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**Bioavailable inhibitors of HIV-1 RNA biogenesis identified through a Rev-based screen**

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Corresponding Author: Dr. José Gallego,

Corresponding Author's Institution: Universidad Catolica de Valencia

First Author: Silvia Prado

Order of Authors: Silvia Prado; Manuela Beltrán; Mayte Coiras; Luis M Bedoya; José Alcamí; José Gallego

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Abstract: New antiretroviral agents with alternative mechanisms are needed to complement the combination therapies used to treat HIV-1 infections. Here we report the identification of bioavailable molecules that interfere with the gene expression processes of HIV-1. The compounds were detected by screening a small library of FDA-approved drugs with an assay based on measuring the displacement of Rev, and essential virus-encoded protein, from its high-affinity RNA binding site. The antiretroviral activity of two hits was based on interference with post-integration steps of the HIV-1 cycle. Both hits inhibited RRE-Rev complex formation in vitro, and blocked LTR-dependent gene expression and viral transcription in cellular assays. The best compound altered the splicing pattern of HIV-1 transcripts in a manner consistent with Rev inhibition. This mechanism of action is different from those used by current antiretroviral agents. The screening hits recognized the Rev binding site in the viral RNA, and the best compound did so with substantial selectivity, allowing the identification of a new RNA-binding scaffold. These results may be used for developing novel antiretroviral drugs.

José Gallego
Facultad de Medicina
Universidad Católica de Valencia
C/Quevedo 2, 46001 Valencia Spain
Tel. +34-963-637412 ext. 52085; e-mail: jose.gallego@ucv.es

Jacques G. Piette
Editor
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*Bioavailable inhibitors of HIV-1 RNA biogenesis identified through a Rev-based screen*

February 12th, 2016

Dear Dr. Piette,

Thank you for editing our manuscript. We were happy to read that both reviewers found the paper interesting and that pending some revision, our article may be accepted for publication.

To address the main concern of reviewer 1 regarding the cellular mechanism of action of the screening hits, we have carried out RT-qPCR experiments to monitor the levels of unspliced, single-spliced and multiple-spliced viral RNA transcripts in cells transfected with an HIV-1 plasmid. These experiments have allowed us to detect changes in HIV-1 splicing patterns induced by our best compound, clomiphene, that are consistent with inhibition of Rev function. Likewise, these assays confirmed that both clomiphene and the second-best hit, cyproheptadine, inhibited HIV-1 transcription. Altogether, these results have led us to slightly change the title of the manuscript, replacing the more general term “inhibitors of HIV-1 gene expression” with “inhibitors of HIV-1 RNA biogenesis”, an expression that encompasses transcription and RNA export.

We provide below point by point answers to the referees’ comments. For her contribution in generating the new results, Mayte Coiras has been included as a coauthor of the manuscript. We thank both reviewers for their constructive criticisms, and hope that you will find the revised version of the manuscript suitable for publication in *Biochemical Pharmacology*.

Sincerely yours

José Gallego

On behalf of the authors

**Reviewer 1**

*"This manuscript describes the discovery of molecules, out of a FDA-approved library, interfering with the gene expression of HIV-1. They were screened against the Rev/IIB interaction in vitro. Two hits inhibit the LTR-driven gene expression in cells. This is an interesting paper describing the identification of Rev/RNA interaction inhibitors."*

*"My major concern is that although the molecules inhibit the LTR-driven gene expression it has not been directly shown that the molecules inhibit the Rev function in cells, although they block the Rev/RNA interaction in vitro."*

To address the major concern of reviewer 1, we have carried out RT-qPCR experiments to monitor the levels of unspliced, single-spliced and multiple-spliced HIV-1 RNA transcripts in treated or untreated cells previously transfected with an HIV-1 plasmid. The changes in HIV-1 splicing patterns induced by clomiphene are consistent with Rev blockage, as explained below in the answer to comment 4 of this reviewer.

*"Specific comments"*

1. *"Page 3 last sentence of 1st and 2nd paragraph: Note that there exist inhibitors of*

*the Rev/Crm1 function with anti-HIV activity being tested in the clinic see Boons et al. EBioMedicine 2015"*

A reference to these inhibitors has been included in the text (p. 3, second paragraph): "Selective Crm1 inhibitors that block Crm1-dependent nuclear export and exert anti-neoplastic and anti-HIV effects are currently being evaluated in clinical studies [13]. However, up to now there are no inhibitors acting on the RRE-Rev system under clinical development."

2. *"2.9 page 10, Mat Methods on cellular anti-HIV activity should be better described; what was the moi used for infection? how many cells were infected?"*

To answer the referee's comment, more detailed data about culture infection have been included in section 2.9 (Evaluation of anti-HIV-1 activity and cellular toxicity) of Materials and Methods (p. 11, second paragraph): "Infectious supernatants were obtained from calcium phosphate transfection of plasmid pNL4.3-Ren on 293T cells. These supernatants were collected and titrated with two different methods: by infecting MT-2 cells ($10^6$ per well) with different concentrations of supernatants and measuring relative luminescence units (RLUs) 48 hours later, and by quantifying HIV-1 antigen p24 with an enzyme-like immunoassay (Innotest$^{TM}$ HIV Ag mAb; Innogenetics, Barcelona Spain). These supernatants were used to infect MT-2 cells ($10^6$ per well) with 100.000 RLUs per well, equivalent to 10 ng p24 per well, in the presence of the compounds to evaluate."

3. *"Page 15, 3.4 Antiviral activity. In the first sentence the authors asked whether the hits block the replication in cell culture. Does this assay as performed here really measure replication of virus? How much virus is used to infect the cells? Read out is luciferase activity driven from the LTR in virus background. Authors must use a reverse transcriptase inhibitor and, even more important because of the post-integration mechanism of action, a protease inhibitor as control in this experiment to show they measure replication in their assay."*

The molecular clones used in both infection and transfection experiments generate fully replicative, multiple-cycle competent viruses as previously described. This statement is now specified in a sentence inserted in section 2.8 (Plasmids, viruses and cells for ex vivo assays) of Materials and Methods (p.10, second paragraph): "These constructs generate replication-competent viruses as previously shown [30]”. Accordingly, treatment with reverse transcriptase or protease inhibitors results in inhibition of luciferase activity. Following the reviewer’s recommendation, the viral titration and input doses are now described in section 2.9 (p.11 of Materials and Methods), and data from zidovudine (a reverse transcriptase inhibitor) and nelfinavir (a protease inhibitor) controls have been included in a footnote to Table 3. The following sentence has also been added to the text of section 3.4 (antiviral activity) of Results (p.17, first paragraph): “A reverse transcriptase inhibitor (zidovudine) and a protease inhibitor (nelfinavir) were used as positive controls of inhibition of HIV-1 replication (Table 3)”.

4. *“Page 17 "Since the expression of the RRE-luciferase and Rev genes used in this assay depends on transcription, the RRE-Rev activities of these two compound may be dominated by a transcriptional effect". This is indeed my major concern. Authors should demonstrate that the mechanism of action in vitro also occurs in cells. For example, authors could directly measure Rev activity in infected cells.”*

We totally agree with the referee. To specifically evaluate the effect on Rev function, we have measured the splicing of HIV-1 RNA transcripts by RT-qPCR in cultured cells as suggested by the referee. Since Rev is involved in the export of unspliced or partially spliced viral transcripts to the cytoplasm where they cannot be spliced, impairment of Rev function by drug inhibition should result in lower levels of unspliced and single-spliced transcripts. This is precisely what is detected when treating the cells with clomiphene. In the case of cyproheptadine, inhibition of HIV-1 transcription may have impeded detection of a direct effect on Rev. The methodology used for carrying out these experiments is described in a new section in p.12 of Material and Methods (2.12. Evaluation of HIV-1 RNA splicing). The results have been summarized in a new figure (Figure 7) and are described in p.19, second paragraph, and p. 20, first paragraph, under section 3.7 (Evaluation of RRE-Rev inhibition) of Results. They are discussed in p. 24 (second paragraph) of the Discussion section.

5. *“Fig 5. (B) ".... luciferase plasmid under the control of the whole genome of HIV-1" Please rephrase. The plasmid is not under control of the HIV genome, neither is the luciferase. The luciferase gene is under the control of the HIV LTR promotor. Basically this is a similar assay as the one described in Fig 6 A, indeed, note that the EC50 values in Table 3 (middle column) and Table 4 (first column) are in the same range between*

*the different compounds"*

According to the referee's suggestion, the sentence in the legend of Figure 5 has been changed to: "(B) Inhibition of HIV-1 post-integration steps: the cells were transfected with a full-length proviral DNA plasmid containing a luciferase reporter gene whose expression is under the control of the HIV-LTR promoter."

