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Small molecule modulators of HIV Rev/Rev response element interaction identified by random screening

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Abstract

A high throughput scintillation proximity assay with biotinylated human immunodeficiency virus (HIV) Rev protein and tritiated Rev response element RNA was used to screen over 500 000 small molecules. Several chemical classes of inhibitors and two chemical classes of enhancers of binding were identified, with the molecular weight range being 400–600. The most common structural motif of inhibitor was an acidic moiety at the end of a linear aromatic system. Most of these modulators had EC_{50} values in the 1–10 μM potency range, with several below 1 μM. Several classes displayed structure–activity relationships suggesting specific molecular interactions between small molecule and macromolecule. Several molecules were confirmed as inhibitors in a gel shift assay and by surface plasmon resonance analysis. Furthermore, one inhibitor was shown to bind the Rev protein with a binding constant equal to its IC_{50} value, consistent with the mechanism of inhibition being binding Rev. Thus, small molecules can modulate this macromolecular protein–RNA interaction in vitro. However, no compound demonstrated HIV antiviral activity in a relevant cell-based assay. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Human immunodeficiency virus (HIV); Rev, Rev response element (RRE); Protein–RNA interaction; High throughput screening (HTS)

1. Introduction

Pharmaceutical research has been highly successful in developing three major classes of drugs aimed at two molecular targets of HIV: nucleoside analog inhibitors of reverse transcriptase, nonnucleoside inhibitors of reverse transcriptase, and protease inhibitors. Whereas combination therapies have had profound impact on the length and quality of life for AIDS patients (Vella and

Abbreviations: HIV, human immunodeficiency virus; RRE, rev responsive element; SPR, surface plasmon resonance; SPA, scintillation proximity assay; SAR, structure–activity relationship.

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Palmisano, 2000), drug resistance remains an issue (Pillay et al., 2000). Resistance can be overcome either by new versions of present drugs or by drugs that work through novel mechanisms.

An unexploited target that is essential to HIV is the interaction between the viral regulatory protein Rev and its RNA binding partner, the rev-responsive element (RRE) (Hope, 1999). Rev binds RRE in unspliced or singly spliced HIV mRNAs. Subsequently, through an interaction of a separate domain of Rev with the nuclear export machinery, Rev transports these bound mRNAs into the cytoplasm for translation into the essential late-stage viral proteins (ex, Gag, Gag-Pol, Env). Inhibition of HIV has been demonstrated with many biological reagents directed at either Rev or RRE (Malim et al., 1992; Duan et al., 1994; Yamada et al., 1996; Selvam et al., 1996).

Few small molecules have been shown to block this protein–RNA interaction, with the aminoglycoside antibiotics (ex, neomycin B) being the most notable class (Zapp et al., 1993; Wang et al., 1997; Lacouciere et al., 2000). A second class of RNA binders, the diphenylfuran cations, have also been shown to inhibit Rev/RRE interaction (Zapp et al., 1997). There have been descriptions of natural product inhibitors of Rev/RRE (Quian-Cutrone et al., 1996), though no mechanism of inhibition was described. To date, only one small molecule has shown antiviral activity in cells. At very high concentrations of neomycin B (>100 μM), p24 antigen expression was suppressed in a chronically HIV-infected cell line (Zapp et al., 1993). However, no data was presented to demonstrate that suppression was due to inhibition of Rev/RRE.

There have been only a few reports of assays developed to identify modulators of Rev/RRE interaction. A filter-binding assay was used in the identification of natural product inhibitors (Quian-Cutrone et al., 1996). A cell-based reporter assay was described where translation of secreted alkaline phosphatase was dependent on Rev/RRE interaction (Tang and Su, 1997), though no screening results were presented. We report here the development of a high throughput assay for Rev/RRE interaction using a scintillation proximity assay (SPA). This assay was used to test over 500 000 compounds for modulation of Rev/RRE binding. We also used SPR analysis to determine whether the identified molecules bound either to Rev or RRE. A number of small molecule modulators were identified in different chemical classes, including the first described inhibitors of Rev/RRE that bind the Rev protein.

2. Materials and methods

2.1. Rev purification and biotinylation

HIV-1 Rev protein (AGRSGDSDED LLKAVRLIKF LYQSNPPPNP EGTRQARRNR RRRWRERQRQ IHSISERILS TYLGRSAEPV PLQLPPLERL TLDCNEDCGT SGTQGVGSPQ ILVESPTVLE SGTKE) was expressed in *E. coli* from a plasmid under the T7 promoter, and purified using a previously described procedure (Cole et al., 1993). The purified protein concentration, based on MW of 13065 and extinction coefficient of 8490, was determined from the OD_{280} to be 3.7 mg/ml. Rev was stored in aliquots at -70 °C. Rev contains two cysteine residues in the C-terminal portion of the protein in a region separate from the known binding site for RRE. For the SPA, these residues were biotinylated with a thiol specific reagent, iodoacetyl-LC-biotin (*N*-iodoacetyl-*N*-biotinylhexylenediamine). A 20 mg of Rev in 8.25 ml of 20 mM HEPES, pH 7.2, containing 375 mM KCl, 1 mM EDTA and 0.67 mM DTT was adjusted to pH 8.0 by the addition of 0.5 ml triethanolamine HCl, pH 8.3. About 1.25 ml of iodoacetyl-LC-biotin (8 mg/ml) in DMSO (a 1.4-fold excess of iodoacetyl groups to total thiol groups) was added and the mixture was incubated at 25 °C for 90 min in the dark. The reaction was stopped by the addition of 10 μl of 100 mM DTT, and free biotin was removed by dialysis against 20 mM HEPES, pH 7.2, containing 300 mM KCl and 1 mM EDTA. The extent of biotinylation was measured according to the procedure of Green (1964) and determined to be 1.5 mole biotin per mole Rev. The modified protein was stored at 4 °C.

