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Inhibition of therapeutically important polymerases with high affinity bis-intercalators

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ABSTRACT

We have previously demonstrated that polymerases such as telomerase can be inhibited by molecules (e.g., intercalators) that target the key RNA/DNA duplex substrate. In this work we show that this also holds true for reverse transcriptase, and show that the lead intercalators can be modified to increase inhibition efficacy. Specifically, we use the strategy of multiple simultaneous intercalation, by linking two intercalators with a variable linker. The rationale behind this design is that a specific linker has the potential to increase affinity and specificity for the target duplex. We have synthesized a library of 45 ethidium bis-intercalators in which the distance between intercalators is systematically varied. We observe that members of the dimer library have improved telomerase and reverse transcriptase inhibition, relative to the monomeric leads. We show that this improvement in inhibition over mono-intercalators is most prominent when non-productive sites of inhibitor binding are limited in the assay mix. When this is done, a 400-fold increase in inhibition efficacy is observed.

Polymerases and other enzymes that process nucleic acids form an important class of therapeutic targets. Members of this class include the anti-cancer target telomerase, and the reverse transcriptase from HIV. Previously, we have developed a strategy to inhibit telomerase by targeting small molecules to the RNA/DNA duplex that forms during telomerase's catalytic cycle.[1] This duplex forms when the template portion of telomerase's RNA subunit binds to the telomeric DNA, acting as a template for the DNA's extension. The rationale behind our approach is that molecules that bind this duplex, for example intercalators, can distort it, thereby interfering with the extension of the DNA and possibly its translocation. It seems reasonable that other polymerases could be inhibited through a similar mechanism. We previously demonstrated that several intercalators act as inhibitors of telomerase, and that their efficacy correlates with their affinity for the telomerase RNA/DNA duplex.[1,2] The most effective intercalator, ethidium, outcompeted other tested intercalators for binding to an isolated RNA/DNA duplex with the telomerase sequence. In addition, Ren and Chaires demonstrated that ethidium, when allowed to compete simultaneously for 15 different nucleic acid structures, bound most avidly to an RNA/DNA duplex.[3] The IC_{50} we observed for ethidium was 3.3 μM. The aim of our subsequent work has been to increase the efficacy of inhibitors against telomerase by increasing their affinity.

While some intercalators are used as drugs, they represent a challenging starting point for drug design. The cell contains high concentrations of nucleic acids that can act as alternative binding sites, leading to reduced concentrations of free intercalator, as well as toxic effects. Our lab is therefore examining multiple strategies for adding specificity to the inherent ability of these molecules to target therapeutically important nucleic acid structures. Starting from a high affinity intercalator, there are multiple strategies for increasing its affinity and specificity. The approach we take in this work is to attempt multiple, simultaneous intercalation. This has the potential to increase affinity, through the multi-dentate effect. In addition, it also has the potential to increase specificity, if the specific distance and geometry of the linker allows for simultaneous (and hence high affinity) intercalation with the *target* nucleic acid, while preventing bis-intercalative binding to unwanted sites (such as genomic DNA). We apply this approach both to telomerase and HIV reverse transcriptase (RT). Specifically, we tested both commercially available bis-intercalators (a bis-ethidium and a bis-acridine), as well as a library of bis-intercalators that we synthesized, where the linker distance and geometry was systematically varied. This resulted in the identification of several low nano-molar inhibitors of reverse transcriptase, and mid-nano-molar inhibitors of telomerase. Interestingly, differences between different library members were only observable when competing nucleic acids were limited, either by reducing substrate concentration in the case of RT, or by purifying telomerase from other cellular components found in cellular homogenate.

To identify potentially high affinity and high specificity bis-intercalator inhibitors of telomerase, we built a library, based on