## Reviewer 2

*"Gallego and co-workers report the screening of about 1,000 FDA-approved bioavaialble small molecules for binding to the RRE RNA from HIV, an RNA structure required for viral replication that is recognized by the viral protein Rev to affect, with host factors, the nucleocytoplasmic transport of the viral RNA. The RRE has been called a good antiviral target for many years, but reviews on its potential have far outweighed any interesting targeting structure. without much progress. Using a fluorescent peptide displacement screen, the authors identify a few (4) compounds that bind to the RNA with low uM activity and varying degrees of specificity."*

*"The binding data and screening are of very high quality. The most interesting observation contained in the manuscript is the identification of a common scaffold that binds to RNA (Fig. 3C). This is a very interesting result, and should be emphasized more because such structures are rare."*

We thank reviewer 2 for his encouraging comments. Following his/her recommendation we have emphasized to a higher degree the finding of a new RNA-binding scaffold by incorporating it in the graphical abstract. Likewise, this result is mentioned in the Abstract, Figure 3C, and Discussion section (second paragraph of p. 23 and concluding paragraph, p. 26).

*"The authors go on to characterize the anti-viral activity of some of the best compounds. I am not convinced that the activity of any of the compounds is through the Rev-RRE mechanism. More virological and cell-based studies would be required to prove this fully, and it is hoped that the authors will do that in the future. I don't mean to say that the data are wrong, just that more tests need to be executed to demonstrate the mechanistic relationship between any effect on viral replication and the Rev-RRE axis; in the past, many TAR binding molecules (all but one so far as this reviewer knows), gave rise to false hopes in these sort of studies because of insufficient*

*characterization in cells. This is for future work, though."*

Although reviewer 2 did not ask for additional tests in this manuscript, we have addressed his/her comment by carrying out the additional experiments described above (see answer to comment 4 of Reviewer 1). The changes in the levels of multiple-spliced versus single-spliced and unspliced HIV-1 transcripts induced by clomiphene were consistent with inhibition of Rev function.

*"As a smaller compliment to the authors, I am heartened by some of the observations of the authors concerning the lack of specificity of some well-known RNA ligands (aminoglycosides) whose identification has led to the publication of considerable amount of garbage in the past. The case of mitoxanthone is more complex, in that it might be structure selective (and therefore provide another scaffold for RNA binding), but it is also true that it is not specific for the RRE, as the authors correctly observe. This attitude is refreshing."*

We thank the reviewer for his/her positive comment. We agree that mitoxantrone may be active against the RRE-Rev system, as suggested by the strong effect detected in the EMSA experiments (Figure 4). Likewise, in this case the $IC_{50}$ values measured in the RRE-Rev transfection assay (Figure 6B) were below the post-integration and LTR inhibitory concentrations, suggesting that RRE-Rev inhibition may contribute to the post-integration activity of this agent. This is mentioned in p.19 of Results, first paragraph. Mitoxantrone's impact on the HIV-1 splicing patterns could not be evaluated because of the toxic effect of this compound at the assay's concentrations (this is explained in p.20 of Results, first paragraph).

# Bioavailable inhibitors of HIV-1 RNA biogenesis identified through a Rev-based screen

Silvia Prado[a], Manuela Beltrán[b], Mayte Coiras[b], Luis M. Bedoya[b,c], José Alcamí[b]* and José Gallego[a]*

[a]Facultad de Medicina, Universidad Católica de Valencia, C/Quevedo 2, 46001 Valencia, Spain

[b]Instituto de Salud Carlos III, Carretera Majadahonda-Pozuelo km 2, 28220 Majadahonda, Spain

[c]Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramón y Cajal s/n, 28040, Madrid, Spain

*Corresponding authors contact data: José Gallego, e-mail: jose.gallego@ucv.es; tel. +34-963-637412. José Alcamí, email: ppalcami@isciii.es; tel +34-918-22393.

## Abstract

New antiretroviral agents with alternative mechanisms are needed to complement the combination therapies used to treat HIV-1 infections. Here we report the identification of bioavailable molecules that interfere with the gene expression processes of HIV-1. The compounds were detected by screening a small library of FDA-approved drugs with an assay based on measuring the displacement of Rev, and essential virus-encoded protein, from its high-affinity RNA binding site. The antiretroviral activity of two hits was based on interference with post-integration steps of the HIV-1 cycle. Both hits inhibited RRE-Rev complex formation *in vitro*, and blocked LTR-dependent gene expression and viral transcription in cellular assays. The best compound altered the splicing pattern of HIV-1 transcripts in a manner consistent with Rev inhibition. This mechanism of action is different from those used by current antiretroviral agents. The screening hits recognized the Rev binding site in the viral RNA, and the best compound did so with substantial selectivity, allowing the identification of a new RNA-binding scaffold. These results may be used for developing novel antiretroviral drugs.

**Keywords:** human immunodeficiency virus type 1; Rev; RNA; screen; transcription

Chemical compounds studied in this article

Ciprofloxacin (PubChem CID: 2764); Clomiphene (PubChem CID: 1548953); Cyproheptadine (PubChem CID: 2913); Homochlorcyclizine (PubChem CID: 3627); Mitoxantrone (PubChem CID: 4212); Neamine (PubChem CID: 72392); Neomycin B (PubChem CID: 8378)

## 1. Introduction

The current antiretroviral combination therapies imply life-long treatments with a strict adherence to dosage regimens to maintain viral suppression [1, 2]. In addition, the virus is capable of acquiring resistance to these treatments, and resistant strains spread through transmission among individuals [3]. Consequently, there is a need to develop new antiretroviral agents with different inhibitory mechanisms relative to those used by existing HIV therapeutics, which act on all of the virus-encoded enzymatic functions as well as on virus entry into the cell [4]. One aspect of the virus life cycle that is not targeted by any of the compounds currently used in the market encompasses the gene expression processes of the virus, including transcription, RNA processing and transport.

Rev is a virally-encoded, 116-amino acid protein with a helix-turn-helix structure [5, 6] that uses an arginine-rich $\alpha$-helix (Rev$_{34-50}$) to enter the cell nucleus and recognize the Rev Recognition Element (RRE), a strongly conserved 350-nucleotide structure located in the second intron of the HIV-1 genomic RNA. Rev$_{34-50}$ forms a high-affinity complex with an internal loop of RRE subdomain IIB [7]. This interaction triggers the cooperative incorporation of additional Rev molecules to the complex through interactions with further sites on the RRE and protein-protein contacts [8, 9] (Figure 1). The subsequent tethering of this RRE-Rev ribonucleoprotein complex to the Crm1 host export factor allows transport of unspliced or incompletely spliced viral RNA molecules to the cytoplasm of the infected cell in the late phase of the virus cycle. In addition to this function, Rev has been reported to enhance RNA translation and packaging [10, 11] and to modulate the nucleocytoplasmatic shuttling of the HIV-1 integrase [12]. Indeed, this small regulatory protein is essential for virus viability. Selective Crm1 inhibitors that block Crm1-dependent nuclear export and exert anti-neoplastic and anti-HIV effects are currently being evaluated in clinical studies [13].

However, up to now there are no inhibitors acting on the RRE-Rev system under clinical development.

Here we report the identification of bioavailable HIV-1 inhibitors that interfere with gene expression processes of the virus. The compounds were detected by screening a small library of FDA-approved drugs with an assay based on measuring the displacement of Rev from its RNA binding site. The antiretroviral activity of two of the hits had not been previously reported and was based on interference with post-integration processes of the virus cycle. Both compounds inhibited RRE-Rev complex formation, and blocked HIV-1 LTR-dependent gene expression and viral transcription in cellular assays. The best hit altered the levels of multiple-spliced versus single-spliced and unspliced HIV-1 transcripts in a manner consistent with inhibition of Rev function. This mechanism of action is different from those used by currently marketed antiretroviral agents. On a broader perspective, all of the screening hits bound to RNA subdomain IIB with different degrees of affinity and specificity, and this study allowed us to identify a new RNA-binding scaffold. This is also important, as the search for RNA-binding therapeutics is relatively underdeveloped but shows increasing promise [14, 15]. The findings described in this report may be useful for the development of new antivirals with alternative mechanisms of action.