2.2. RRE transcription template and synthesis of [^{3}H]RRE$_{209}$ for SPA

The T7 RNA polymerase promoter and the DNA sequence corresponding to residues 70–279 of HIV RRE (Mann et al., 1994) were cloned into pUC12 between the *Eco*RI and *Bam*HI sites. Prior to synthesis of [^{3}H]RRE, the plasmid was linearized by digestion with *Bam*HI. For a 100 μl synthesis of [^{3}H]RRE$_{209}$, 120 μl 48 Ci/mmol [^{3}H]UTP (Amersham) was reduced to dryness, then resuspended with nuclease free H_2O to yield a final concentration of 25 μM. The following were added to the resuspended [^{3}H]UTP: 10 μl 10× Synthesis Buffer (Ambion MaxiScript T7 Kit), 5 μl each 10 mM ATP, CTP, GTP, 5 μl 1 mM UTP, 5 μg linear cDNA template, and 10 μl T7 RNA polymerase (MaxiScript T7 Kit, Ambion Inc., Austin, TX). The reaction was kept at room temperature for 2–3 h, heated to 90 °C for 2 min, transferred to ice for 2 min, and then 5 μl DNase was added and incubated at 37 °C for 15 min. The following RNA sequence was produced:

gggaCCUUGG GUUCUUGGGA GCAGCAGGAA GCACUAUG**GG** CGCAGUGUCA UUGACGCUGA CGGUACAGGC CAGACAAUUA UUGUCUGGUA UAGUGCAACA GCAGAACAAU UUGCUGAGGG CUAUUGAGGC GCAACAACAU CUGUUGCAAC UCACAGUCUG GGGCAUCAAG CAGCUCCAGG CAAGAGUCCU GGCUGUGGAA ACAUACCUAA AGGuccg.

Residues in lower case are derived from the plasmid sequence, residues in capital case are residues 70–279 from HIV RRE (Mann et al., 1994). The GG bolded and underlined are the two G nucleotides within bubble site which forms the high affinity binding site for Rev (Heaphy et al., 1990) and are deleted to make $\Delta G_{104-105}$ RRE. [^{3}H]RRE was used in the SPA with no purification. The quality of synthesis was determined by running the product on a 6% TBE–urea gel and visualizing a single band of radioactivity at the correct molecular weight. The labeled RRE could be kept at 4 °C approximately 3 weeks, after which smaller molecular weight bands could be visualized by gel analysis.

2.3. RRE transcription template and synthesis of RRE for SPR analysis

The 244 base reverse transcription template was created by PCR using the same cDNA used to generate [^{3}H]RRE, a primer homologous to the T7 promoter (TAATACGACTCACTATAGGG), and a primer homologous to residues 227–244 of RRE (CTCAGCTGGACGTCGGGT). RNA was synthesized with the MEGAshortscript kit (Ambion, Inc., Austin, TX). For immobilization onto streptavidin sensor chips, the resultant RRE RNA was annealed to a 5′-biotinylated oligo, which was identical in nucleotide sequence to the RRE227-244 used for the PCR reaction.

2.4. SPA for Rev/RRE interaction

The SPA for Rev/RRE interaction was developed in clear-bottom white 96-well SPA plates (Costar #3632). The standard assay (50 μl) consisted of 5 nM Rev protein, 0.2 nM [^{3}H]RRE in assay buffer (20 mM HEPES (pH 7.3), 300 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, and 0.2% NaAzide). The typical order of addition was Rev then RRE then incubation at room temperature for 15 min before addition of Streptavidin SPA beads (Amersham, Piscataway, NJ). Final assay concentration for each Streptavidin SPA bead lot was determined by titrating against current REV–biotin stock. SPA beads were reconstituted with assay buffer minus BSA. A typical final bead concentration was 1 mg/ml in the assay. After addition of beads, plates were incubated overnight at 4 °C, and bound radioactivity was counted for 30 s per well in a Wallac 1450 Microbeta counter. There was no difference in signal between plates incubated after addition of beads for 4 versus 24 h. For modulation of binding, compounds were added first to wells in DMSO. Rev/RRE binding could tolerate up to a final concentration of 20% DMSO; typical DMSO concentration was 3%. For screening, compounds were first tested in pools of ten compounds with individual compounds at approximately 10 μM. Individual compounds were titrated with dilution of compound in DMSO typically starting at 90 μM compound and doing ten 2-fold dilutions.

2.5. Gel shift assay for analysis of Rev/RRE modulators

Gel shift analysis was preformed by incubating 1 μl compound with 2 μl 5 × reaction buffer (700 mM KCl, 50 mM K_2HPO_4, 50 mM $Na_2H_2PO_4$ (pH 7.0)), 1 ul RNAsin (Promega, Madison, WI) and 3 μl 50 nM [^{3}H]-RRE (which had been diluted from stock in water) for 10 min at room temperature. Then 3 μl Rev–biotin was added at 10 molar excess over [^{3}H]RRE and incubated for an additional 10 min followed by the addition of 0.5 μl loading buffer. The reaction mix was loaded onto 6% 1/2 × TBE gel (Novex, San Diego, CA) and run at 100 V for 2 h. Autoradiography was preformed following fixation for 30 min (25% isopropanol, 10% acetic acid), incubation for 30 min with Enhance (Amersham) and drying under vacuum.