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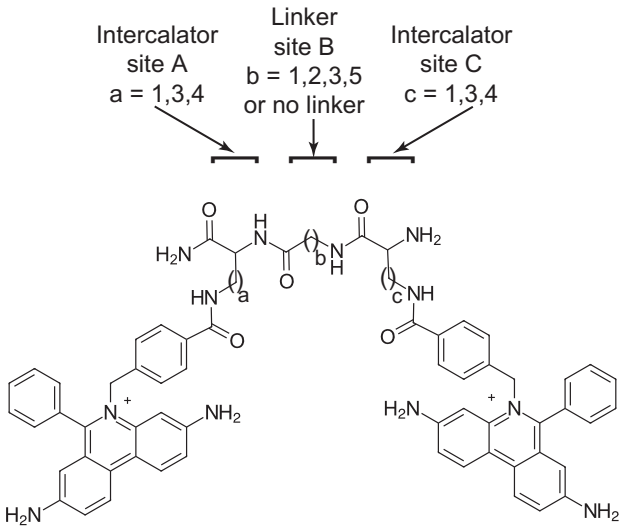
our lead, ethidium. Specifically, we used ethidium benzylic acid (EBA), a monomer developed in our lab that allows for easy conjugation to amines.[7] We previously have shown that both the parent EBA and simple derivatives maintain telomerase inhibition ability. We constructed a library of scaffolds that present two amines to be acylated by EBA (Scheme 1). The library of 45 compounds was designed to vary both the distance between the intercalators as well as the potential conformations. The first and third positions were either di-amino propionic acid (Dap), ornithine (Orn), or lysine (Lys). The second or linker position was either glycine (Gly), beta alanine (β-Ala), gamma amino butyric acid (Gaba), epsilon amino hexanoic acid (Ehx), or no linker. We used an orthogonal Mtt protection scheme that allowed us to construct the scaffold on resin, and in the late stages of the synthesis install the EBA. Representatives of the library were characterized by ESI-MS, and showed the expected molecular weights (see Supplementary data for these ESI-MS and complete experimental detail, including assay setup).

The library was then screened using a single point PCR assay of telomerase activity.[4] The results show a narrow range of low micro-molar estimated IC_{50} values from 2.1 to 13.6 μM, similar to the parent EBA inhibition value (data not shown). This was somewhat surprising, as we expected that molecules capable of bis-intercalation would have greater efficacy compared to mono-intercalators if in fact they bis-intercalated. To confirm the low affinity indicated during single point screening, we determined a full IC_{50} of eight of the compounds. The determined IC_{50} values for the eight compounds varied from 0.58 to 1.81 μM, somewhat lower than the single point estimates, but close to the original lead mono-intercalator. A representative inhibition curve of compound **1**, Dap-β-Ala-Lys, with an IC_{50} of 0.60 μM is shown in Figure 1a. One explanation for the lack of improvement in these bis-intercalators over the lead mono-intercalator was that the library members were simply incapable of simultaneous bis-intercalation. This seemed less likely as the library covered many possible distances and conformations. A second explanation was that the putative concentration of free bis-intercalator in the system was actually significantly less than the amount added, due to non-specific binding of the bis-intercaltor by non-telomerase species in the assay. This hypothesis was supported by the shape of the inhibition curve, which showed signs of tight binding (sharp transition in the curve). The assay as performed uses HeLa cell homogenates that include many components of the cellular contents, including other nucleic acids. These could potentially bind to the bis-intercalator in the assay mix, and reduce their concentration.



Scheme 1.