## 2. Materials and Methods

*2.1. Preparation of RNA, DNA, peptide and protein samples*. The $IIB_h$ RNA oligonucleotide (Figure 1D) used in NMR spectroscopy and fluorescence anisotropy experiments was purchased from Dharmacon (ThermoFisher, Wilmington USA). After removing the 2'-ACE protecting groups, this sequence was purified on denaturing 20% polyacrylamide gels containing 8 M urea, and subsequently electroeluted from the gel, ethanol-precipitated and desalted. For the fluorescence intensity experiments, two fluorescent $IIB_h$ constructs were purchased HPLC-purified from Microsynth AG and desalted. In the $IIB_h$-19ap system, 2-amino purine replaced adenine at unpaired loop nucleotide A19. Construct $IIB_h$-23fl contained a fluorescein (FITC) probe linked to the extra-helical loop nucleotide U23 (Figure 1D). Full-length RRE RNA (Figure 1B) was synthesized by run-off transcription from a pUC19 vector linearized with SmaI. This vector was generated by cloning a 231-nt portion of an RRE sequence obtained from plasmid pDM628 between the SmaI and EcoRI sites of pUC19. After transcription, the RRE RNA was purified using a gradient of NaCl in sodium phosphate buffer in a 26/60 anion exchange column (GE Healthcare, Little Chalfont UK), ethanol-precipitated and desalted. *Escherichia coli* $tRNA^{Cys}$ was transcribed *in vitro* from a BstNI-digested pUC19 plasmid, a generous gift from M. Eugenia Armengod (CIPF, Valencia Spain) and purified following a protocol based on phenol-chloroform extraction and anion-exchange chromatography [16]. The 26-nt d(GGCGGGACTTTCCGGAAAGTCCCGCC) self-complementary DNA oligonucleotide ($LTR_d$) containing NF-κB and Sp-1 binding sequences present in the LTR promoter of HIV-1 was purchased from Microsynth AG and purified as described for $IIB_h$.

An FITC-Ahx-GTRQARRNRRRRWRERQRAAAAR-am peptide (frevp) containing a fluorescein (FITC) moiety attached to the $N_t$ Gly residue was purchased HPLC-purified from Genscript (Piscataway USA) and used for the fluorescence anisotropy experiments. A similar but unlabeled succynyl-TRQARRNRRRRWRERQRAAAAR-am peptide (revp) was also purchased from Genscript. Both peptides contain the Arg-rich $Rev_{34\text{-}50}$ residue tract forming the RNA-binding α-helix of Rev, together with an additional $C_t$ AAAAR segment to stabilize the α-helix [17]. The full-length Rev protein was expressed in BL21 E. coli cells using a pET11d plasmid containing a Rev gene (HIV-1 BH10 strain) engineered to avoid usage of low abundance Arg codons in E. coli [18] (kindly provided by F. Blanco and R. Tycko). Purification of Rev was performed in the presence of 6 M urea essentially as described [18], passing the protein through anion and cation exchange columns followed by polishing on a 16/70 gel filtration column (GE Healthcare). Protein purity was assessed by SDS electrophoresis. To avoid aggregation, a purified stock dissolved in 6 M urea was microdialyzed at protein concentrations below 25 µM in an EMSA buffer containing 10 mM HEPES pH 7.5, 300 mM KCl, 1 mM $MgCl_2$ and 0.5 mM EDTA.

*2.2. Compounds*. The primary fluorescence anisotropy screen used a collection of 1,120 FDA-approved drugs dissolved in DMSO at a concentration of 5 mM, acquired from Prestwick Chemical (Illkirch France). According to the manufacturer, the compounds were selected for their high chemical and pharmacological diversity and accessible information on bioavailability and safety in humans. For subsequent experiments, neomycin B, clomiphene and ciprofloxacin were obtained from Sigma-Aldrich (St. Louis USA), neamine from Toronto Research (Toronto Canada), and cyproheptadine and mitoxantrone from Santa Cruz Biotechnology (Dallas USA). Additional amounts of other compounds were obtained from the Prestwick stocks. The antiretroviral drugs zidovudine and nelfinavir used as positive controls in the anti-HIV activity assay were obtained through the AIDS Reagent Program (NIAID, NIH USA).

*2.3. Fluorescence anisotropy*. These experiments were conducted at 25 °C using 96-well plates and Victor X3 or Victor X5 (PerkinElmer, Waltham USA) plate readers set up with 480 and 535 nm excitation and emission filters, respectively. The fluorescence anisotropy buffer contained 30 mM HEPES (pH 6.8), 100 mM KCl, 10 mM sodium phosphate, 10 mM ammonium acetate, 10 mM guanidinium chloride, 2 mM $MgCl_2$, 20mM NaCl, 0.5 mM EDTA, and 0.001 % (v/v) Triton X-100 [19].

The primary screen used a Tecan robot to incubate frevp (at a concentration of 10 nM) and $IIB_h$ (2 nM) with each of the 1,120 drugs dissolved at a concentration of 100 μM in the 200 μL assay mixture. Each 96-well plate experiment had one positive (a mixture of $IIB_h$ and frevp, equivalent to 0% inhibition) and two negative (isolated frevp as well as a mixture of $IIB_h$, frevp and unlabeled revp) controls. In order to ensure that there was no spectral overlap with FITC, anisotropy data were collected for each of the compounds that inhibited more than 50% $IIB_h$-$Rev_{34-50}$ complex formation. We only accepted hits that did not fluoresce at the assay conditions.

Subsequent dose-response fluorescence anisotropy experiments used 10 nM frevp and 60 nM $IIB_{h.}$ As in the primary screen, each experiment had one positive and two negative controls, and we only accepted experiments were the anisotropy reached the expected minimum value at the highest concentrations of inhibitor. 50% inhibitory concentration ($IC_{50}$) values were calculated by fitting observed anisotropy (A) to the following equation with Prism (GraphPad Software, San Diego USA):

$$A = A_f + \frac{(A_b - A_f)}{1 + (\frac{C}{IC_{50}})^m}$$

where $A_f$ and $A_b$ are the anisotropy values measured for free and $IIB_h$-bound frevp, respectively, C is the total concentration of small molecule inhibitor and m is the slope of the linear portion of the sigmoidal curve. $IC_{50}$ and m were allowed to float during the fitting process. Each fluorescence anisotropy experiment was repeated at least two times. This assay had been previously validated in our laboratory by measuring the inhibitory activity of revp and neomycin B [20].

*2.4. Fluorescence intensity*. Depending on the absorbance spectra of the ligands, these experiments measured association to $IIB_h$-19ap or $IIB_h$-23fl RNA constructs labeled with 2-aminopurine and fluorescein at unpaired loop IIB residues A19 and U23, respectively (Figure 1D). The RNA (at 100 nM concentration) was pre-equilibrated for 5 minutes in a buffer containing 10 mM sodium phosphate pH 6.6 and 0.1 mM EDTA after ligand addition. For experiments involving $IIB_h$-19ap, fluorescence intensity was measured using a SPECTRA GEMINI XPS plate reader (Molecular Devices, Sunnyvale USA), with excitation and emission wavelengths of 310 and 385 nm, respectively. Experiments involving $IIB_h$-23fl were carried out in a Victor X5 plate reader (Perkin Elmer), using excitation and emission wavelengths of 485 and 520 nm, respectively. The equilibrium dissociation constants $K_d$ were determined by fitting the observed fluorescence intensities ($I$) to the following equation with Prism:

$$I = 1 + (I_{\min} - 1)\left(\frac{(K_d + C_{IIB} + C) - \sqrt{(K_d + C_{IIB} + C)^2 - 4C_{IIB}C}}{2C_{IIB}}\right)$$

where $C_{IIB}$ is $IIB_h$ concentration (100 nM), C ligand concentration, and $I_{min}$ fluorescence intensity in the absence of compound. The RNA and DNA specificity of the interactions was assessed by duplicating the experiments in the presence of a 100-fold molar excess (10 µM) of either $tRNA^{Cys}$ or DNA duplex $LTR_d$. These experiments were validated using the reference antibiotic neomycin B, for which we measured a $K_d$ of 0.84 ± 0.17 µM together with $K_d(IIB_h)/K_d(IIB_h$+tRNA) and $K_d(IIB_h)/K_d(IIB_h+LTR_d)$ selectivity ratios of 0.01 and 0.02 (Table 2 and Figure 2D). Neomycin's $IIB_h$ affinity and limited specificity were consistent with our previous SPR observations [20] and other literature reports [21-23].