2.6. SPR analysis of Rev/RRE modulators

SPR analysis was performed using the Biacore 3000 instrument (Biacore Inc.). The running buffer was 25 mM Hepes, pH 7.4, 400 mM NaCl, 2 mM EDTA, and 0.1% Triton X-100. For experiments using compounds, 5% DMSO was included. Biotinylated Rev or RRE were immobilized on Streptavidin-coated sensor chips (Biacore Inc.) and blocked with 5 mM free biotin. For direct binding of compounds to Rev, Rev was covalently immobilized to the sensor chip by amine-coupling according to the manufacturers instructions. For specificity, binding was ascertained to another nucleic acid-binding protein HPV E2 under same conditions. The sensor chip surface was regenerated by a 1 min pulse of 0.1% SDS in running buffer. Inhibition experiments were conducted by preincubating the compounds with 25 nM Rev in running buffer at room temperature for 30 min. Twenty microliters of the Rev/compound mixture was injected at 10 μl/min onto a chip immobilized with ~1000 RU of RRE. Free Rev was determined from the binding response 20 s after the end of the association phase. The binding response for Rev in the absence of compounds was found to be linear for Rev concentrations below 50 nM.

2.7. HIV MT4 antiviral cell assay

Antiviral HIV activity and compound-induced cytotoxicity were measured in parallel by means of a propidium iodide-based procedure in the human T-cell lymphotropic virus transformed cell line MT4 (Daluge et al., 1994).

2.8. Data analysis

IC_{50} or EC_{50} values (concentrations which produced 50% inhibition or effect) were determined from nonlinear least squares fits of the data to the simple competitive binding model of Eq. (1).

$$(\mathrm{CPM}_{\mathrm{sample},X} - \mathrm{CPM}_{\mathrm{background}}) = \frac{(\mathrm{CPM}_{\mathrm{max}})X}{\mathrm{EC}_{50} + X} \quad (1)$$

where $\mathrm{CPM}_{\mathrm{sample},X}$ was the experimentally observed count rate at sample compound concentration X (at ten different concentrations of compound), $\mathrm{CPM}_{\mathrm{max}}$ the best fit value for the maximum amplitude of the concentration–response curve and $\mathrm{CPM}_{\mathrm{background}}$ the count rate observed in the absence of Rev–biotin.

3. Results

3.1. SPA development and screening

When biotinylated Rev protein (5 nM) was mixed with [^{3}H]RRE (0.2 nM) and then captured with streptavidin SPA beads, a signal of 2500 cpm above a background of 25 cpm (no Rev protein) was observed. When the concentration of biotinylated Rev was varied at constant [^{3}H]RRE (0.2 nM), a concentration-dependent increase in radioactivity which saturated was measured with an apparent EC_{50} value of 5 nM (Fig. 1A). When [^{3}H]$\Delta G_{104-105}$ RRE was used in the SPA, the apparent EC_{50} value was 20 nM (Fig. 1A), a shift in potency consistent with the literature (Iwai et al., 1992). The increase in signal could be potently inhibited with unlabelled RRE (IC_{50} of 3 nM) and 500-fold more weakly inhibited with yeast tRNA (IC_{50} of 1.6 μM) (Fig. 1B). Inhibition by yeast tRNA appeared to be dominanted by nonspecific charge interactions as the apparent IC_{50} value was

dependent on the salt concentration in the assay: 20 nM at 50 mM NaCl, 300 nM at 100 mM NaCl, 700 nM at 200 mM NaCl, and 1600 nM at 300 mM NaCl. The apparent IC_{50} value for unlabelled RRE did not vary with salt concentration (data not shown). Finally, neomycin B inhibited the SPA with an apparent IC_{50} value of 6 μM, which was consistent with potencies of neomycin B (1–6 μM) reported in the literature (Zapp et al., 1993; Wang et al., 1997; Kirk et al., 2000).

For high throughput screening, a concentration equal to the EC_{50} value (5 nM) of Rev was chosen both to allow for sensitivity of inhibition and also to detect enhancement of binding (the biological rationale for this will be addressed in the Discussion). Approximately 550 000 compounds were tested as pools of ten (individual concentration of 10 μM). After initial screening, decoding, and retesting by full titrations, 248 discrete compounds (0.04%) had IC_{50} values less to or equal to 10 μM.

A number of wells in the primary data from the pooled compounds had significantly *higher* signal than the control. Following decoding and titrations, two discrete compounds demonstrated a dose-dependent increase in signal.

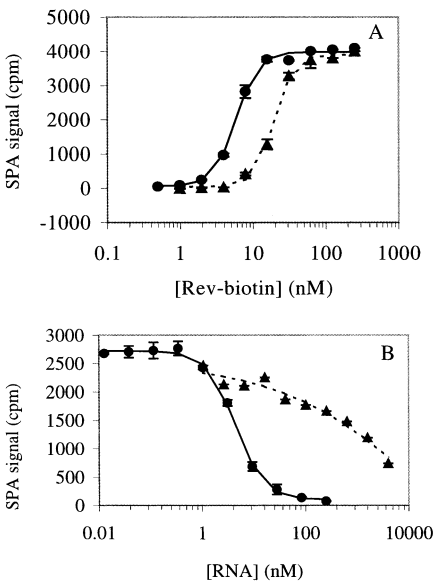


Fig. 1. Specificity of Rev/RRE binding in SPA. (A) The concentration of biotinylated Rev protein was varied in the SPA under conditions described in Experimental Procedures. Circular data points and solid line represent Rev binding to wildtype RRE, and triangular data points and dashed line represent binding to $\Delta G_{104-105}$ RRE. Both RNAs were at 0.2 nM. (B) Inhibition of Rev binding to wildtype [^{3}H]RRE by either unlabeled wildtype RRE (circle/solid line) or yeast tRNA (triangles/dashed line). Rev concentration was 5 nM, and [^{3}H]RRE concentration was 0.2 nM. Data points are averages of four experiments with error bars representing standard deviations, except for inhibition by yeast tRNA (single determination). Lines were calculated from the fit of the parameters in Eq. (1) to the data.

