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Multiple weak intercalation as a strategy for the inhibition of polymerases

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ABSTRACT

In this work we have developed specific inhibitors of HIV-1 reverse transcriptase by targeting the RNA/DNA duplex that is a principal substrate of the enzyme. To accomplish this, we have developed what we are calling the "weak intercalator" approach, wherein we attempt to simultaneously bind multiple weak intercalators to critical polymerase nucleic acids. We define weak intercalators as planar sp^2 hybridized molecules with only two cycles, that have poor binding affinity individually and can only bind with high affinity if two or more weak intercalation events can take place. Using this approach, we have identified linear and cyclic molecules that present two weak intercalators that can inhibit HIV-1-RT 50 to 100 times more effectively than single weak intercalators. Specifically, a cyclic peptide motif that presents two quinoxaline rings inhibits HIV-1-RT at low μM concentration, shows no inhibition of DNA polymerase and in addition maintains a majority of its inhibitory power in the presence of 90,000 fold excess duplex DNA. These results suggest that the weak intercalator approach may prove effective as a way of targeting increasingly complex nucleic acid structures in a highly specific manner.

Introduction

We have previously explored the use of intercalators to inhibit therapeutically important targets such as telomerase and reverse transcriptase.[1,2] Duplex nucleic acids are key substrates of polymerases, and our hypothesis is that intercalators through binding to these duplexes can inhibit the polymerase catalytic activity. Possible mechanisms include the distortion of the duplex, leading to altered binding affinity, or distorted alignment of critical functionalities in the enzyme bound duplex (Fig. 1). The advantage of the intercalator strategy is the high likelihood that inhibition of a target polymerase will be realized. The major challenge is how to achieve specificity for the target polymerase duplex over other duplex sites in a cell.

The principle problem that we face is that the intercalation site i.e. a stacked base pair, appears to be a relatively generic binding site which is present throughout the genome and at the heart of other polymerase and nucleic acid binding enzymes. We envision two major solutions to this problem: In the first, the intercalator can act as a base, upon which we can introduce new moieties that interact with unique, target-specific sites. By "target-specific sites" we are referring to amino-acid side chains presented by the polymerase that are close to the nucleic acid portion of the polymerase. In the case of targets such as telomerase, the nucleic acid and protein components are subunits of the enzyme. In the case of a target such as reverse transcriptase, the nucleic acid is a substrate of the enzyme. The new interactions introduced between the intercalator base, and these unique polymerase sites can increase affinity as well as specificity, as the unique interactions will only be available with the target.

In the second solution, one explored in this work, the route to specificity is based on multiple simultaneous intercalation events (a type of multivalency).[3–6] A single intercalator has relatively few constraints on its binding to duplex sites. For a scaffold that presents two intercalators however, there has to be a match between the geometry of the intercalators presented, and the spatial relationship of the two intercalative sites. If the match isn't present, at best only a single intercalation event can take place, with substantially lower affinity. Because of this requirement for matching of the multiple intercalative scaffold, and of the binding sites on the duplex, there is a greater potential for differentiation between duplexes, as there can be variation in duplex geometry. There is a danger however, when using traditional intercalators, as even single intercalation events may be too high in affinity, leading to off-target binding. Our solution to this issue is to use what we are calling "weak intercalators" that mimic the structural features required of intercalation (i.e. sp^2 hybridized planar rings), but are detuned to reduce affinity. With this reduction in affinity of an individual weak intercalator, we can only get binding when two or more of these are permitted to bind by the scaffold. An individual weak intercalator has too little binding energy to bind by itself.