*2.5. NMR spectroscopy*. NMR spectra were acquired in a Bruker Avance III 500 MHz spectrometer, and analyzed using Topspin 1.3 (Bruker Biospin, Billerica USA) and Sparky 3.110 [24]. The $IIB_h$ RNA samples were previously microdialyzed in an aqueous solution containing 10 mM sodium phosphate (pH 6.0) and 0.1 mM EDTA. The interaction of 40-60 μM (5-8 ODs) $IIB_{h,}$ samples with the small molecule ligands was monitored at 27 $^{o}$C using two-dimensional (TOCSY) and/or one-dimensional experiments at increasing ligand:RNA molar ratios: 1:1, 2:1, 4:1 and, depending on the observed spectral changes, 6:1.

*2.6. Electrophoretic mobility shift assays (EMSA)*. These experiments utilized 78 nM full-length RRE and 1.32 μM full-length Rev dissolved in 10 mM HEPES pH 7.5, 300 mM KCl, 1 mM $MgCl_2$ and 0.5 mM EDTA binding buffer, and increasing concentrations of each compound [9]. The reactions were incubated at room temperature for 20 minutes and loaded onto 8% polyacrylamide gels with TB running buffer. Gels were run at 4ºC for 1–4 hours at 150 V, and the bands were stained with SYBR gold (Life Technologies, ThermoFisher, Wilmington USA) and quantified with Image J (W.S. Rasband, NIH Maryland, USA). $IC_{50}$ values were determined by fitting the data to a simple competitive binding model with Prism:

$$a - a_i = \frac{a_{\max} C}{IC_{50} + C}$$

where *a* is the area of the band corresponding to unbound RRE species at compound concentration *C*, $a_{max}$ the best-fit value for the maximum area of each concentration–response curve, and $a_i$ the area of unbound RRE band in the absence of compound [25]. These experiments were repeated at least two times for each compound. The assay was validated by examining the inhibitory activity of neomycin B, for which we obtained an $IC_{50}$ of 7 ± 1 μM. This value was in very good agreement with previously reported data [25-27].

*2.7. Molecular modeling*. Three-dimensional models of complexes of loop IIB with the screening hits were built by docking the ligands into the subdomain IIB RNA structure (PDB code 1ETG) [7] using Gold 5.2 [28]. The ligand binding site was defined with a very large 60 Å radius around nucleotide C20. All calculations were unrestrained, employed the GoldScore fitness function [28] and generated 20 solutions for each ligand with maximum search efficiency. In all cases, the compounds were predicted to bind to the major groove side of loop IIB forming Rev's high-affinity site. For cyproheptadine and homochlorcyclizine, the docking runs resulted in converged sets of 11 (55%) and 12 (60%) solutions that had root-mean-square (rms) deviations lower than 1.4 and 0.7 Å, respectively, and included the best-scored poses. Clomiphene had higher scores but poorer convergence: 12 (60%) solutions including the best scored poses were distributed in five different sets with rms deviation lower than 1.1 Å.

*2.8. Plasmids, viruses and cells for ex vivo assays*. Vectors pNL4.3-Luc and pNL4.3-Ren were generated by cloning the luciferase and renilla genes, respectively, in the nef site of HIV-1 proviral clone pNL4.3 [29]. These constructs generate replication-competent viruses as previously shown [30]. pCMV-Rev expresses Rev, and pDM628 is a Rev-dependent luciferase-based reporter plasmid in which the RRE and a luciferase-coding sequence have been cloned. The LTR-luc plasmid carries a luciferase gene under the control of the HIV-1 LTR [31]. In the 3-enh-κB-ConA-luc plasmid, luciferase expression depends on three synthetic copies of the κB consensus of the immunoglobulin k-chain promoter cloned into the BamHI site located upstream from the conalbumin transcription start site [32]. pCMV-Rev and pDM628 were generous gifts from Dr. Cecilia Cabrera (IrsiCaixa Foundation, Barcelona Spain). MT-2 [33] cells (American Type Culture Collection, Rockville USA) were cultured in DMEM medium containing 10% (v/v) fetal bovine serum in standard conditions. 293T cells (American Type Culture Collection) were cultured in DMEM medium containing 10% (v/v) fetal bovine serum in standard conditions and were split twice a week.

*2.9. Evaluation of anti-HIV-1 activity and cellular toxicity*. Infectious supernatants were obtained from calcium phosphate transfection of plasmid pNL4.3-Ren on 293T cells. These supernatants were collected and titrated with two different methods: by infecting MT-2 cells ($10^6$ per well) with different concentrations of supernatants and measuring relative luminescence units (RLUs) 48 hours later, and by quantifying HIV-1 antigen p24 with an enzyme-like immunoassay (Innotest$^{TM}$ HIV Ag mAb; Innogenetics, Barcelona Spain). These supernatants were used to infect MT-2 cells ($10^6$ per well) with 100.000 RLUs per well, equivalent to 10 ng p24 per well, in the presence of the compounds to evaluate. Anti-HIV activity quantification was performed 48 h post-infection by determining luciferase activity in cell lysates using the Renilla-Luciferase Assay System Kit (Promega, Madison USA). RLUs were obtained in a luminometer (Berthold Detection Systems, Pforzheim Germany). Cellular viability was evaluated in mock infected cells similarly treated with the same concentrations of compounds using the CellTiter Glo (Promega) assay. 50% inhibitory ($EC_{50}$) and cytotoxic ($CC_{50}$) concentrations were calculated with Prism 6 using log(inhibitor) vs response non-linear regression analyses. The results represent the average of at least three independent experiments.

*2.10. Cellular transfection assays*. MT-2 cells were maintained in culture without stimuli and prior to assay were collected in 350 μL of RPMI without serum and antibiotics and pulsed at 320 V, 1500 μF and maximum resistance with a full-length proviral DNA plasmid containing a luciferase reporter gene whose expression is under the control of the HIV-1 LTR promoter (NL4.3-luc), or with luciferase plasmids under the control of the HIV-1 LTR (LTR-luc) or NF-κB (3-enh-κB-ConA-luc), at a concentration of 1 μg/$10^6$ cells, using an Easyject plus

Electroporator (Equibio, Middlesex UK). After transfection, cells were immediately cultured in RPMI with 10% fetal calf serum and antibiotics, treated or not with different concentrations of compound and harvested 48 h later. Luciferase activity (RLUs) was measured in a luminometer. The $EC_{50}$ values were calculated with Prism 6 as above.

*2.11. Rev-dependent RNA transport assay*. To analyze RRE-Rev inhibition, we employed pCMV-Rev and pDM628 plasmids, encoding Rev and a luciferase gene under the control of the RRE, respectively. Transcripts produced upon transcription of pDM628 contained the RRE and the luciferase gene, where both elements were situated between a splicing donor and a splicing acceptor. In the presence of Rev, the RRE-Rev interaction enables export of the transcript to the cytoplasm, resulting in luciferase expression. Experiments were performed with a constant concentration of pDM628 (500 ng/well) and 2-4 decreasing concentrations of pCMV-Rev. The $EC_{50}$ values were calculated with Prism 6 as explained above.

*2.12. Evaluation of HIV-1 RNA splicing*. MT-2 cells were transfected with a pNL4.3 plasmid and treated with two different concentrations of clomiphene (5 and 10 μM) or cyproheptadine (5 and 50 μM) for 72 or 96 hours. The compound concentrations were chosen on the basis of the observed RRE IIB $K_d$'s and cellular $EC_{50}$ and $CC_{50}$ values. Total cellular RNA was isolated with an RNeasy mini kit (Qiagen, Hilden Germany) and quantified with a Nanodrop-1000 spectrophotometer (ThermoFisher). After treatment with DNase I (Ambion, ThermoFisher), reverse transcription was performed on 500 ng of RNA with the GoScript cDNA synthesis kit (Promega, Wisconsin USA). Unspliced, single-spliced and multiple-spliced HIV-1 RNA transcripts were quantified by qPCR (StepOne Plus, Applied Biosystems, ThermoFisher) using the primers and probes described by Mohammadi *et al*. [34]. Briefly, cDNA synthesis was carried out in a C1000 thermocycler (Bio-Rad, Hercules USA) for 60 minutes at 45ºC, immediately followed by qPCR with the following cycling conditions: 10 minutes at 95ºC, followed by 50 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. qPCR was performed by the comparative CT method relative to an untreated reference control. GAPDH was used as endogenous control.

## 3. Results

### 3.1. High-throughput screen and inhibition of RRE subdomain IIB-Rev$_{34\text{-}50}$ interaction

Aiming to identify bioavailable inhibitors of the RRE-Rev interaction, we screened a diverse library of 1,120 small-molecule drugs approved for clinical use. The assay was based on detecting the displacement of a FITC-labeled Rev$_{34\text{-}50}$ α-helix from its high-affinity site in RRE subdomain IIB by fluorescence anisotropy.