3.2. Structure and activity of Rev/RRE modulators

Examples of structures of Rev/RRE modulators are shown in Fig. 2. Of the 248 small molecule inhibitors, 180 ($\sim$75%) had a commonality of multiple aromatic rings tethered in a linear array (Fig. 2A). All of the more potent compounds (IC_{50} values less than 2 μM) displayed this general feature. These structures could be divided into the following subclasses.

Eighty-five of the aromatic-rich compounds had free carboxylates (71 monoacids and 14 diacids) and are represented by compounds **1**–**3** (Fig. 2A and Table 1). All diacids were symmetrical with each acid at the ends of a linear system of aromatic rings usually connected by amide bonds (ex, **4**). An additional 15 compounds exemplified by compound **5** had sulfate moieties (10 had two and 5 had four sulfates). Therefore, 40% of all inhibitors less than 10 μM had at least one fully negative charge.

The simple presence of a charged carboxylate was not sufficient for inhibition, as the 85 carboxylates represented only 0.1% of total number of free carboxylates in the collection screened. The juxtaposition of a carboxylate with an aromatic system also did not necessarily produce inhibition. Several carboxylate series displayed structure–activity relationships where potency relied at least as much on substitutions distal to the carboxylate than on the presence of a negative charge. One series was exemplified by compound **2** (Table 2). There were 13-fold differences in potencies between **2** and **10** or **11** with only the differences being well removed from the carboxylate [*meta* nitro (**2**) vs. *ortho* nitro (**10**) vs. no functionality (**11**)]. Conversely, there was only a

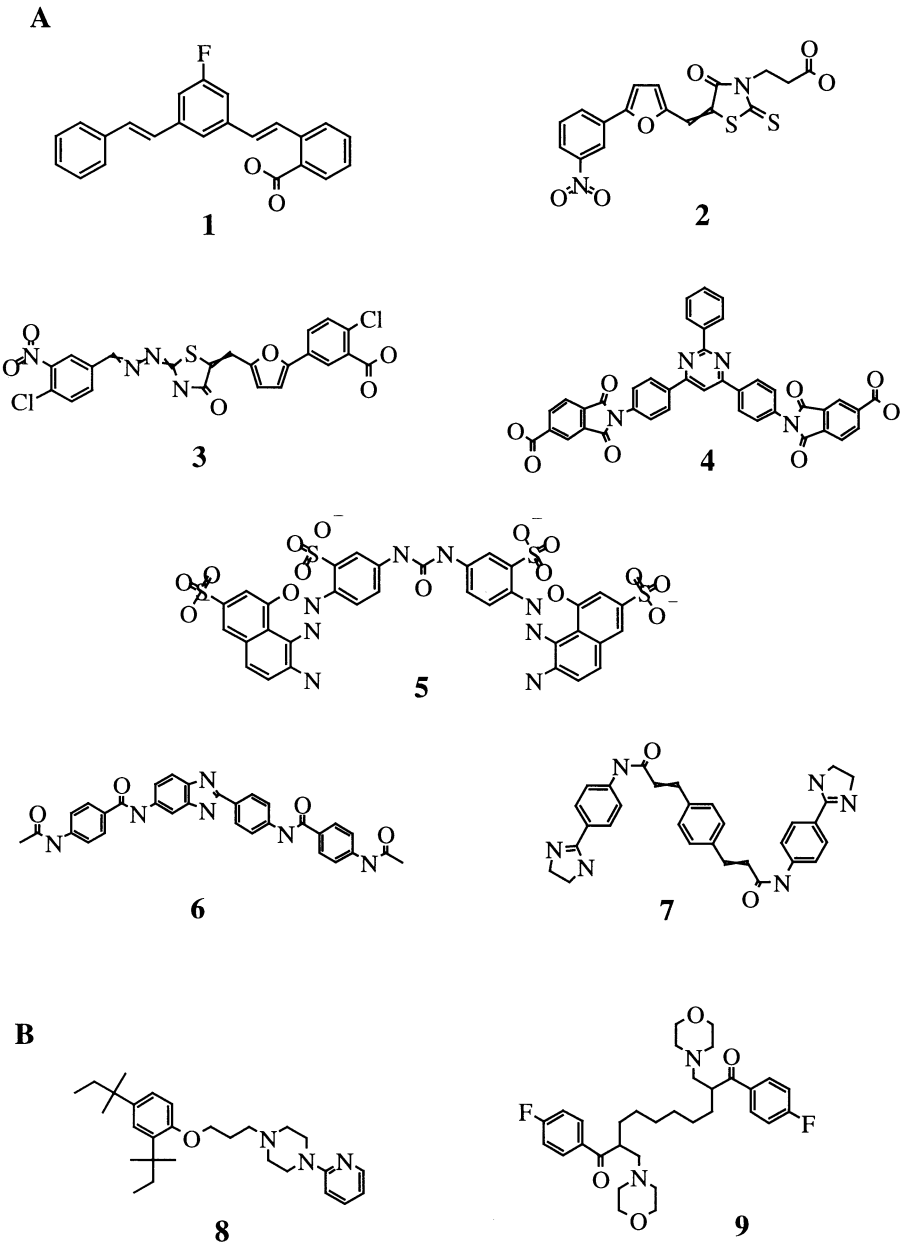


Fig. 2. Structures of representative modulators of Rev/RRE interaction. (A) Structures of inhibitors of Rev/RRE interaction. IC_{50} values for each compound is given in Table 1. (B) Structures of the two enhancers of Rev/RRE interaction. Effect and potency are shown in Fig. 3.

2-fold difference in potency between **12** and **13** where the negative charge was removed, with the two oxygens of the carboxylate (**12**) in potentially the similar orientation in the sulfone derivative (**13**). Finally, the weakness of inhibition of **14** and **15** was further evidence that potency was not simply a negative charge next to any linear aromatic hydrophobicity.