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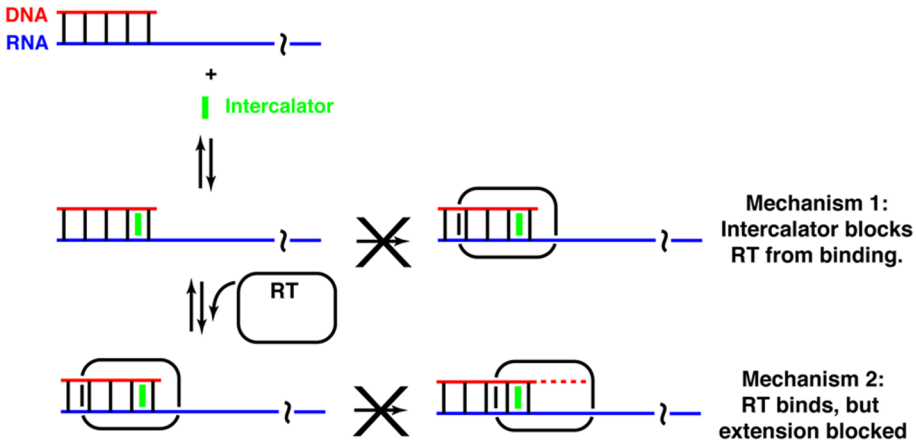

Fig. 1. Potential polymerase inhibition modes by intercalators. a) Intercalator binding to duplex prevents distorted duplex from binding polymerase, b) Intercalator binding tolerated by polymerase, but prevents substrate extension.

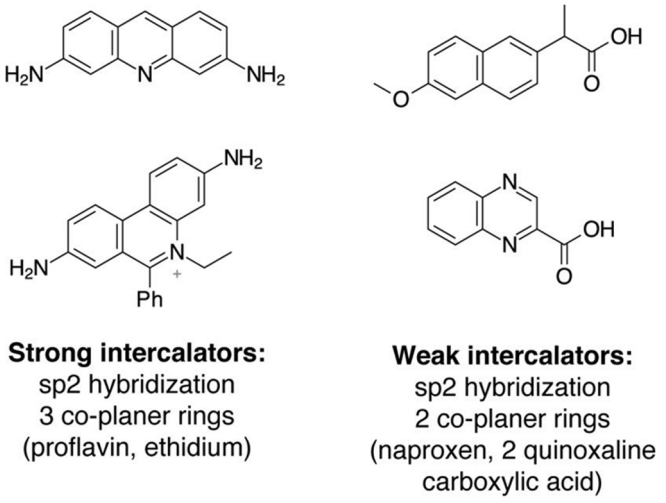

Fig. 2. Strong versus weak intercalators. Classic intercalators (left) with 3–4 rings of sp^2 hybridized atoms versus weak intercalators (right) with 2 rings of sp^2 hybridized atoms.

Our strategy for creating weak intercalators is straightforward: Rather than using a 3 or 4 ring sp^2 hybridized system as is typical (e.g. ethidium, acridine, daunomycin),[7] we use 2 ring sp^2 hybridized cycles (Fig. 2). This eliminates a third of the binding interactions, and thus strongly reduces binding affinity while maintaining intercalative potential.[8] In this work we explore a range of weak intercalators, and synthesize families of scaffolds that are able to vary the geometry that defines the spatial relationship between multiple weak intercalators. Using this approach, we were able to convert weak intercalators with poor inhibition ability (undetectable or mM IC_{50} values) into potentially simultaneously binding multiple weak intercalators with higher affinity and specificity. In addition, we identified a cyclic peptide that presents two quinoxaline rings and inhibits reverse transcriptase in the low micromolar range. It does so even in the presence of large amounts of competitor duplex DNA and shows no signs of promiscuous inhibition. In addition it shows minimal inhibition against DNA polymerase alpha. These results suggest a new path for more specifically targeting nucleic acids, through the simultaneous binding of specifically presented weak intercalators.

Results and discussion

The first molecules that we examined for RT inhibition via simultaneous weak intercalation were based on tryptophan. Tryptophan has multiple appealing features as the basis for a weak intercalator: It is a non-toxic ubiquitous amino acid, it can be efficiently incorporated into libraries of compounds, and it contains a two ring planar sp^2 hybridized indole, potentially capable of weak intercalation. We created a library in which two tryptophans were bridged with either one linker or no linker (in other words directly connected) (Fig. 3). The bridging monomers were glycine, beta-alanine, gamma amino butyric acid, epsilon amino hexanoic acid, and D and L asparagine. These allowed for the systematic variation of the distance between the two tryptophans. We used both L and D tryptophan to increase structural diversity, so for each linker, there were four subspecies in the library (LL, DD, LD, DL).