A single-concentration, high-throughput screen detected eleven hits that inhibited more than 90% complex formation at 100 μM without exhibiting fluorescence at the assay conditions. Further analyses of these hits with dose-response experiments allowed us to identify four compounds with low inhibitory IC$_{50}$ values (between 2.8 and 4.2 μM): mitoxantrone, clomiphene, ciprofloxacin and cyproheptadine (Table 1 and Figure 2). Homochlorcyclizine and the aminoglycoside antibiotics neomycin B and neamine were likewise among the eleven initial hits. Neomycin and neamine also had low IC$_{50}$'s (6.4 and 5.2 μM, respectively), whereas homochlorcyclizine exhibited weaker IIB-Rev$_{34\text{-}50}$ inhibitory activity (47.6 μM). Neomycin B did not inhibit HIV-1 replication in our cell infection assay at concentrations below 100 μM [20], but its RRE binding and RRE-Rev inhibition capacity are very well characterized [20-23, 25-27, 35, 36]. We therefore used both aminoglycoside antibiotics together with homochlorcyclizine as controls in subsequent experiments *in vitro*. The remaining hits had significantly higher IC$_{50}$ values.

### 3.2. RRE subdomain IIB RNA binding

Since the screening hits inhibit the formation of the complex between internal loop IIB and Rev$_{34-50}$, it is possible that they do so by binding to the RNA loop. To investigate RNA binding we measured subdomain IIB association with fluorescence intensity experiments, using IIB$_h$-19ap or IIB$_h$-23fl RNA hairpin constructs containing 2-amino purine and fluorescein probes at unpaired loop IIB residues A19 and U23, respectively (Figure 1D). The RNA specificity of the IIB$_h$ interaction was assessed by duplicating the experiments in the presence of a 100-fold molar excess of tRNA$^{Cys}$. We also evaluated specificity relative to double-stranded DNA by additionally carrying out the experiments with a 100-fold molar excess of a 26-base pair DNA duplex (hereafter identified as LTR$_d$). This duplex contained binding sites of transcription factors NF-κB and Sp-1 in the HIV-1 LTR promoter. These two factors are essential for virus replication [37].

All of the compounds bound to the IIB$_h$ RNA hairpin, but exhibited significant differences in affinity and specificity (Table 2 and Figures 2C and 2D). Mitoxantrone had the strongest affinity for IIB$_h$ (K$_d$ 0.25 μM), but the interaction was unspecific relative to both $_t$RNA$^{Cys}$ and LTR$_d$. Cyproheptadine and ciprofloxacin also exhibited low K$_d$ values (1.8 and 2.5 μM, respectively) but limited RNA and DNA specificity, the selectivity ratios being particularly low for ciprofloxacin. Clomiphene (with a K$_d$ of 12.4 μM) and the homochlorcyclizine control (30 μM), associated to IIB$_h$ with less affinity, but the interaction was significantly more specific in both cases (Table 2 and Figures 2C and 2D). The reference antibiotics neomycin B and neamine exhibited IIB$_h$ K$_d$ values of 0.84 and 27 μM, respectively, and were unspecific in relation to the RNA and DNA controls (Table 2 and Figure 2D). The binding parameters of neomycin B and neamine were in accordance with previous literature reports [21-23, 36] and with our SPR observations for neomycin [20]. In general, the IIB$_h$ affinities approximately followed the trend observed for the $\mathrm{IIB_h}$-$\mathrm{Rev_{34\text{-}50}}$ inhibitory $\mathrm{IC_{50}}$ values (Table 1).

In order to examine the location of the binding site(s) of each compound in the RNA hairpin we also monitored the titration of $\mathrm{IIB_h}$ with the selected hits using one- and two-dimensional NMR spectroscopy. Figure 3A shows superpositions of two-dimensional TOCSY spectra allowing visual inspection of the effect of drug association on the RNA signals. Clomiphene and cyproheptadine induced broadening or chemical shift variations that mainly affected the resonances of internal loop nucleotides only (Figure 3A). This indicated that the interaction of these compounds was loop IIB-specific within the $\mathrm{IIB_h}$ hairpin. In this respect, a model of a complex between loop IIB and clomiphene is shown in Figure 3B. In this model, built with unrestrained docking calculations and compatible with the NMR chemical shift displacements, the inhibitor is located in the major groove side of the loop recognized by the Rev protein. In contrast, mitoxantrone, and to a lesser extent ciprofloxacin, broadened or shifted the resonances of many $\mathrm{IIB_h}$ nucleotides at low ligand:RNA molar ratios. This was indicative of strong but unspecific binding. The association of the homochlorcyclizine control was more specific, but affected nucleotides located both inside and outside the internal loop (data not shown). We showed before that neomycin B also induced chemical shift perturbations in $\mathrm{IIB_h}$ stem nucleotides outside the internal loop [20]. Altogether, these NMR observations were approximately consistent with the affinities and specificities quantified by fluorescence experiments.

### 3.3. Interference with full-length RRE-Rev complex formation

In a process triggered by the high-affinity interaction between internal loop IIB and the $\mathrm{Rev_{34\text{-}50}}$ $\alpha$-helix, the RRE-Rev complex is formed by the incorporation of additional Rev monomers that bind to other sites in the RRE and to other Rev monomers through RNA-protein and protein-protein contacts mediated by α-helices [5, 6, 8] (Figure 1B). We have shown above that the screening hits bind to the high-affinity site formed by subdomain IIB and inhibit its interaction with $Rev_{34-50}$. Using an electrophoretic mobility shift assay (EMSA) involving full-length RRE and Rev, we next evaluated whether they were also capable of interfering with the formation of the complete RRE-Rev ribonucleoprotein.

The results indicated that all of the selected compounds inhibited the RRE-Rev interaction. They did so with $IC_{50}$ values that were in the same range as those measured for IIB-$Rev_{34-50}$ inhibition (Table 1 and Figure 4). The most potent compounds in this assay were cyproheptadine ($IC_{50}$ 3 μM) and clomiphene (5 μM). Mitoxantrone also blocked significantly the RRE-Rev interaction but could only be tested up to 10 μM probably because this agent interfered with the conformation of RRE at higher concentrations (Figure 4). Ciprofloxacin, in contrast, exhibited a higher $IC_{50}$ in this assay, 20 μM. Likewise, the homochlorcyclizine control, which had less $IIB_h$-$Rev_{34-50}$ inhibitory activity and less $IIB_h$ affinity, did not inhibit full-length RRE-Rev complex formation at the concentrations tested in the assay (up to 50 μM). The $IC_{50}$ values obtained in this assay for the reference antibiotics neomycin B and neamine (7 and 11 μM, respectively) agreed very well with previously reported values [25-27, 35].

### 3.4. Antiviral activity

We next checked whether the selected hits were able to block HIV-1 replication in cell cultures. All four compounds turned out to have antiviral activity in infection assays. The most potent inhibitor was mitoxantrone, with an $EC_{50}$ value of 54 nM, followed by ciprofloxacin and clomiphene (4.2 and 4.3 μM, respectively), and cyproheptadine (17.5 μM) (Table 3 and Figure 5A). As described in previous reports [20, 25], the neomycin B control did not exhibit antiviral activity at concentrations below 100 μM. A reverse transcriptase inhibitor (zidovudine) and a protease inhibitor (nelfinavir) were used as positive controls of inhibition of HIV-1 replication (Table 3).

### 3.5. Antiviral mechanism of action

In order to pinpoint the location of the main target, or set of targets, of each of the selected hits in the virus life cycle, we next carried out an assay based on transfecting a full-length competent HIV-1 clone. In this experiment, the entry, reverse transcription and DNA integration steps of the virus cycle are bypassed, so that only transcriptional or post-transcriptional processes occur and can be blocked by the inhibitor being evaluated.

The $EC_{50}$ values obtained with this transfection assay for clomiphene (4.3 μM) and cyproheptadine (25.6 μM) were similar relative to those obtained with the infection experiment (Table 3 and Figure 5). This indicates that the main target (or set of targets) for these molecules is likely contained in transcriptional or post-transcriptional steps of the virus infectious cycle. In contrast, the post-integration $EC_{50}$'s of mitoxantrone and ciprofloxacin were 570-fold and more than 25-fold higher, respectively, relative to the $EC_{50}$ values measured in the infection assay, implying that these two drugs mainly acted on pre-transcriptional processes. Mitoxantrone, which had high activity in the infection assay ($EC_{50}$ 54 nM) still retained significant post-integration activity (30.8 μM). On the contrary, the post-integration effect of ciprofloxacin was not detected at the highest concentration tested of 100 μM (Table 3 and Figure 5B).