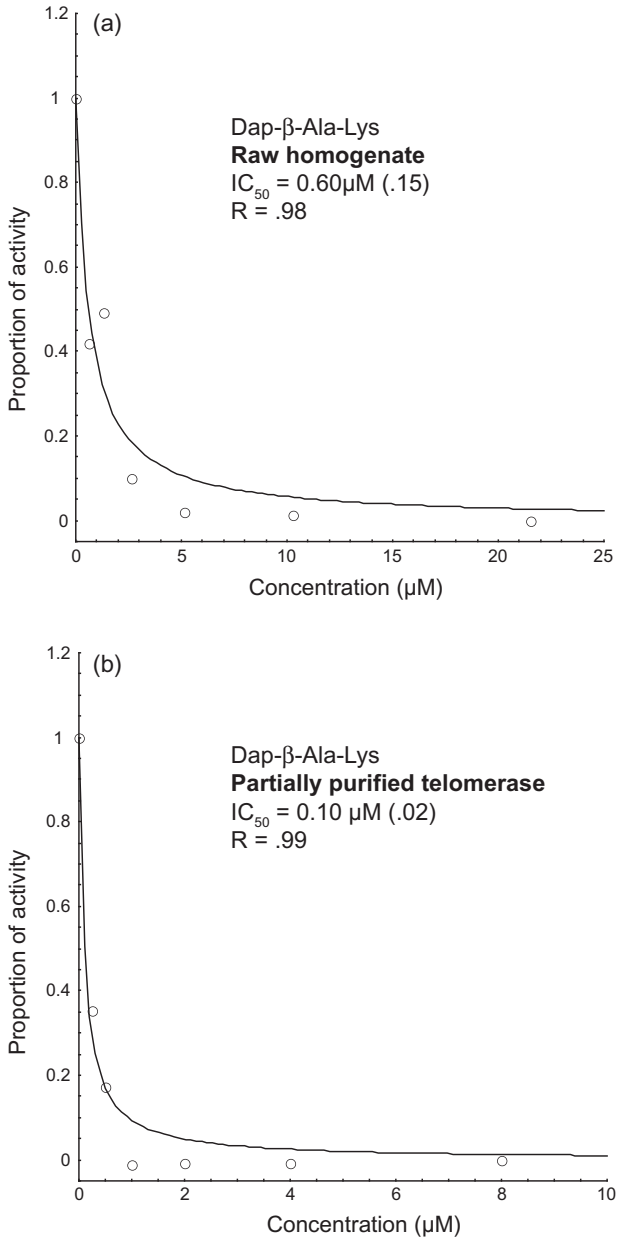


Figure 1. Telomerase inhibition assay of compound **1**, Dap-β-Ala-Lys, using whole cell homogenate (a, top), or partially purified telomerase (b, bottom). Error values are indicated in parenthesis.

To test for this effect, we then repeated the inhibition assay using partially purified telomerase, prepared using a procedure derived from Szatmari et al.[5] This method likely limits the amount of extra nucleic acids present. When we retested the IC_{50} of compound **1** it showed a significantly lower IC_{50} value of 103 nM (Fig. 1b).

In a parallel effort, we also examined the ability of bis-intercalators to inhibit reverse transcriptase from HIV-1. Because HIV-RT also depends on a duplex substrate, it seemed reasonable that it could also be inhibited by intercalators. The distortion of the duplex substrate that would result from intercalator binding was anticipated to limit enzyme activity.

We initially screened a range of 14 mono-intercalators using a single point assay of HIV-RT activity.[6] This showed estimated IC_{50} values of 2 μM and above (Table 1). This was consistent with our previous screening results of mono-intercalators against telomerase. It is of interest to note that ethidium had the greatest inhibition

efficacy, as it previously showed the greatest efficacy against telomerase. With both HIV-RT and telomerase, our target is an RNA/DNA heteroduplex. Previously Ren and Chaires have shown that of all the common intercalators, ethidium has the greatest preference for RNA/DNA duplexes over other nucleic acid structures.[3] In addition, we have shown that when multiple intercalators simultaneously compete for an RNA/DNA duplex, ethidium preferentially binds.[2]

To improve the inhibition of HIV-RT by ethidium, we then tested a commercially available ethidium dimer (Fig. 2, ethidium homodimer-1). The rationale behind this approach was to increase inhibition through bis-intercalation. While the binding curve was not particularly 'clean' it gave an IC_{50} fit value of 2.3 μM (Fig. 3a). This was surprising, as we originally anticipated that a molecule capable of bis-intercalation would have improved inhibition efficacy through higher affinity. Because of the poor fit of the binding curve, we suspected that we were in a tight-binding situation, where the actual concentration of ligand in solution was in fact much lower than the theoretical, due to sequestering of the ligand by high concentrations of binding sites in the assay mix. A potential cause of this was the high concentration of nucleobases found in our standard HIV-RT RNA/DNA substrate. Intercalators can bind both to duplex and single stranded basepairs affecting the true free concentration of intercalator. The standard conditions used for this substrate are 25 μg/mL which corresponds to a total concentration of substrate of 64.5 μM single strand bases, and 1.86 μM duplex base pairs, a concentration high enough to impact the true free concentration of ligand in solution.