A second subset of molecules was exemplified by compound **6** (Fig. 2A), which was the most potent inhibitor found in HTS with an IC_{50} value of 0.3 μM (Table 1). These molecules were closely related to the diacids (**4**) in being a string of aromatic rings in a symmetrical or near symmetrical display, however, these did not contain carboxylates. The SAR of analogs indicated a specific interaction that was dependent on the correct type and placement of functionality on the terminal rings (Table 3). The terminal amide functionality *ortho* relative to the rest of the molecule (**16**) was inactive at 90 μM and thus was greater than 1000-fold less potent. Other functionalities in the *para* position were tolerated, though less potent: chloro (**17**) 8-fold, an aldehyde (**18**) 22-fold, and the amine (**19**) 25-fold less potent than **6**. Some functionality was absolutely required as the unsubstituted terminal phenyl ring (**20**) was inactive at 90 μM. Finally, it appeared the distance (or chemical identity) between terminal functionalities was critical. Compound **21** which had the *para* amides separated by a slightly shorter distance (19 atoms) versus compound **6** (21 atoms) was 15-fold less potent, and compound **22** which was approximately one half the length of **6** was inactive at 90 μM.

We found eight molecules (ex, **7**, Fig. 2A) that were similar to the diphenyl furan cations previously described in the literature as RRE binders and Rev/RRE inhibitors (Zapp et al., 1997). Each of these compounds was symmetrical with terminal positive charges. The micromolar potencies of these molecules (**7**, Table 1) were similar to the reported potencies of the diphenyl furan cations (Zapp et al., 1997).

The structures of the two compounds that *enhanced* Rev/RRE binding are shown in Fig. 2B, and the effects of these compounds in the SPA are shown in Fig. 3. Both compounds *increased* binding relative to control in similar dose-dependent fashions. Compound **8** maximally increased the SPA signal 72% above control with an apparent EC_{50} value of 4 μM, and compound **9** increased the signal 44% with an apparent EC_{50} value of 7 μM. A limited number of compounds similar in structure to compound **9** were available to probe potential SAR. The analog of **9** with a five carbon spacer between symmetrical units increased binding 28% with an EC_{50} value of 20 μM. In con-

Table 1
Biological activity of small molecule inhibitors of Rev/RRE

Compound number	In vitro binding assays (IC_{50}, μM)			HIV MT4 cell assay (IC_{50}, μM)	
	SPA[a]	Gel shift[b]	SPR[c]	Antiviral activity	Cell toxicity
1	5.2 ± 0.3	20	25	n.d.[d]	63
2	2.9 ± 0.5	2	33	n.d.	36
3	1.1 ± 0.3	4	1.0	n.d.	40
4	0.5	4	8	20	n.d. at 20 μM
5	1.0 ± 0.4	1	0.4	n.d.	8
6	0.3 ± 0.1	–[e]	–	n.d.	n.d. at 10 μM
7	1.2	–	–	n.d.	n.d. at 10 μM
Neomycin B	6 ± 2	1	–	970	n.d. at 2 mM

[a] IC_{50} values in the SPA were determined as described in Experimental Procedures section. Error in determination of individual IC_{50} value was <10%. Titrations of compounds **2**, **3**, **4**, **6**, **8**, **9**, and neomycin B were performed two or more times, and value is the average IC_{50} value ± S.D.

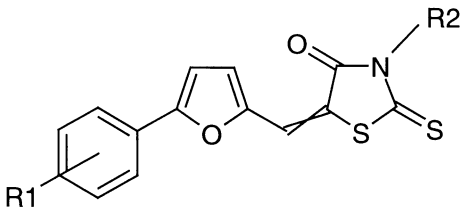
[b] IC_{50} values in the gel shift assay were determined by quantification of the Rev/RRE complexes exemplified in lane 3 in Fig. 4, using ImageQuant 5.0 (Molecular Dynamics) software and fitting data to parameters of Eq. (1). Error in IC_{50} values was approximately 50%.

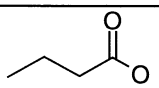
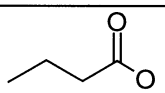
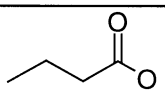
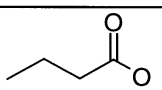
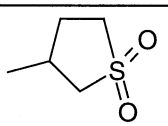
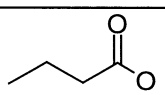
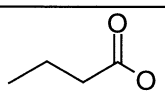
[c] IC_{50} values in the SPR assay were determined by measuring signal of Rev binding to immobilized RRE in the presence of various concentrations of compound and fitting data to parameters of Eq. (1). Error in IC_{50} values was <10%.

[d] Not detected.

[e] Not performed.

Table 2
Structure-activity relationship of an aromatic carboxylate Rev/RRE inhibitor series



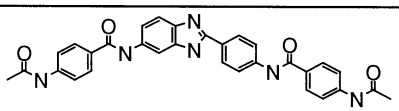
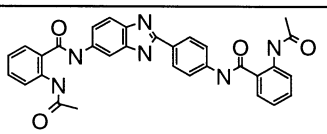
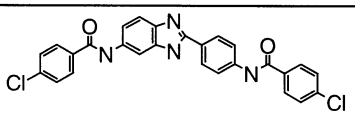
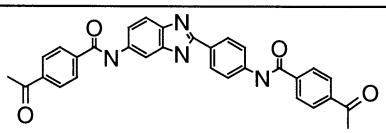
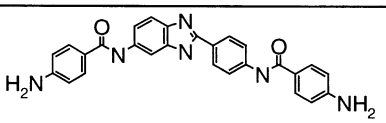
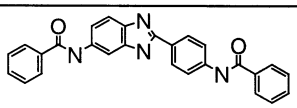
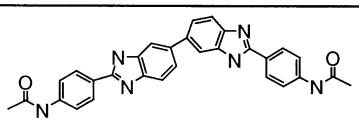
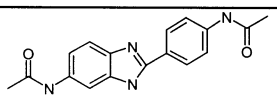
Cmpd #	R1	R2	SPA IC_{50}, μM
2	3-nitro		3
10	2-nitro		40
11	unsubstituted		40
12	2,5-dichloro		4
13	2,5-dichloro		10
14	2-chloro		90
15	4-sulfonamide		90