### 3.6. Inhibition of LTR- and NF-κB-dependent gene expression

To further characterize the mechanism of action of the screening hits, we used a cellular assay based on transfecting a plasmid encoding a luciferase gene whose expression depends on the LTR promoter of the virus [31] (Table 4 and Figure 6A). Clomiphene and cyproheptadine inhibited LTR-dependent gene expression with $IC_{50}$ values that were very similar to those obtained in the infection and post-integration experiments (see Tables 3 and 4, and Figures 5 and 6A). The LTR inhibitory activity of mitoxantrone was in the same range as the value registered in the post-integration test, confirming that this agent was acting on post-integration events of the virus cycle at higher concentrations than it did in pre-transcriptional steps. Ciprofloxacin did not inhibit LTR-dependent expression as expected, since this compound was inactive in the post-integration experiments.

The $LTR_d$ DNA duplex used as a control in fluorescence experiments contained binding sites of transcription factors NF-κB and Sp-1, which are essential for LTR-dependent transcription and virus replication [37]. To test whether the screening hits specifically interfered with the activity of NF-κB, we also evaluated inhibitory activity in cells transfected with a luciferase construct under the control of this transcription factor (Table 4 and Figure 6A). Clomiphene, cyproheptadine and mitoxantrone were able to decrease luciferase expression through inhibition of NF-κB activity with $IC_{50}$ values that were approximately similar relative to those obtained with the LTR reporter assay. As observed with the LTR system, ciprofloxacin was inactive.

### 3.7. Inhibition of RRE-Rev function

The RRE-Rev interaction allows transport of unspliced or partially spliced viral transcripts to the cytoplasm, a post-transcriptional process absolutely needed by the virus to complete its replication cycle. Although with different degrees of selectivity, we have shown that all of the screening hits bound to loop IIB and inhibited the high-affinity interaction between subdomain IIB and $Rev_{34-50}$ (Tables 1 and 2). Using EMSA’s, we also found that all four selected hits hampered the formation of the full-length RRE-Rev complex *in vitro*, although the inhibitory activity of ciprofloxacin was significantly weaker (Table 1 and Figure 4). To test whether the compounds acted on this system in a cellular context, we first used an assay based on transfecting plasmids encoding Rev and an RRE-luciferase reporter system (Table 5 and Figure 6B). Once again, ciprofloxacin had no activity, in agreement with the absence of post-integration activity and the weaker EMSA effect exhibited by this agent. Clomiphene and cyproheptadine were active in this assay, and their inhibitory concentrations were similar to those measured in the infection and post-integration assays involving full-length viruses. However, they also paralleled the $IC_{50}$ values registered in the LTR and NFκ-B experiments (see Tables 3, 4 and 5). Since the expression of the RRE-luciferase and Rev genes used in this assay depends on transcription, the RRE-Rev activities registered by this assay may be dominated by a transcriptional effect. Mitoxantrone also inhibited RRE-Rev function. In this case, the $IC_{50}$ values were below the post-integration and LTR inhibitory concentrations, suggesting that RRE-Rev inhibition may contribute to the post-integration activity of this agent. The RRE-Rev $IC_{50}$ values of all hits were only slightly affected by differences in the concentration of the Rev-encoding plasmid used in the assays (Table 5 and Figure 6B).

To clarify whether clomiphene and cyproheptadine inhibited Rev function, we subsequently transfected cells with a full-length proviral DNA plasmid and quantified the amount of unspliced, single-spliced and multiple-spliced HIV-1 RNA transcripts present in the cells with RT-qPCR. The results obtained with an untreated control were compared with those observed upon exposing the cells to the compounds at two different concentrations and incubation time periods after transfection. Since Rev transports unspliced or partially spliced viral transcripts to the cytoplasm where they cannot be spliced, inhibition of Rev function should result in lower levels of unspliced and single-spliced transcripts. At a concentration of 5 µM, clomiphene similarly reduced the amount of all types of HIV-1 RNA transcripts by approximately half relative to the untreated control (0.4 vs. 1). This result was fully consistent with the $EC_{50}$ values obtained for this compound in infection and transfection assays (between

3 and 6 μM; Tables 3 and 4) and confirmed that this agent blocked HIV-1 transcription, but did not suggest Rev inhibition. At 10 μM, however, clomiphene diminished the levels of unspliced and single-spliced HIV-1 RNA transcripts to a higher degree relative to multiple-spliced species. This result suggests that in these conditions this agent blocked Rev function in addition to viral transcription. In contrast, cyproheptadine did not induce significant differences in the levels of HIV-1 RNA splice variants at either concentration, although a small effect suggestive of Rev inhibition was observed at 5 μM and 96 hours of incubation (Figure 7). This agent only reduced the levels of all HIV-1 transcripts at 50 μM, in agreement with the $EC_{50}$ values determined in infection and transfection experiments (Tables 3 and 4). This confirmed that cyproheptadine had an effect on HIV-1 transcription. Mitoxantrone's impact on HIV-1 splicing could not be evaluated because of the toxic effect of this agent (see below) at the assay concentrations.

### 3.8. Cellular toxicity

We also evaluated the cellular toxicity of the compounds (Table 3 and Figure 5A) and compared this parameter with the HIV inhibitory activities (Tables 3, 4 and 5). Cyproheptadine and ciprofloxacin were not toxic at the concentrations tested in the assays (up to 100 μM). In contrast, clomiphene and mitoxantrone did exhibit some cellular toxicity. The $CC_{50}$ of clomiphene (17.4 μM) was approximately 2- to 6-fold above the inhibitory concentrations registered for this compound in the infection experiment and all of the transfection assays (between 3.3 and 8.7 μM). The $CC_{50}$ value of mitoxantrone (25-50 μM) was much higher than its activity against HIV-1 infection (54 nM), but similar to its post-integration and LTR $IC_{50}$'s (30.8 and 42.2 μM, respectively). This suggested that these effects may be related with the unspecific cellular toxicity of this agent.

## 4. Discussion

This study had a double objective: first, identify bioavailable inhibitors of HIV-1 acting on a viral target not used by current antiretroviral agents and second, find new RNA-binding scaffolds and explore their biological effect in a cellular context. To achieve these goals, we screened a diverse set of 1,120 bioavailable drugs approved for clinical use with an assay based on detecting the displacement the $\text{Rev}_{34\text{-}50}$ $\alpha$-helix from its RRE subdomain IIB high-affinity site, and selected four inhibitor hits. The RRE-Rev inhibition properties and RNA subdomain IIB binding affinity and selectivity of these compounds were subsequently analyzed, together with their antiviral effect, cellular toxicity and mechanism of action.

All four hits selected on the basis of the RRE IIB-$\text{Rev}_{34\text{-}50}$ screen turned out to have anti-HIV-1 activity. Ciprofloxacin, however, was a false positive in the sense that it had no post-integration activity in cellular assays (Table 3 and Figure 5). This compound inhibited the $\text{IIB}_h$-$\text{Rev}_{34\text{-}50}$ interaction and bound to the $\text{IIB}_h$ RNA hairpin with a low $K_d$ (2.5 μM) but limited RNA and DNA specificity (Tables 1 and 2 and Figure 2). The full-length RRE-Rev inhibition capacity of this compound detected by EMSA (20 μM) was weaker than that measured for the rest of the hits (Table 1 and Figure 4). Ciprofloxacin belongs to the quinolone family of compounds widely used as antibacterials, and is in fact employed to treat opportunistic infections affecting AIDS patients. The quinolone antibiotics inhibit the action of prokaryotic topoisomerases by binding to the complexes formed by these enzymes and DNA. Several quinolone compounds have more recently been reported to have antiretroviral activity, with a mechanism of action proposed to be based on integrase inhibition or transcriptional blockage [38, 39]. Our results support the former mechanism for ciprofloxacin, since this compound inhibited HIV-1 replication in cell infection assays but lacked activity in post-integration experiments. Actually we demonstrate that ciprofloxacin has no detectable effect on LTR-dependent transcription, NF-κB activity or RRE-Rev function at concentrations up to 100 μM (Tables 3, 4 and 5).