To test this hypothesis, we repeated the analysis of the ethidium dimer in the presence of 500-fold lower concentration duplex substrate (0.05 μg/mL). At this concentration of substrate there are 3.7 nM duplex base pairs, and 130 nM single stranded bases present. This lower substrate concentration gave a much cleaner inhibition curve and a fit value of 10.8 nM, 213-fold lower than the apparent inhibition constant at the higher substrate concentration (Fig. 3b). In addition, we tested the ethidium dimer at an intermediate concentration of duplex substrate (0.5 μg/mL) and it gave an intermediate fit value (85 nM, data not shown). To confirm that the improvement in apparent inhibition was due to the hypothesized mechanism of tight binding and not something else, we also tested the inhibition of monomeric ethidium under all three duplex substrate conditions. Ethidium gave an IC_{50} value that was essentially unchanged at all three substrate conditions (2.2 μM, 1.8 μM, 2.1 μM, respectively at 25 μg/mL, 0.5 μg/mL, and 0.05 μg/mL. Fig. 4 depicts high and low substrate conditions). A second commercially available bis-intercalator, acridine dimer (Fig. 2), was tested and showed a pattern of improvement similar to ethidium dimer. Specifically it showed a duplex substrate concentration dependent inhibition, with fit IC_{50} values of 12.9 μM, 400 nM and 221 nM at 25 μg/mL, 0.5 μg/mL, and 0.05 μg/mL, respectively (Fig. 5 depicts high and low substrate conditions).

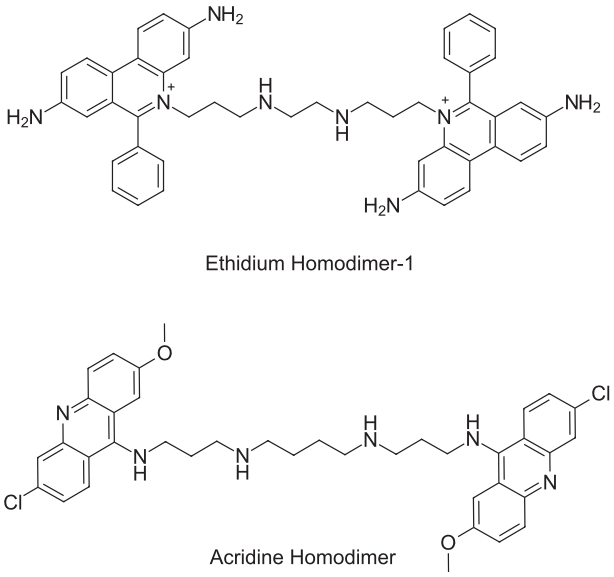


Figure 2. Structures of commercially available bis-intercalators tested.

Given this improvement in HIV-RT inhibition with bis-intercalation, we then examined whether or not we could identify unique bis-intercalators from our library that had particularly high efficacy due to a close match of the bis-intercalator structural properties (e.g., distance between the intercalators, conformation) with the structural properties of the target duplex (e.g., pitch, helicity). To examine this, we screened the library of bis-intercalators we previously synthesized to target telomerase. This collection of 45 different molecules was analyzed in single point assays at 100 nM concentration of drug and 0.05 μg/mL of RNA/DNA substrate. We observed a very similar range of estimated IC_{50} values ranging from 45 to 173 nM. Because of the inherent limitation of single point screening, we retested five of these using full concentration range IC_{50} determinations. These included compounds with two of the best estimated IC_{50} values, two of the worst, and one intermediate. The IC_{50} values for these and their identities are shown in Table 2. The best inhibition was observed with Orn-Ehx-Orn which gave an IC_{50} of 4.9 nM (Fig. 6). The consistency of low nM inhibition indicates that the range of structural parameters explored in the library were all capable of effecting bis-intercalation. In addition, the shape

Table 1
Estimated IC_{50} values for mono-intercalators against reverse transcriptase.

S. No.	Molecule	IC_{50} estimates
1	Ethidium bromide	2 μM
2	Propidium iodide	3 μM
3	Rivanol	20 μM
4	Acridine yellow	32 μM
5	Doxorubicin	52 μM
6	7-amino actinomycin D	93 μM
7	Daunorubicin	139 μM
8	Ellipticine	182 μM
9	Acridine orange	200 μM
10	Actinomycin D	270 μM
11	9-Amino-6-chloro-2-methoxy acridine (ACMA)	No inhibition observed
12	2-Anthracene carboxylic acid	No inhibition observed
13	1-Anthracene carboxylic acid	No inhibition observed
14	9-Acridine carboxylic acid	No inhibition observed