These were synthesized readily via solid phase peptide synthesis as the C-terminal carboxamides, and showed correct masses by ESI-MS (see Supporting Information). The inhibition potential of all molecules was assessed using an HIV-1 reverse transcriptase assay, adapted from prior work.[9–11] This utilized a poly rA/oligo-dT as the substrate for HIV-1 reverse transcriptase. We monitored RT's incorporation of tritiated deoxy thymidine into the substrate by filtering the product, and quantitating via scintillation counting.

The resulting 28 species were screened in a single point assay at 300 μM concentration. The single point assay is meant as a rapid assessment of inhibition potential in large numbers of compounds. The best compounds from this screen could then be analyzed using full concentration range IC_{50} determinations, which is inherently more accurate. The single point proportion of reverse transcriptase activity (shown in Table 1) ranged from 0.19 to 0.78, with the control mono weak intercalator L-tryptophan carboxamide showing 0.94 activity. We selected the top four compounds, #18, #20, #24 and #26, for full IC_{50}

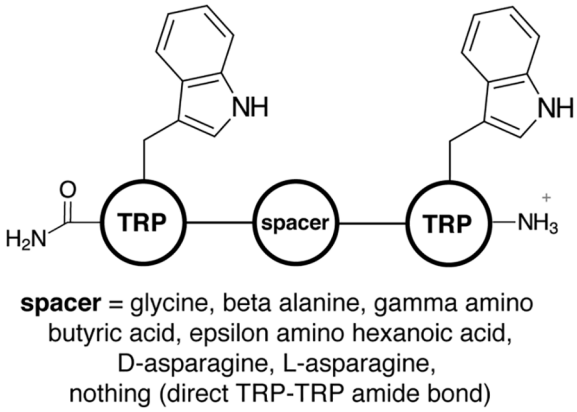

Fig. 3. Tryptophan library design. Two tryptophan moieties are joined by linkers of varying length to probe critical distance required for binding.

Table 1
Single point screening of tryptophan library at 300 μM: Proportion of control reverse transcriptase activity indicated.

Molecule	Description	Proportion value
1	W_D-W_D	0.70
2	W_D-W_L	0.45
3	W_L-W_D	0.61
4	W_L-W_L	0.65
5	W_D-(Gly)-W_D	0.76
6*	W_D-(β-Ala)-W_D	0.39
7	W_D-(γ-Abu)-W_D	0.36
8	W_D-(ε-Ahx)-W_D	0.48
9	W_D-(D-Asn)-W_D	0.47
10	W_D-(L-Asn)-W_D	0.69
11	W_D-(Gly)-W_L	0.74
12	W_D-(β-Ala)-W_L	0.46
13*	W_D-(γ-Abu)-W_L	0.78
14*	W_D-(ε-Ahx)-W_L	0.76
15	W_D-(D-Asn)-W_L	0.35
16	W_D-(L-Asn)-W_L	0.44
17	W_L-(Gly)-W_D	0.63
18	W_L-(β-Ala)-W_D	0.30
19	W_L-(γ-Abu)-W_D	0.43
20	W_L-(ε-Ahx)-W_D	0.31
21	W_L-(D-Asn)-W_D	0.34
22	W_L-(L-Asn)-W_D	0.69
23	W_L-(Gly)-W_L	0.40
24	W_L-(β-Ala)-W_L	0.25
25*	W_L-(γ-Abu)-W_L	0.55
26	W_L-(ε-Ahx)-W_L	0.19
27	W_L-(D-Asn)-W_L	0.60
28	W_L-(L-Asn)-W_L	0.62
Control	L-Tryptophan amide	0.94

* Molecules 6, 13, 14 and 25 were screened at ~100 μM due to low concentration of these molecules and 5% DMSO limit.