trast, the analogs with four or two carbon spacers did not alter the SPA signal at concentrations up to 90 μM. Thus, the linker length was critical for this enhancement activity. In addition, the analog that differed only in replacing the two fluorine atoms with hydrogens increased binding 20% with an EC_{50} value of 5 μM. No molecules were available that were as similar to compound **8** (as the analogs of **9** were to **9**). However, three analogs were tested that had the same right-hand side of the molecule (i.e. 2-(4-propylpiperazinyl)pyridine) with very different left-hand sides (structures not shown) and each was inactive at 90 μM. These data argue that the right-hand side was not sufficient for enhancement activity.

3.3. Gel-shift assay for Rev/RRE interaction

To validate the ability of the SPA to identify modulators of Rev/RRE interaction, several compounds were examined for their ability to block the Rev/RRE complex observed by gel shift analysis. In the absence of modulator and at a ratios 6 and 60 of Rev to RRE, the mobility of [^{3}H]RRE was altered such that multiple slower-migrating RRE species were observed when compared with control (control lane 1 vs. 3 and 7 in Fig. 4A and B). In the absence of yeast tRNA and at the higher ratio of 60, much of the complexed RRE migrated very slowly in a diffuse pattern (lane 7 in Fig. 4A). This mobility has been previously attributed to nonspecific Rev/RNA interactions (Iwai et al., 1992). When yeast tRNA was included, this pattern significantly shifted to more

Table 3
Structure–activity relationship of a linear aromatic Rev/RRE inhibitor series

Cmpd #	Structure	SPA IC_{50}, μM
6		0.3
16		>90
17		2.2
18		6.7
19		7.7
20		>90
21		4.6
22		>90

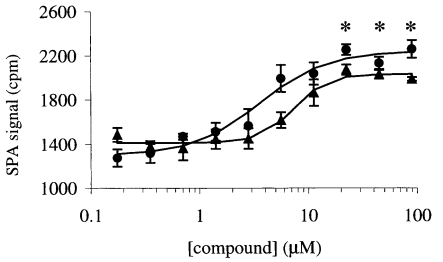


Fig. 3. Dependence on enhancer concentration of the *increase* of the Rev/RRE SPA signal. The concentration of compound **8** (circle) and **9** (triangle) was varied in the SPA at 5 nM Rev protein under condition described in Experimental Procedures. Lines were calculated from the fits of the parameters in Eq. (1) to the data. Data points are mean ± S.D. from four determinations of each concentration of compound. Asterisk indicates that value of signal at indicated concentration has a P value < 0.002 versus no compound controls (for both compounds **8** and **9**) as determined by the Student's t-test.

discrete faster migrating species (lane 7 in Fig. 4B) most likely reflecting specific Rev/RRE interactions. Compound **5** was tested at both Rev/RRE ratios and with or without tRNA and in each experiment inhibited the formation of the Rev/RRE complexes at concentrations between 1 and 10 μM (Fig. 4A and B). Compounds **1**–**5** and neomycin B were fully titrated at a Rev/RRE ratio of 10 in the absence of tRNA (the Rev/RRE migration was identical to lane 3 in Fig. 4A), and the IC_{50} values are presented in Table 1. Furthermore, compounds **8** and **9** *increased* the intensity of the major Rev/RRE band relative to control (data not shown). Due to the small increase in intensity and large standard deviation in measuring the increase ($37 \pm 14\%$ increase for **8** and a $31 \pm 10\%$ for **9**), the EC_{50} values could not be determined accurately. Finally, it is noted that no Rev/RRE modulator other than neomycin B caused a detectable mobility change in RRE (ex, lane 2 in Fig. 4A and B).

3.4. SPR analyses of Rev/RRE modulators

Previous reports described using SPR to study Rev/RRE interaction (West and Ramsdale, 1996–1997; Van Ryk and Venketesan, 1999). Similarly, we observed Rev binding specifically to immobilized RRE and not immobilized yeast tRNA (Fig. 5A). Stoichiometries of greater than 10 Rev monomers to one RRE were observed at high Rev concentrations, which is similar to data recently reported (Van Ryk and Venketesan, 1999). Due to this multimerization, the affinity constant for Rev binding to immobilized RRE could not be accurately determined. This binding could be effectively competed in solution when nonbiotinylated RRE was added (IC_{50} value of 4 nM; Fig. 5B). This value agreed well with the value deter-

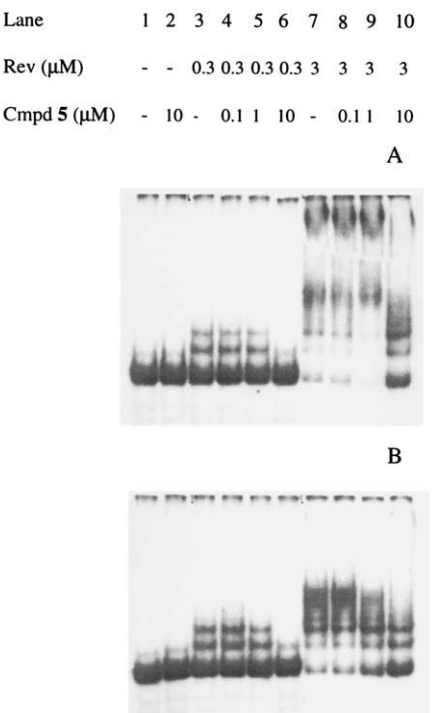


Fig. 4. Inhibition of Rev/RRE complex as monitored by the gel shift assay. Rev and [^{3}H]-RRE were incubated and the complex and RNA were separated as described in Section 2. All lanes contain 50 nM [^{3}H]-RRE. Lanes 3–6 contain 0.3 μM Rev, and lanes 7–10 contain 3 μM Rev. Lane 2 contains 10 μM compound **5**, with lanes 4–6 and 8–10 containing 0.1, 1, and 10 μM compound **5**, respectively. Binding reactions were done in the absence (A) or presence (B) of 4 μM yeast tRNA.

