 112]. Detergent was not included in the pilot screen because the compounds in the LOPAC library are all pharmacologically active, and it wasn't expected that more than a few hits would be promiscuous. It is possible that because the editing complex contains many protein components and has a large surface area, aggregation is more likely to occur with certain compounds. Indeed, those non-specific editing inhibitors that were removed by 0.01% Triton X-100 had little or no affect on the β-galactosidase assay and chymotrysin assay, suggesting they are not overly "promiscuous".

The mitochondrial extract that was used for the drug screens was prepared from *Leishmania tarentolae*. This is a non-human pathogen that has been widely used to characterize the Leishmania editing reaction. In addition to being classified as a biosafetey level 1 organism, the major advantage of using this organism is that it can be grown to to high densities ($\approx 2 \times 10^8$ cells/ml) in relatively inexpensive media that does not require serum. The human pathogenic species such as *Leishmania donovani* and

Leishmania major do not grow to anywhere near the same densities even with the use of expensive media additives, and as a result the drug screen would not be feasible with these species. The disadvantage of using extract prepared from *Leishmania tarentolae* is that it is possible that some of the identified drugs may not work on the human pathogens. However, most of the *Leishmania tarentolae* editing complex proteins have a high amino acid identity with the homologs from the pathogenic species, and as a result many of the drugs identified from the screen would be expected to be inhibitory, and these drugs could be readily identified from secondary screens using the pathogenic species.

MATERIALS AND METHODS

1. The stripey assays

Stripey assays and the LOPAC screens were performed in streptavidin-coated, standard-capacity 384-well ECL plates (Meso Scale Discovery). Plates were pre-blocked with Superblock buffer (Thermo Scientific) and washed three times prior to use with 0.05% Tween 20 in 50 mM NaCl and 25 mM Tris (pH 8.0, 27°C) and one time with binding buffer (4 mM $MgCl_2$, 50 mM KCl and 5 mM Tris at pH 8.0). Plate washing was performed with a Flexdrop (Perkin-Elmer).

The stripey plate contained four columns of 16 wells that were replicated six times across the entire plate (Fig. 4.3). The positive control column (+extract/+UTP) contained the reporter RNA, editing extract, and the editing co-factors ATP and UTP. The minus UTP column (+extract/-UTP) contained the reporter RNA, the editing extract, and the editing co-factor ATP but no UTP. The minus extract column (-extract/+UTP) contained the reporter RNA, ATP, UTP but no editing extract. The buffer only column contained 12 μl of binding buffer. For the positive control and the minus UTP columns, pre-edited reporter RNA was denatured for 5 min at 65°C in 2.4 volumes of denaturing buffer containing 0.2 mM EDTA and 25 mM Tris (pH 8.0, 27°C). The RNA was then

mixed with 3 volumes of buffer containing 4 mM ATP, 4 mM dithiothreitol, 20 μg/ml leupeptin, 12 mM $MgCl_2$, and 2 mg/ml Pefabloc SC, and then incubated for 10 min at room temperature. Editing extract, in 3.6 volumes containing 16.7 units of editing activity per micro-liter, was added to the reaction mix. Nine microliters of the reaction mix were delivered into each microtitre well, and 3 μl UTP solution (4 mM UTP, 8 mM $MgCl_2$) or dH_2O was then added to wells of column 1 or 2, respectively. Column 3 was the same as column 2 except 3.6 volumes of dilution buffer (25mM Hepes, pH7.5, 10 mM $MgCl_2$, 1mM EDTA, 10% glycerol, 225 mM KCl, 1 mM dithiothreitol, 5 μg/ml leupeptin, and 0.5 mg/ml Pefabloc SC) instead of the editing extract, were added to the reaction mix. Liquid dispensing of 12 μl , 9 μl and 3 μl was with a Multidrop Combi (Thermo Scientific) dispenser. Microtitre plates were then incubated at 27°C for 4 hr followed by washing four times with wash buffer (4 mM $MgCl_2$, 150 mM KCl and 5 mM Tris at pH 8.0). Washing was performed with a Perkin-Elmer Flexdrop. To avoid differences in dissociation caused by read buffer delivery times, a Biomek FX Laboratory Automation Workstation (Beckman Counter) was used to simultaneously add 35 μl of read buffer T plus surfactant (Meso Scale Discovery) to each microtitre well. The ECL signals were recorded using a SECTOR Imager 6000 Reader (Meso Scale Discovery).

2. The LOPAC screens

For the LOPAC screens, 12 nl of DMSO or 10 mM compound in DMSO was transferred to designated microtitre wells by an Echo liquid handler (Labcyte). RNA was denatured and refolded the same as for column 1 of the stripey plate. After the reporter RNA and editing extract were mixed, 9μl were delivered to each microtiter well and incubated with the previously delivered chemical compounds at room temperature for 15 min. The reaction of the positive control and assay wells were initiated with 3 μl UTP solution (4 mM UTP, 8 mM $MgCl_2$), and 3 μl dH_2O was added to negative control wells. Liquid dispensing of 9 μl and 3 μl was performed by Multidrop Combi (Thermo Scientific).

3. The chymotrypsin assays

For chymotrypsin assays, the enzyme (10 nM) was incubated with either DMSO or chemical compounds (1-1000 μM) at room temperature for 15 min, and the reaction was initiated with 200 μM of the succinyl-ala-ala-pro-phe-p-nitroanilide substrate. Reaction progress was monitored at 405 nm at 37°C. The inhibitory effect was determined by comparing the reaction rate of each test compound reactions with that of the DMSO control.

4. The β–galactosidase assays

For β–galactosidase assays, the enzyme (1.5 nM) was incubated with either DMSO or the chemical compounds (1-1000 μM) at room temperature for 15 min, and then the reaction was initiated with 1 mM *ortho*-Nitrophenyl-β-galactoside. Reaction progress was monitored at 405 nm at room temperature. The inhibitory effect was determined by comparing the reaction rate of the test compound reactions with the DMSO control.

5. IC50 determination

To determine the IC50 of the selected compounds for the editing reaction, 0.4 pmol of a radiolabled editing substrate RNA was denatured for 5 min at 65°C in 10 μl denaturing buffer containing 0.2 mM EDTA and 25 mM Tris (pH 8.0, 27°C). The RNA was then mixed with 12.5 μl buffer containing 4 mM ATP, 4 mM dithiothreitol, 20 μg/mL leupeptin, 12 mM $MgCl_2$, and 2 mg/mL Pefabloc SC, and it was incubated for 10 min at room temperature. Fifteen microlitre of editing extract (16.7 units of editing activity per micro-liter) was added, and the reaction mix was incubated with either DMSO or a chemical compound at indicated concentration for 15 min at room temperature. The reaction was initiated by 12.5 μl of UTP solution (4 mM UTP, 8 mM

$MgCl_2$). Edited RNA was resolved from the non-edited one by denaturing gel-electrophoresis, and the editing effeciency was quantified by PhosphorImage analysis.

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