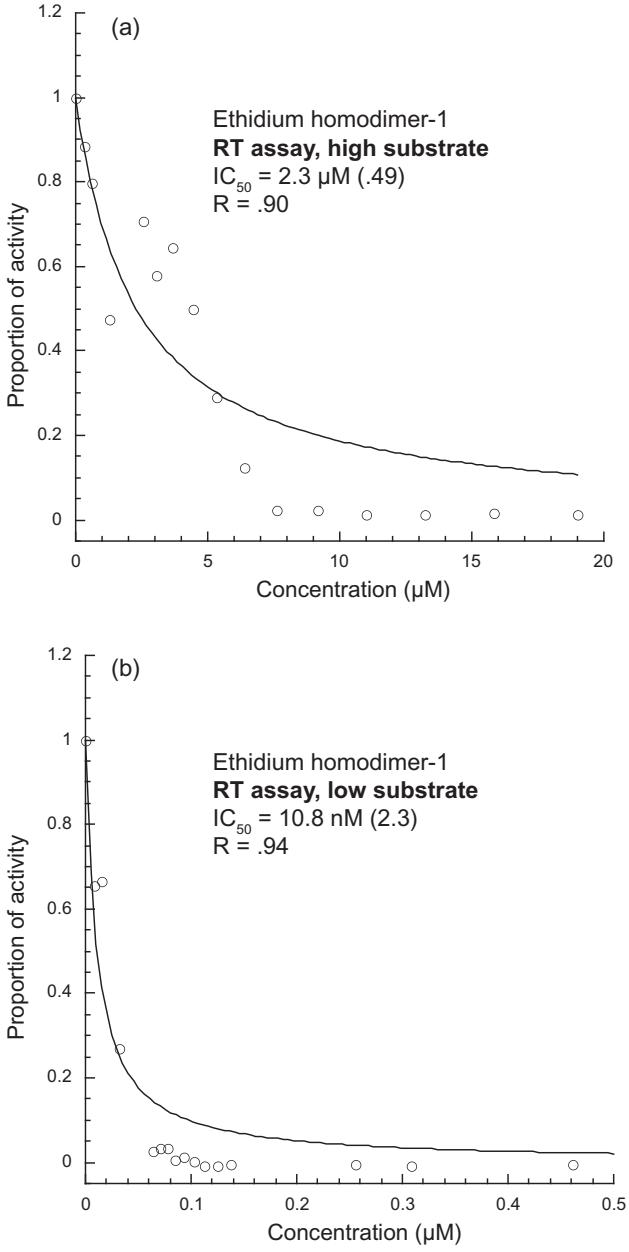


Figure 3. Reverse transcriptase inhibition by ethidium homodimer-1 at high substrate (a) and low substrate (b) concentrations. Error values are indicated in parenthesis.