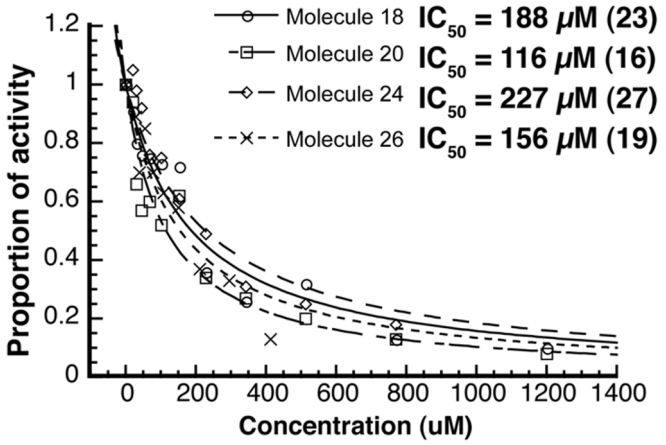

Fig. 4. Reverse transcriptase inhibition curves for top four tryptophan library members. IC_{50} values indicated for molecules #18, #20, #24 and #26. Data was fit to $p = 1/(1 + [I]/IC_{50})$. Standard error for the fit is indicated in parenthesis.

determination. These results are shown in Fig. 4. They show the library members with well fit inhibition curves and IC_{50} values that vary between 116 μM and 188 μM. This is in comparison with the mono-intercalator control L-tryptophan amide (Supplementary data Fig. s7), which showed an IC_{50} of 9.9 mM. This shows an improvement of 50–100 fold in inhibition efficacy when two weak intercalators were linked.

Because there is a possibility for promiscuous inhibition at such high concentrations of molecules, we retested one of the compounds, #26 with and without triton X-100, a detergent found to be diagnostic for promiscuous inhibition.[12,13] The results are shown in Supporting Information. They show that there is only a small difference in IC_{50} in the presence vs absence of Triton X-100 (236 μM vs 156 μM). This small difference was also seen with our mono-intercalator control, tryptophan amide, when it was tested in the presence and absence of Triton X-100 (IC_{50} of 15.7 mM vs 9.9 mM). These results suggest that the inhibition observed is not due to non-specific promiscuous inhibition, and is due to the specific interactions of the molecules with target.

The tryptophan libraries supported the idea of multiple simultaneous weak intercalation as being a driving force for higher affinity, showing a 50–100 fold increase in efficacy when comparing molecules capable of a single versus a double intercalation event. In absolute terms however, we were interested in finding molecules with greater efficacy, i.e. lower IC_{50} values. To this end, we broadened our examination of the range of potential weak intercalators we could incorporate into our libraries. Our first step was to analyze potential two ring sp^2 hybridized weak intercalators for potential reverse transcriptase inhibition. We examined both FDA approved drugs (naproxen and primaquine, as well as other 2 ring systems that could ultimately be easily incorporated into combinatorial synthetic schemes (3 quinoline carboxylic acid, 4 quinoline carboxylic acid and 2 quinoxaline carboxylic acid). These were initially screened at high concentration in single point assays, and all, showing inhibition were then assessed over a wide concentration range to determine full IC_{50} values. The resultant IC_{50} values are summarized in Table 2. Most of the species show low mM inhibition, which is consistent with expectation. Two ring systems should have severely limited inhibition because 1/3rd of the interacting surfaces and hence binding energy found in normal intercalators is missing. Of the species tested, quinoxaline 2 carboxylic acid (Q2CA) gave the best IC_{50} value of 0.32 mM, as well as the cleanest fit to an IC_{50} curve (Fig. 5). We used Q2CA as the basis for subsequent optimization efforts because of this performance. In addition Q2CA is also the key intercalative moiety found in the bis-intercalative natural products echinomycin and triostin[14,15], suggesting that it might be possible to use it in the context of targeting reverse transcriptase.

We initially incorporated Q2CA into a library of unconstrained linear peptides that allowed the distance between two Q2CA groups to be systematically varied. The intention was to identify a species with a "special" distance that allowed bis-intercalation and hence higher inhibition efficacy. The library members had two ornithines, linked by two linker amino acids which varied the number of methylenes and hence distance between the two ornithines (glycine, beta-alanine, gamma amino butyric acid and epsilon amino hexanoic acid.) The side chain amino group of each ornithine was reacted with Q2CA to make the final 16 library members (Supplementary data Fig. s1). Library members were synthesized in ~90% purity, and confirmed by ESI-MS. They were then tested for inhibition of reverse transcriptase in single point assays at 100 μM. None of the 16 showed inhibition at this concentration, which was just a factor of 3 less than the IC_{50} of unmodified Q2CA.