Mitoxantrone inhibited subdomain $IIB_h$-$Rev_{34-50}$ and full-length RRE-Rev interactions and, like ciprofloxacin, bound to $IIB_h$ with high affinity (0.35 μM) but low DNA and RNA specificity (Tables 1 and 2 and Figure 2). Not surprisingly, mitoxantrone has also been described to associate with several other RNA targets [40-43]. This compound intercalates into double-helical DNA, disrupting DNA and RNA synthesis and topoisomerase II function, and is used as an anticancer and immunosuppressive drug [44]. It has also been reported to inhibit HIV-1 integrase activity at low micromolar concentrations [45]. Like in the ciprofloxacin case, the interference with the integration step may be related to the DNA-binding properties of the compound. In our experiments, mitoxantrone inhibited HIV-1 replication at nanomolar concentrations in the cell infection assay, and had an $EC_{50}$ of 30.8 μM in the post-integration experiment (Table 3 and Figure 5). This indicated that the main target of the drug was contained in integration and/or pre-integration steps of the virus cycle, as observed for ciprofloxacin. The post-integration effect of mitoxantrone, however, was still significant. The compound interfered with HIV-1 post-integration processes and LTR-dependent expression at concentrations close to its $CC_{50}$ value (Tables 3 and 4). This suggests that these effects are related with the cytotoxicity of the compound, and are likely connected to transcriptional blockage in accordance with previous studies [44]. The NF-κB and cellular RRE-Rev inhibitory concentrations were below the $CC_{50}$ and the LTR and post-integration $EC_{50}$´s (Tables 3, 4 and 5). DNA binding probably contributed to the observed effect on NF-κB-dependent expression, since we indirectly detected mitoxantrone association to an $LTR_d$ duplex containing NF-κB binding sites (Table 2 and Figure 2). Likewise, RRE-Rev inhibition may participate in the post-integration activity of mitoxantrone. Nevertheless, the combined $EC_{50}$ and $CC_{50}$ data of this agent favor its usage for inhibiting pre-integration or integration steps of the viral cycle.

In contrast to ciprofloxacin and mitoxantrone, no nucleic acid binding and no antiretroviral activity had been reported to date for the two remaining hits. Clomiphene, the most potent compound of the pair ($EC_{50}$ 4.4 μM in the infection assay), is a selective estrogen receptor modulator used to treat infertility [46], whereas cyproheptadine is an $H_1$ antihistamine agent [47]. In contrast to the previous drugs, these agents exhibited similar activities in the HIV-1 infection and post-integration assays (Table 3 and Figure 5). This indicated that their main target was likely contained in transcriptional or post-transcriptional steps of the virus infectious cycle. *In vitro*, both drugs inhibited $IIB_h$-$Rev_{34-50}$ and RRE-Rev complex formation with similar $IC_{50}$'s (between 3 and 5 μM; Table 1 and Figures 2 and 4), and associated to RRE subdomain IIB with low μM affinities (12.4 and 1.8 μM; Table 2 and Figures 2C and 2D). Subdomain IIB recognition by clomiphene was substantially specific. This was demonstrated by fluorescence control experiments employing a 100-fold molar excess of $tRNA^{Cys}$ or $LTR_d$ DNA duplex, as well as by $IIB_h$ titration experiments monitored by NMR, which indicated specific recognition of loop IIB nucleotides by this ligand (Figures 2 and 3). Cyproheptadine associated to $IIB_h$ with more affinity but less specificity (Table 2 and Figure 2). The specificity factors, however, were above those observed for ciprofloxacin, mitoxantrone or the aminoglycoside antibiotics, and the NMR analyses indicated specific binding to loop IIB nucleotides (Figure 3A).

Docking calculations revealed that clomiphene fitted well in the widened major groove of RNA loop IIB (Figure 3B). Moreover, the non-planar triphenylethylene and dibenzocycloheptene scaffolds of clomiphene and cyproheptadine, respectively, superposed surprisingly well, exhibiting approximately similar orientations of the aromatic rings and positively-charged groups (Figure 3C). Homochlorcyclizine, the weaker $IIB_h$-$Rev_{34-50}$ inhibitor hit used as a control in experiments *in vitro,* also superposed well with the other two ligands. This compound, another $H_1$ antihistamine agent related to cyproheptadine, recognized

$IIB_h$ with less affinity but significant selectivity (Table 2 and Figure 2D). The less specific ciprofloxacin and mitoxantrone hits, in contrast, have a more planar structure and a likely different RNA-binding mode based on stacking and/or intercalation [40]. Interestingly, clomiphene and the two antihistamine hits share homologies regarding the nature of their binding sites in their preferred estrogen and histamine $H_1$ receptor targets. In both cases, the drug binding sites are quite rich in $\alpha$-helices and relatively hydrophobic [48, 49].

When the mechanism of antiretroviral action of clomiphene and cyproheptadine was evaluated, we found that both hits inhibited luciferase expression mediated by the HIV-1 LTR promoter and NF-κB. For each compound, the LTR and NF-κB inhibitory concentrations had similar values, which were close to the $EC_{50}$'s determined in the infection and post-integration experiments (see Tables 3 and 4). The RRE-Rev inhibitory concentrations of both hits followed a similar trend (Table 5). However, since the expression of the RRE-luciferase and Rev genes used in the RNA transport assay depended on transcription, it is possible that the RRE-Rev activities obtained in this assay were dominated by transcriptional blockage. In this respect, varying the concentration of Rev-encoding plasmid affected only slightly the RRE-Rev $IC_{50}$ values (Figure 6B), in contrast to what was observed with recently designed Rev mimics [20]. To investigate whether clomiphene and cyproheptadine had an effect on Rev function, we conducted experiments based on monitoring the levels of unspliced, single-spliced and multiple-spliced HIV-1 RNA transcripts. Both compounds reduced the amount of all viral transcripts at concentrations close to their infection and transfection $EC_{50}$'s (Figure 7 and Tables 3 and 4), confirming an effect on HIV-1 transcription. At a concentration of 10 μM, clomiphene reduced the levels of unspliced and incompletely spliced HIV-1 transcripts to a greater extent relative to multiple-spliced RNA species (Figure 7). This suggests that at least at this concentration, the antiviral activity of this agent is based on RRE-Rev inhibition in addition to transcriptional blockage. In contrast, cyproheptadine did not induce significant differences in the patterns of HIV-1 RNA splicing, although at the higher concentration transcriptional blockage might have hidden the effect on splicing patterns (Figure 7).

It is unlikely that the HIV-1 transcriptional inhibitory activity of clomiphene has a DNA-based mechanism, since this agent showed very weak LTR DNA association (Table 2 and Figure 2C). Rather, this effect may be due to RNA binding or to interference with the estrogen receptor or other nuclear receptor factors involved in LTR transcription [50, 51]. Interference with Rev function likely contributes to the antiviral action of this agent, as suggested by the effect on RRE-Rev association detected *in vitro* and by the changes in HIV-1 splicing patterns observed in cellular assays. It is relevant to note in this respect that the processes of transcription and RNA export are tightly coupled[52, 53]. Interestingly, clomiphene and other estrogen receptor modulators have been recently reported to inhibit Ebola virus replication [54, 55]. In this case, however, the mechanism was reported to be based on blockage of virus entry [54].

Several groups have studied the RRE-binding and RRE-Rev inhibition properties of aminoglycoside antibiotics and conjugates [19, 21-23, 26, 35, 36], diphenylfurans [56], acridines [57], metal complexes [19] and peptide boronic acids [58], but either these molecules lacked antiviral activity, or their activity and/or mechanism was not reported. To our knowledge, there is only one precedent of a small-molecule, high-throughput RRE-Rev screen based on the usage of purified RRE and Rev species: a Glaxo team tested over 500,000 compounds with an scintillation proximity assay [25]. Several classes of RRE-Rev inhibitors with low µM potencies were identified, but most of them lacked antiviral activity. On the other hand, Shuck-Lee *et al*. conducted an RRE-Rev screen of 40,000 compounds based on a cellular assay [27]. These authors identified three HIV post-integration inhibitors that interfered with RRE-Rev function. However, these molecules did not inhibit RRE-Rev binding *in vitro*, and thus may act on some other step of the Rev pathways [27]. The lack of antiviral activity of the compounds identified by Glaxo might have been influenced by their cellular penetration properties: 40% of those hits were negatively charged, and all of the tested molecules associated to Rev rather than RRE. In our case, the positively-charged RRE-Rev inhibitor hits associated to RRE, and the post-integration $EC_{50}$'s of the hits were similar to the $K_d$'s of nucleic acid association. This suggests that these compounds have better cell and nucleus penetration properties.