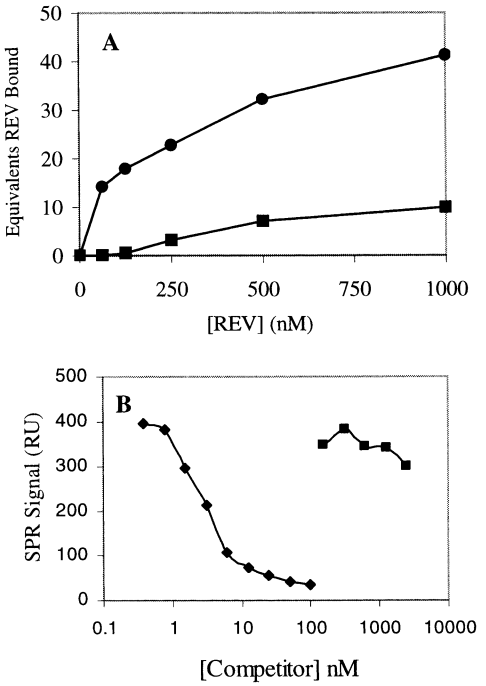


Fig. 5. Specificity of Rev binding for RRE in the SPR Assay. (A) Binding of REV to immobilized RRE (circles) or yeast tRNA (squares). (B) Inhibition of 50 nM REV binding to immobilized RRE by solution concentrations of RRE (diamonds) or yeast tRNA (squares).

mined in the analogous SPA experiment (3 nM). Also consistent with the SPA results, yeast tRNA weakly inhibited ($<30\%$) when added to solutions at concentrations above 1 μM (Fig. 5B). As was noted above with the SPA, when salt concentration was lowered, the nonspecific binding of Rev binding to RNA increased as witnessed by an increase in inhibition by yeast tRNA at lower concentrations of NaCl (data not shown).

The interaction between Rev protein and immobilized RRE could be blocked in solution by several inhibitors described above (Table 1). Of the five compounds examined, the inhibitory potencies measured by SPR varied to differing degrees from the other two assays: **3** and **5** compared very well, **1** and **4** compared moderately well, and **2** less well. It is noted that **6** and **7** had poor aqueous solubility (~ 1 μM) and their effects could not be measured in this assay.

To ascertain whether the small molecule inhibitors bound to either Rev or RRE, we examined the binding of compounds to immobilized RRE or Rev. Neomycin B was the only molecule that bound directly to immobilized RRE, and it did not bind to Rev protein (data not shown). Both compound **3** and **5** bound to immobilized Rev protein (Fig. 6), but did not bind to immobilized RRE (data not shown). In addition, **3** and **5** binding was specific to Rev as these compounds only weakly bound to another nucleic acid-binding protein, HPV E2 (Fig. 6). Interestingly, the binding levels were significantly higher than one would expect for a 1:1 interaction (~ 5 compound **5**/Rev and ~ 8 compound **3**/Rev). A recent report has shown that the refractive index increment of small molecules can alter the amplitude of the SPR signal (Davis and Wilson, 2000). Whether the high signal observed for binding to Rev was due to multiple binding sites or refractive index differences in the compounds is unclear. Compounds **1**, **2**, **4**, **8**, and **9** all produced selective signals binding to Rev at the concentration tested

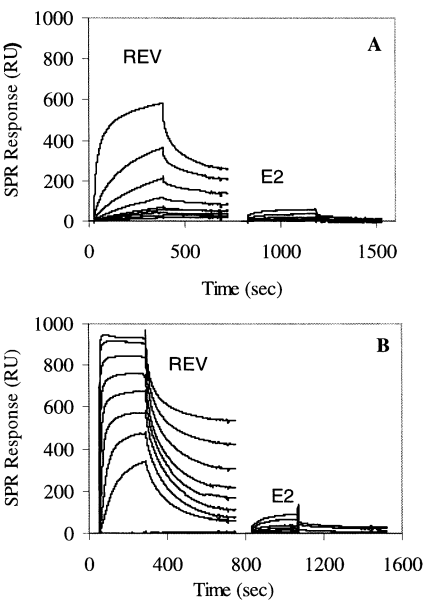


Fig. 6. Direct binding of compounds **3** and **5** to immobilized Rev or HPV E2. Sensorgrams for the binding of various concentrations (60, 30, 15, 7.5, 3.75, 1.88, 0.938, 0.47 μM) of compound **3** (A) or compound **5** (B) to 2500 RU of immobilized Rev (left) or HPV E2 (right).

(25 μM) versus both the RRE RNA and HPV E2 (data not shown), but full titrations could not be performed due to solubility limitations.

3.5. Antiviral activity of Rev modulators

The MT4 HIV IIIb assay (Daluge et al., 1994) is sensitive to HIV inhibitors effecting multiple points of the viral life cycle: fusion, reverse transcription, viral gene transcription, and HIV protease-dependent maturation (data not shown).