Given this lack of inhibition, we then considered using a cyclic scaffold to present the two moieties. Cyclic scaffolds can severely limit the conformational entropy loss associated with the binding of highly flexible molecules and in so doing, increase the overall binding affinity and inhibition efficacy. The strategy was similar with the linear motif:

Table 2
Inhibition of reverse transcriptase by weak intercalators. IC_{50} values were determined by fitting enzyme activity as a function of intercalator concentration. Standard errors for the fit are indicated.

Molecule	IC_{50} against HIV-1 RT
Primaquine di phosphate	0.1 mM*
Naproxen sodium	6.7 mM (3.7)
3-Quinoline carboxylic acid	2.4 mM (0.7)
4-Quinoline carboxylic acid	9.6 mM (4.7)
2-Quinoxaline carboxylic acid	0.32 mM (0.04)

* Single point IC_{50} estimate.

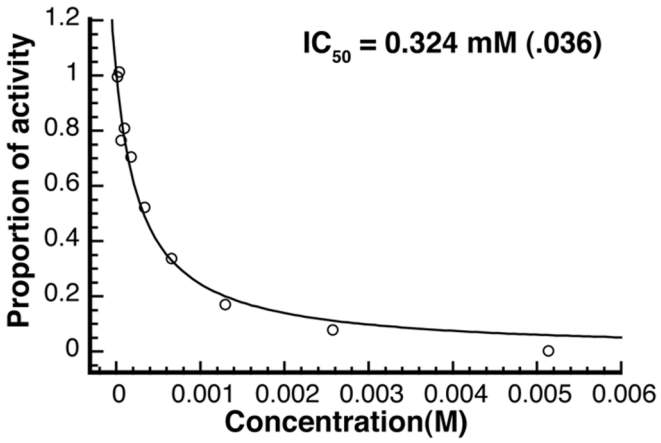

Fig. 5. Reverse transcriptase inhibition curve for quinoxaline 2 carboxylic acid (Q2CA).

incorporate amino acids with amine side chains that allow for condensation with the Q2CA during synthesis. Specifically, we used diamino propionic acid to limit the conformational entropy associated with the longer ornithine side chain. We create the cycle by reacting a side chain carboxylic acid presented by aspartic acid with the terminal amine. Fig. 6 shows the final motif which contains seven amino acids total. Two amino acids are involved in making the ultimate cycle, two or three are involved in presenting the Q2CA groups and two or three (glycines) are used to vary the spacing available to the intercalative moieties. The development of this highly orthogonal synthesis and the characterization of the molecules has previously been published.[16] We synthesized the ten possible bis-intercalators and the ten possible tris-intercalators.

This library was screened in single point at 20 μM, and the results are shown in Table 3. Most of these molecules in the bis and tris weak intercalator library showed significant inhibition of reverse transcriptase at this significantly lower concentration of inhibitor. Molecules 8 and 5 were the bis modified compounds with the lowest proportion of RT activity and were thus selected and retested in full IC_{50}. These results are shown in Fig. 7 and Supplementary data Fig. s9 respectively. Because of the possibility of inhibition due to so called 'promiscuous inhibition', we retested these compounds in the presence of the detergent TX-100, and found similar results, indicating the reduction in enzyme activity was not due to non-specific aggregates inducing inhibition.[12,13] The IC_{50} values of 6.1 μM and 6.5 μM respectively for compounds **5** and **8** represents an over 50 fold increase in efficacy over the starting Q2CA. As our most stringent control, we synthesized compound **1**, which had the identical cyclic framework with a single Q2CA. This showed an IC_{50} of 43 μM (Supplementary data Fig. s10), indicating that the bis intercalator compounds **5** and **8** represent a 7 fold improvement, due to the presence of the second intercalator.

We also determined a full IC_{50} for the best tris-intercalator identified in the screen, compound 15 (Supplementary data Fig. s11). This showed a modest improvement in inhibition efficacy over compounds **5** and **8**, with an IC_{50} of 3.8 μM. As before, the IC_{50} in the presence of TX-100 was very similar, supporting the idea that these compounds are not promiscuous inhibitors but are driven by a specific interactions.