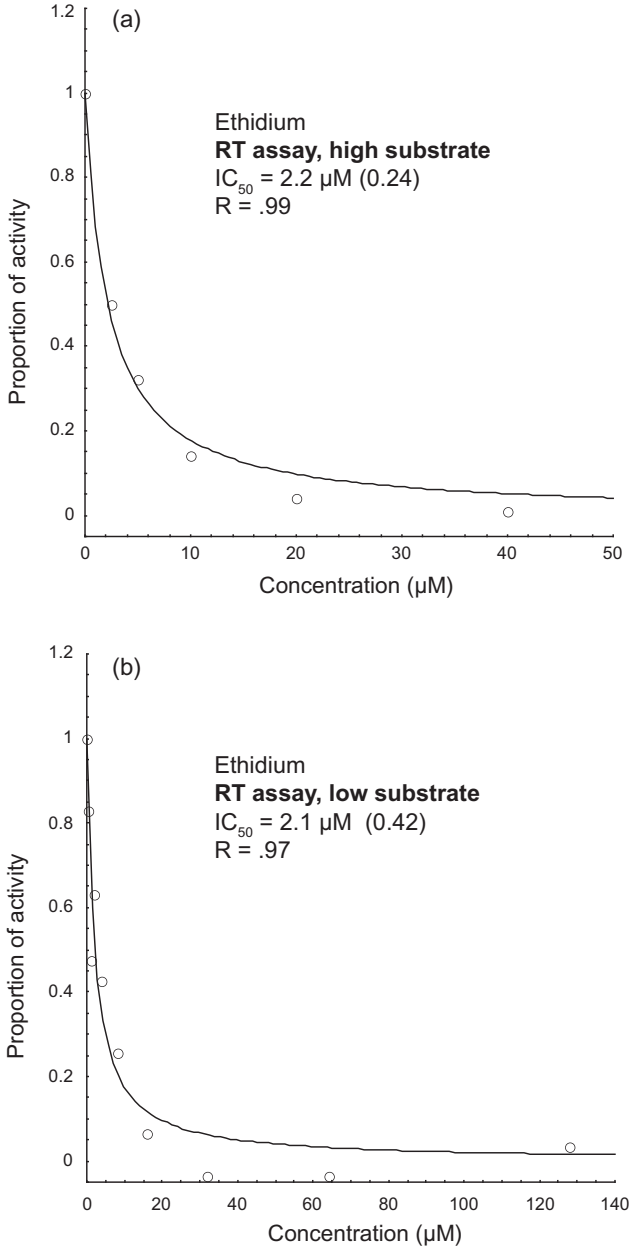


Figure 4. Reverse transcriptase inhibition by ethidium at high substrate (a, top) and low substrate (b, bottom) concentrations. Error values are indicated in parenthesis.

of the inhibition curve still suggests tight binding. This is consistent with the actual concentration of 3.7 nM duplex base pairs found in the duplex substrate, as if this is the target of binding, we could not observe an apparent binding constant lower than this number. It may well be that there are significant binding/inhibition differences between individual members of the library, however they have been effectively leveled by the relatively high concentration of substrate needed to practically assay RT activity.

Finally, we tested the ethidium dimer inhibition efficacy against HIV-RT in the presence of competitor calf thymus DNA. The purpose of this DNA was to simulate the effect that competitor cellular nucleic acids would have on the inhibition efficacy of bis-intercaltors. As higher duplex *substrate* concentrations 'leveled' the apparent inhibition constant of bis-intercalators, we expected that under cellular conditions, genomic DNA as well as RNA of different forms could act as reservoirs and soak up the ligands leading to lower apparent efficacy. We selected a concentration of 340 μM base pairs of calf thymus DNA, as this is ~1/10th the total concentration of genomic DNA in a cell and would represent a significant challenge to a completely non-specific nucleic acid binding compound.[8] In addition, higher concentrations of calf thymus DNA introduced solubility issues. The IC_{50} curve for ethidium dimer produced a fit value of 8.5 μM (data not shown). While this does confirm a degree of a 'leveling' effect of the competitor DNA, it does suggest some specificity of the bis-intercalator for the HIV-RT RNA/DNA duplex over the DNA/DNA competitor duplex. This is illustrated by the fact that a >90,000-fold molar excess of competitor DNA/DNA duplex base pairs to RNA/DNA substrate duplex base pairs did not eliminate inhibition, which would be expected if the two nucleic acid forms had identical affinity for the intercalator.

The aim of this work was to explore the strategy of bis-intercalation for improving the efficacy of intercalator based