None of the in vitro modulators of Rev/RRE binding discovered through screening displayed antiviral activity separate from cell toxicity that could be attributed to inhibition of Rev/RRE (Table 1). Neomycin B did inhibit HIV, though potency was very weak (IC_{50} value ~ 1 mM). Many of the compounds displayed cell toxicity, which would interfere with observation of antiviral activity. Two compounds as mentioned above (**6** and **7**) had limited aqueous solubility and could only be tested at concentrations near their in vitro IC_{50} values. Only the diacid subclass of Rev/RRE inhibitor showed any antiviral activity separate from cell toxicity (ex, **4**); three other similar diacids showed similar antiviral activity (data not shown). All of the diacid inhibitors of Rev/RRE were also tested in a cell-based assay that measures HIV-host cell fusion. There was good correlation between whether or not these molecules inhibited fusion and inhibition of HIV in the MT4 cell assay and the potencies of inhibition (unpublished data, R. Ferris). Therefore, the simplest explanation for this antiviral activity was inhibition of cell fusion. Finally, neither of the two enhancers produced antiviral activity; **8** was toxic (IC_{50} value of 4 μM) and **9** had no effect at concentrations up to 20 μM.

4. Discussion

A scintillation proximity assay was used to identify modulators of HIV Rev/RRE interaction through random screening. The relevance of the SPA was validated by: (1) the apparent binding constant of Rev (5 nM) being consistent with literature values (Kjems et al., 1992; West and Ramsdale, 1996–1997; Kirk et al., 2000); (2) binding inhibited by unlabelled RRE (3 nM) but 500-fold by yeast tRNA (1.6 μM); (3) binding of Rev to the $\Delta G_{104-105}$ RRE being weaker than to the wildtype RRE as was previously described (Iwai et al., 1992); and (4) the IC_{50} value of neomycin (6 μM) being similar to values measured by various techniques (Zapp et al., 1993; Wang et al., 1997; Kirk et al., 2000). Subsequently, several modulators were shown to effect Rev/RRE binding in two other assays (a gel shift assay and surface plasmon resonance analysis) in a similar manner and with similar potency, thus ruling out the possibility that these compounds were effecting the biotin/streptavidin binding in the SPA.

We used SPR analysis to determine if the inhibitors of Rev/RRE interaction bound either the Rev protein or RRE RNA. We observed binding of neomycin to the immobilized RNA. No other small molecule inhibitor examined altered the SPR signal of the RRE. In contrast, both compound **3** and **5** produced a concentration-dependent increase in signal for binding to the immobilized Rev. Compound **5** binding reached an equilibrium, and the apparent binding constant was measured to be 1 μM, which was the same as its inhibition potency. Thus, these data were consistent with the mechanism of inhibition being binding to the Rev protein. Consistent with these compounds not binding RRE was that yeast tRNA, at a concentration that did not effect the SPA signal in the absence of compound (100 nM), affected the potency of neomycin B (IC_{50} value shifted 10-fold higher; data not shown) but not any of the small molecules listed in Table 1. The binding of negatively-charged small molecules to Rev is consistent with the negatively-charged RNA binding to Rev with positively-charged amino acid residues of Rev involved in that binding (Battiste et al., 1996).

We chose to screen at the EC_{50} value of Rev to sensitize the assay for inhibition and to allow for enhancement of binding. Our reasoning to look for enhancers or stabilizers was based on (1) literature precedence for small molecule stabilizers of protein complexes which had pharmacological activity (Robineau et al., 2000; Smith et al., 1986);

and (2) the theoretical possibility that a stabilized Rev/RRE complex may negatively alter an essential function for viral replication (ex, block Rev/RRE nuclear export, interfere with translation, block nuclear re-entry of Rev). Compounds **8** and **9** increased both the SPA and gel shift assay signal in a dose-dependent fashion. Furthermore, analogs of **9** also displayed this activity and suggested that a critical structure–activity relationship was the length of the central methylene tether. However, because of the lack of antiviral effect, the mechanism of the in vitro stabilization was not pursued.

The identification of small molecular weight molecules that effect the nanomolar binding of a 14 kDa protein and a structured RNA of over 200 bases with micromolar and submicromolar binding is noteworthy. Inhibitors of other macromolecular interactions have been reported [protein–DNA (Hajduk et al., 1997), protein–RNA (Mei et al., 1998), and protein–protein (Li et al., 1997; Perrier et al., 2000)]. What has been lacking to date is the progression of initial micromolar potencies to nanomolar potencies typically needed to observe pharmaceutical effects. A common prelude to increasing potency is SAR to guide the medicinal chemistry work. Several of these modulators of Rev/RRE displayed specific SAR. Furthermore, data from the collection of aromatic molecules that contain a single carboxylate were used to generate a three-dimensional pharmacophore model using the Catalyst software (unpublished data, D. Vanderwall, R. Chapman, and E. Garvey). This model was then used to search a three-dimensional databases of commercially available compounds, and 431 were purchased and tested in the SPA. The model was validated by finding 30 compounds (7% of total tested) with IC_{50} values less than 10 μM, which was 175 times the success rate of finding such potency in the initial random screening (0.04%).

A serious limitation in this work was the lack of small molecule standards to compare in in vitro and cell assays. Thus, it was unknown what in vitro potency was needed to observe inhibition of viral replication in a cell assay. If neomycin B's antiviral activity is due to inhibition of Rev/RRE in infected cells, then there is a 100-fold differential between its in vitro and whole cell potency (Zapp et al., 1993 and this study). If this reflects a general differential, we simply did not achieve the in vitro potency that would translate into antiviral activity. Regardless of whether the 100-fold differential is accurate, because of the critical lack of antiviral activity, none of these compounds were progressed for further study, and this work was concluded.

Acknowledgements

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