The assays are performed using a substrate concentration of 0.05 μg/ml, conditions at which the initial duplex concentration (RNA/DNA) is 3.7 nM, and the concentration of single stranded (template) RNA is 129 nM. The motivation for this work was to target this RNA/DNA duplex. As previously stated, binding of this duplex can create inhibition through a variety of sub-mechanisms: a) The intercalator binding can distort the duplex, lowering its affinity for reverse transcriptase. b) The intercalator binding to duplex could still permit binding of duplex to RT, but cause a misalignment of the duplex substrate with the catalytic machinery of RT. In either case, the potential applicability of the identified compounds depends on their specificity

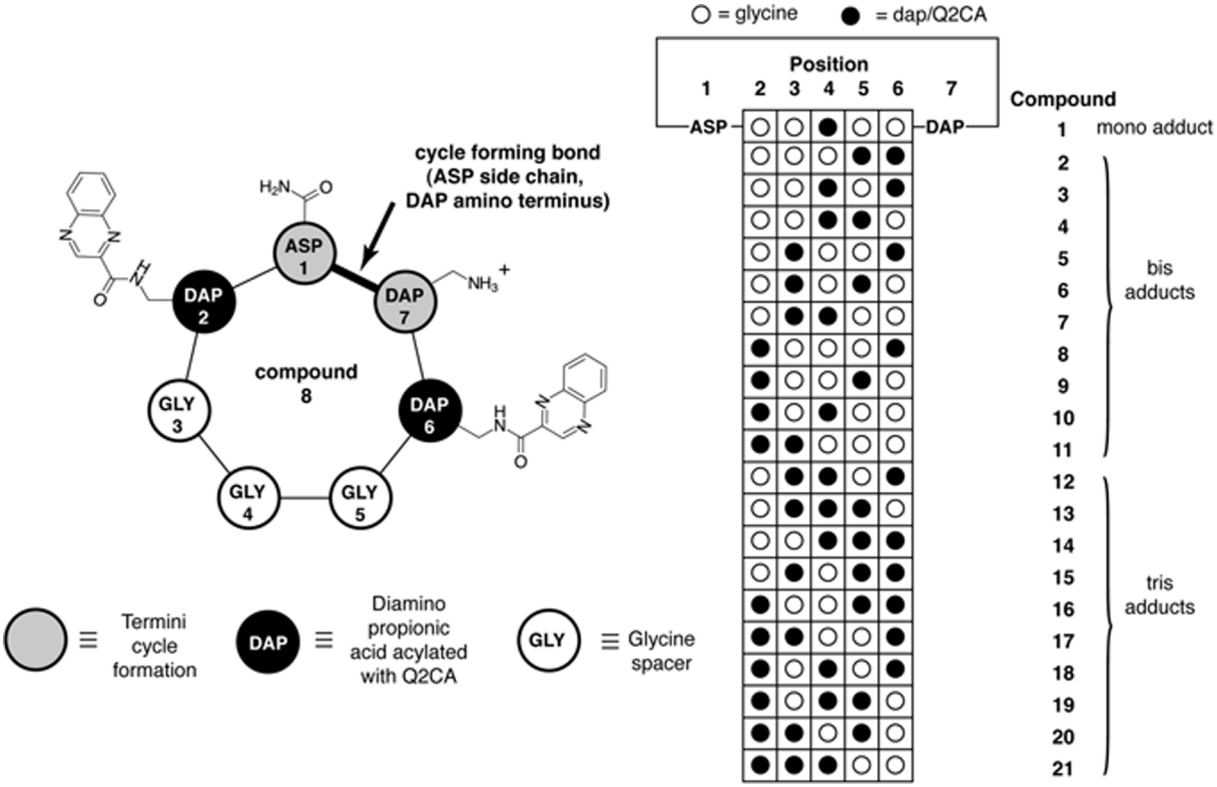

Fig. 6. Design of cyclic heptamer motif. Q2CA moieties are presented by diamino propionic acid residues at all possible positions in the cycle. The cycle is formed through amide bond linkage between the terminal amine and the aspartic acid side chain in the first position.