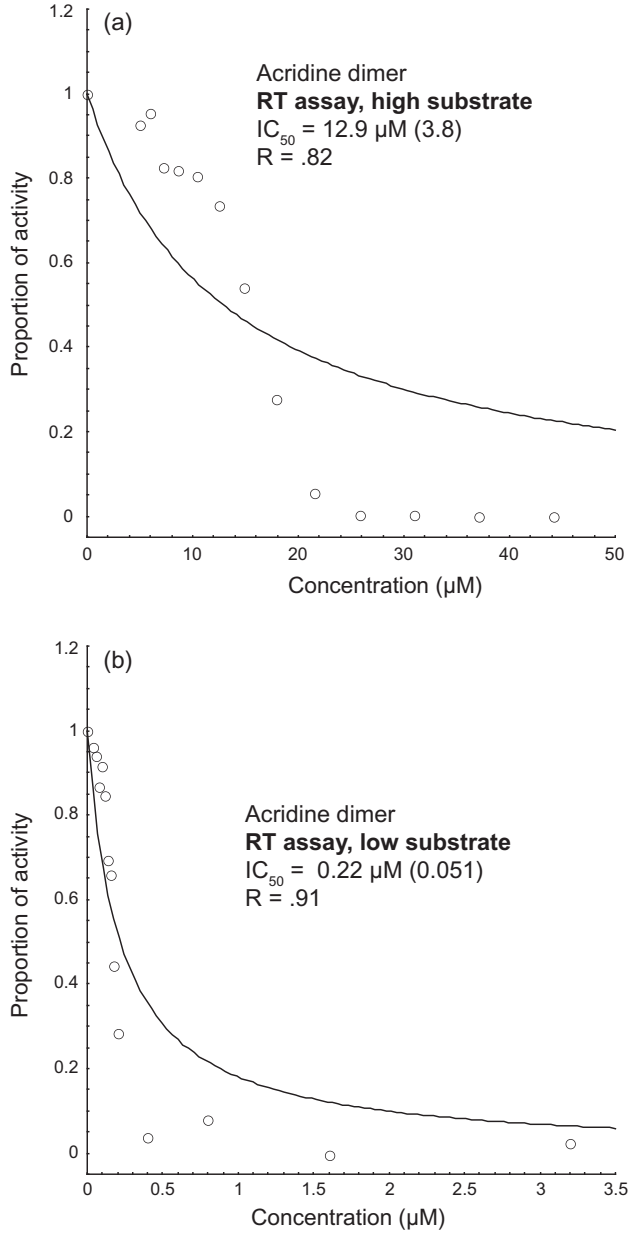


Figure 5. Reverse transcriptase inhibition by acridine dimer at high substrate (a, top) and low substrate (b, bottom) concentrations. Error values are indicated in parenthesis.

Table 2
IC_{50} values from full inhibition studies, determined against reverse transcriptase for five members of bis-intercalator library

S. No.	Molecule	IC_{50} (nM)
1	EBA-Orn-Ehx-Orn-EBA	4.9 (1.8)
2	EBA-Orn-β-Ala-Dap-EBA	9.2 (3.2)
3	EBA-Lys-γ-Abu-Lys-EBA	10.5 (3.7)
4	EBA-Dap-Orn-EBA	22.3 (9.4)
5	EBA-Dap-Ehx-Lys-EBA	30.7 (17.3)

inhibitors of telomerase and HIV-RT. By testing with both commercially available intercalator dimers and a library of ethidium

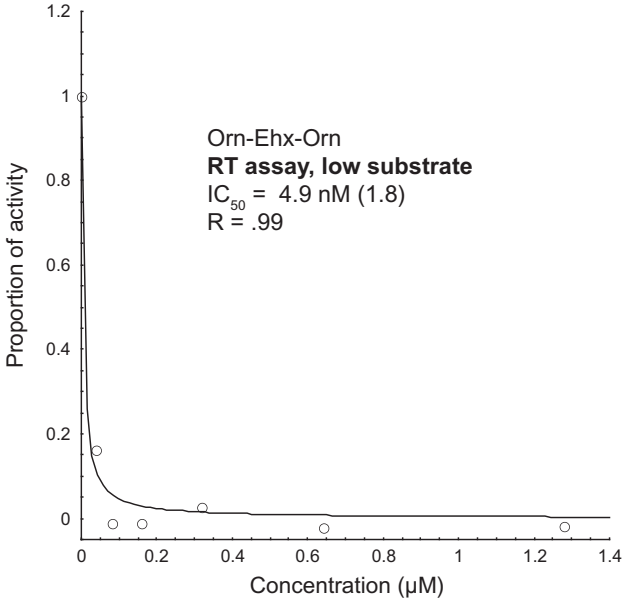


Figure 6. Reverse transcriptase inhibition at low substrate of Orn-Ehx-Orn from the bis-intercalator library. Error value is indicated in parenthesis.

dimers, we demonstrated that we could increase the efficacy of inhibition by greater than 400-fold. This improvement was only seen when we eliminated extraneous sources of intercalator binding sites from the assay mixture. These included cellular homogenate components in the case of telomerase assay, and duplex substrate in the case of HIV-RT assay. Within our library members, most showed improved efficacy in comparison to a monomeric intercalator. This was likely due to the relatively high concentration of substrate duplex. The fact that they can retain inhibition efficacy even in the presence of >90,000-fold molar excess of competitor DNA/DNA duplexes suggests that they have structural specificity for the target RNA/DNA duplex. It is possible that this differential affinity can be further optimized to result in molecules with even greater specificity for the important therapeutic target of the RNA/DNA duplex.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.05.041.

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