Table 3
Single point screening of cyclic library members at 20 μM. Sequence of library member indicated. Dap indicates diamino propionic acid, the site of Q2CA attachment. Reverse transcriptase activity as a proportion of control is indicated.

Molecule	Description	Type of intercalator	Proportion value against HIV-1 RT
1	**Asp**-Gly-Gly-Dap-Gly-Gly-**Dap**	Mono	0.66
2	**Asp**-Gly-Gly-Gly-Dap-Dap-**Dap**	Bis	0.30
3	**Asp**-Gly-Gly-Dap-Gly-Dap-**Dap**	Bis	0.11
4	**Asp**-Gly-Gly-Dap-Dap-Gly-**Dap**	Bis	0.38
5	**Asp**-Gly-Dap-Gly-Gly-Dap-**Dap**	Bis	0.02
6	**Asp**-Gly-Dap-Gly-Dap-Gly-**Dap**	Bis	0.09
7	**Asp**-Gly-Dap-Dap-Gly-Gly-**Dap**	Bis	0.57
8	**Asp**-Dap-Gly-Gly-Gly-Dap-**Dap**	Bis	0.09
9	**Asp**-Dap-Gly-Gly-Dap-Gly-**Dap**	Bis	0.13
10	**Asp**-Dap-Gly-Dap-Gly-Gly-**Dap**	Bis	0.18
11	**Asp**-Dap-Dap-Gly-Gly-Gly-**Dap**	Bis	0.71
12	**Asp**-Gly-Dap-Dap-Gly-Dap-**Dap**	Tris	0.09
13	**Asp**-Gly-Dap-Dap-Dap-Gly-**Dap**	Tris	0.08
14	**Asp**-Gly-Gly-Dap-Dap-Dap-**Dap**	Tris	0.04
15	**Asp**-Gly-Dap-Gly-Dap-Dap-**Dap**	Tris	0.03
16	**Asp**-Dap-Gly-Gly-Dap-Dap-**Dap**	Tris	0.06
17	**Asp**-Dap-Dap-Gly-Gly-Dap-**Dap**	Tris	0.13
18	**Asp**-Dap-Gly-Dap-Gly-Dap-**Dap**	Tris	0.18
19	**Asp**-Dap-Gly-Dap-Dap-Gly-**Dap**	Tris	0.14
20	**Asp**-Dap-Dap-Gly-Dap-Gly-**Dap**	Tris	0.15
21	**Asp**-Dap-Dap-Dap-Gly-Gly-**Dap**	Tris	0.16

Bold residues = handle for cyclization.
Dap = attachment point for quinoxaline 2 carboxylic acid (Q2CA).

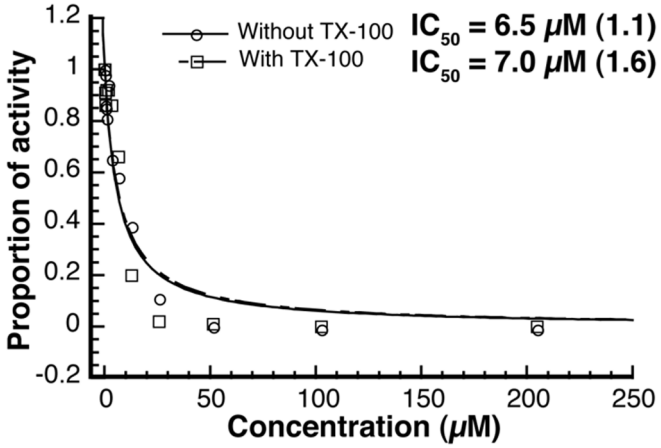

Fig. 7. Reverse transcriptase inhibition curve for bis-intercalator cyclic molecule 8. Two curves are shown, in the presence or absence of 0.01% triton X-100. This is to assess the potential for promiscuous inhibition.

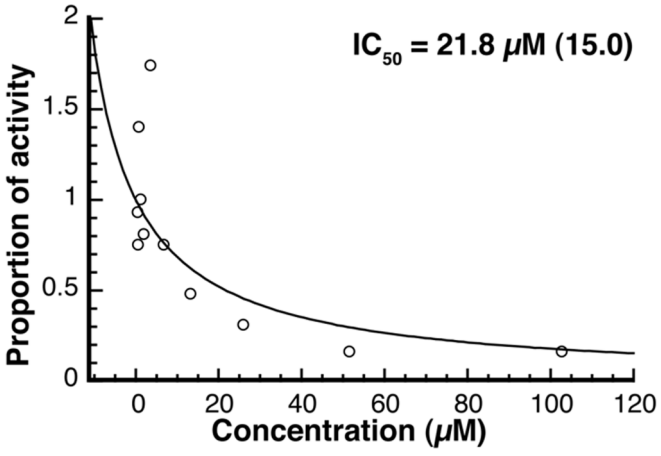

Fig. 8. Retesting of bis-intercalator cyclic molecule 8 in the presence of competitor ct-DNA. 340 μM ctDNA was used in the assay representing a 90,000 fold excess of competitor base pairs.

for the RNA/DNA duplex that acts as the substrate of RT. If the compounds have generic duplex-binding ability, it could lead to a) off target inhibition or b) loss of efficacy as the large excess of non-RNA/DNA duplex sites (e.g. genomic DNA) acts as a reservoir to absorb the compounds. To examine this possibility, we repeated the assay of our best bis-intercalator, compound 8 but in the presence of 340 μM base pairs of calf thymus DNA. This is about 1/10th the approximate concentration of genomic DNA found in a typically sized cell, and was near the solubility limit for our assay conditions. The result is shown in Fig. 8. It shows that despite a > 90,000 fold excess of DNA duplex sites vs RNA/DNA substrate sites, the compound retains its ability to inhibit RT, with only a 3.5 fold loss in efficacy. This strongly supports the inhibition of RT being rooted in a specific structural feature associated with it.

As a final test of this specificity, we examined the ability of molecule 8 to inhibit human DNA polymerase alpha, using a similar assay adapted for this enzyme.[17] Specifically, we determined the incorporation of radioactive TTP into an activated DNA substrate in the presence and absence of compound 8. Even with compound 8 at a concentration of 600 μM, we observed 89% DNA polymerase activity, further supporting the specificity of these compounds for RT.

Conclusions

In this work, we have explored for the first time the approach of using weak intercalators as the basis for the development of inhibitors that target duplex elements of therapeutically important polymerases. Intercalators have features that both strongly favor and disfavor their use in this arena. Their strong and highly predictable binding to duplex elements increases the likelihood that inhibition will take place, as the binding of intercalators to duplex elements should induce distortion. This strong binding however also disfavors their use, as binding is highly non-specific, with little to differentiate one intercalation site from another. In this work we have explored a solution to this conundrum: Weaken intercalation, so that there is less binding affinity for any generic site, but then present multiple weak intercalators in geometries defined by a semi-rigid scaffold. Only an ideal match of the scaffold and the specific geometry of the desired duplex target will allow both weak intercalators to bind, and the significantly higher affinity expected from multi-dentate binding. The low micro molar IC_{50}

values we observe with our molecules are significantly higher than those of clinically used reverse transcriptase inhibitors, which are typically in the low nano molar range. In addition, their peptidic nature may introduce challenges with clinical application (e.g. lower bioavailability). Given this, their purpose in this work was to explore an approach: that of using multiple weak intercalation as a strategy to inhibit polymerases. With the approach supported by this work, we and others can explore other possibly more bioactive motifs that are driven by the same philosophy.

In this work we have targeted the RNA/DNA duplex found in reverse transcriptase and have compelling evidence that specific inhibition resulted. It remains to be seen if this specificity is due to a specific structural difference in these two duplexes (e.g. helicity, pitch, groove width etc.) or other features. We expect that the multiple weak intercalator approach will be particularly fruitful when targeting folded RNA, as the geometrical relationships between different intercalative sites can be very different. The presentation of multiple weak intercalators by semi-rigid scaffolds may allow the rapid targeting of a wide range of therapeutically important nucleic acids, through the matching of intercalator geometry with the geometry of the nucleic acid target.

Material and methods

Detailed synthetic procedures, characterization and biochemical analysis procedures are described in the Supplemental Information.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2018.12.027.

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