In conclusion, by screening one thousand FDA-approved drugs according to their ability to displace $Rev_{34-50}$ from its RRE subdomain IIB site, we have identified two bioavailable drugs, clomiphene and cyproheptadine, that are capable of inhibiting the post-integration stage of HIV-1. Both compounds bound to RRE subdomain IIB and blocked RRE-Rev complex formation at low µM concentrations similar to their cellular $EC_{50}$ values, and RNA loop IIB recognition by clomiphene was substantially specific. Remarkably, no antiretroviral activity and no nucleic acid binding had been previously reported for these two agents. Although the anti-HIV activities of both drugs were apparently dominated by inhibition of LTR-dependent transcription, the observed blockage of RRE-Rev binding may also contribute to their antiviral effect. For clomiphene, this mechanism was supported by the detection of changes of HIV-1 splicing patterns consistent with Rev inhibition. In this regard, these hits have defined a new RNA-binding and RRE-Rev inhibition motif that may serve as a starting point for the development of HIV-1 gene-regulation inhibitors.

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## Figure Legends

**Figure 1.** The RRE-Rev interaction of HIV-1. (A) Three-dimensional structure of a Rev monomer [5, 6]. The arginine-rich α-helix (Rev$_{34-50}$) is shown in red. (B) Model of full-length RRE-Rev complex. The location of the main Rev binding site in subdomain IIB is indicated.

(C) Three-dimensional view of the high-affinity complex formed by an internal loop within subdomain IIB of the RRE (grey) and $Rev_{34\text{-}50}$ (red) [7]. (D) Secondary structure of hairpin $IIB_h$, containing the internal loop forming the high-affinity Rev binding site. This construct was used for fluorescence anisotropy and NMR experiments. For fluorescence intensity assays we employed two labeled $IIB_h$ constructs: $IIB_h$-19ap, where 2-aminopurine replaced adenine at unpaired loop residue A19 (marked with an asterisk); and $IIB_h$-23fl, containing a fluorescein probe linked to the extra-helical loop residue U23 (marked with two asterisks).

**Figure 2.** Inhibition of the $IIB_h$-$Rev_{34\text{-}50}$ interaction and recognition of RNA hairpin $IIB_h$ by the screening hits. (A) Chemical structure of the small-molecule inhibitors identified in the fluorescence anisotropy screen. The weaker inhibitor homochlorcyclizine was used as a control in experiments *in vitro*, together with the aminoglycoside antibiotics neomycin B and neamine (not shown). (B) Representative curves of $IIB_h$-$Rev_{34\text{-}50}$ inhibition by the best hits, clomiphene and cyproheptadine, obtained with fluorescence anisotropy experiments. (C) $IIB_h$ binding curves of clomiphene, cyproheptadine and mitoxantrone obtained with fluorescence intensity experiments in the absence (light blue) and presence of a 100-fold molar excess of unlabeled competitor RNA ($tRNA^{Cys}$; dark blue) or unlabeled competitor double-helical DNA ($LTR_d$; green). (D) Comparison of the $IIB_h$ affinities (left) and specificities (relative to $tRNA^{Cys}$ and $LTR_d$; right) of all screening hits. Note that the $K_d$ and specificity scales are logarithmic. In (B) and (C), the error bars represent standard deviations of at least two independent experiments.

**Figure 3**. Internal loop IIB recognition by screening hits. (A) RNA hairpin $IIB_h$ interaction with the best hits, clomiphene and cyproheptadine, monitored by NMR spectroscopy. The H5-H6 region of the TOCSY spectrum (60 ms mixing time, 27 °C) of hairpin $IIB_h$ (blue) is superposed on the spectra of complexes with increasing RNA:drug molar ratios, color-coded as indicated in the graph. On the right, the location of the ligand binding site in the $IIB_h$ hairpin is indicated by highlighting in red the nucleotides whose aromatic protons underwent broadening or chemical shift variations greater than 0.1 ppm upon the addition of one or two equivalents of drug. Nucleotides with overlapped aromatic resonances are black-colored, and residues whose aromatic signals were not affected by ligand binding are colored grey. The chemical shift variations were monitored with one-dimensional and TOCSY experiments. (B) Best-scored model of a complex between internal loop IIB (grey) and clomiphene (green carbon atoms), built by docking the ligand into the structure of subdomain IIB (PDB code 1ETG) [7]. (C) Superposition of the chemical structures of clomiphene, cyproheptadine and homochlorcyclizine. The drugs are depicted with green, orange and yellow carbon atoms, respectively.

**Figure 4.** Inhibition of full-length RRE-Rev complex formation by the screening hits analyzed by EMSA. (A) EMSA results. All lanes contained 78 nM RRE, 1.32 μM Rev and the indicated concentration of compound. We did not detect inhibition by homochlorcyclizine at the assay concentrations. (B) Plots of unbound RRE band area as a function of compound concentration. The error bars represent the standard deviation of at least two independent EMSA experiments.

**Figure 5.** Cellular assays of selected screening hits. (A) Antiviral activity in HIV-1 cell infection experiments and cellular toxicity. (B) Inhibition of HIV-1 post-integration steps: the cells were transfected with a full-length proviral DNA plasmid containing a luciferase reporter gene whose expression is under the control of the HIV-LTR promoter. In all cases, results are expressed as percentage of relative luminescence units (RLUs), where 100% is the level of viral replication obtained in the presence of the vehicle used to dissolve the compounds.

**Figure 6.** Mechanism of antiviral action of selected screening hits. (A) Inhibition of LTR- and NF-κB-dependent luciferase expression. (B) Inhibition of Rev-mediated transport of RRE-containing RNA into the cytoplasm, evaluated at different concentrations of the plasmid encoding Rev (200, 100, 20 and 10 ng/well). In (A) and (B), results are expressed as percentage of RLUs, where 100% is the level of viral replication obtained in the presence of the vehicle used to dissolve the compounds.

**Figure 7**. Effect of clomiphene and cyproheptadine on HIV-1 RNA splicing. Unspliced, single-spliced and multiple-spliced HIV-1 RNA transcripts were quantified by RT-qPCR after RNA isolation from treated or untreated MT-2 cells transfected with a wild-type HIV-1 plasmid (pNL4.3). Results are expressed as RNA relative quantity (RQ), using untreated cells as a reference control (RQ=1) and GADPH as endogenous control.

**Table 3 with marked changes**

**Table 3.** Anti-HIV activity in infection ($EC_{50}$, infection) and transfection ($EC_{50}$, post-integration) experiments, and cellular toxicity ($CC_{50}$), of selected screening hits.

| compound[a] | $EC_{50}$ *(infection)*[b] (M·$10^6$) | $EC_{50}$ *(post-integration)* (M·$10^6$) | $CC_{50}$ (M·$10^6$) |
|---|---|---|---|
| Clomiphene | 4.3<br>(1.1-10.2, 0.9486) | 4.3<br>(2.8-6.6, 0.8373) | 17.4<br>(6.6-45.7, 0.7475) |
| Cyproheptadine | 17.5<br>(7.9-38.5, 0.9315) | 25.6<br>(16.5-39.7, 0.8527) | >100 |
| Mitoxantrone | 0.054<br>(0.031-0.092, 0.8018) | 30.8<br>(16.3-58.3, 0.6967) | >25<50 |
| Ciprofloxacin | 4.2<br>(2.0-8.9, 0.592) | >100 | >100 |

[a] Confidence intervals and $R^2$ values are shown in parentheses when applicable.

[b] The $EC_{50}$ (infection) values obtained for the control antiretrovirals zidovudine and nelfinavir in the infection assay were 0.0083 µM (0.0056-0.0123 µM, $R^2 = 0.9677$) and 0.0021 µM (0.0010-0.0044 µM, $R^2 = 0.9056$), respectively.

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# Bioavailable inhibitors of HIV-1 RNA biogenesis identified through a Rev-based screen

Silvia Prado[a], Manuela Beltrán[b], Mayte Coiras[b], Luis M. Bedoya[b,c], José Alcamí[b]* and José Gallego[a]*

[a]Facultad de Medicina, Universidad Católica de Valencia, C/Quevedo 2, 46001 Valencia, Spain

[b]Instituto de Salud Carlos III, Carretera Majadahonda-Pozuelo km 2, 28220 Majadahonda, Spain

[c]Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramón y Cajal s/n, 28040, Madrid, Spain

*Corresponding authors contact data: José Gallego, e-mail: jose.gallego@ucv.es; tel. +34-963-637412. José Alcamí, email: ppalcami@isciii.es; tel +34-918-22393.

## Abstract

New antiretroviral agents with alternative mechanisms are needed to complement the combination therapies used to treat HIV-1 infections. Here we report the identification of bioavailable molecules that interfere with the gene expression processes of HIV-1. The compounds were detected by screening a small library of FDA-approved drugs with an assay based on measuring the displacement of Rev, and essential virus-encoded protein, from its high-affinity RNA binding site. The antiretroviral activity of two hits was based on interference with post-integration steps of the HIV-1 cycle. Both hits inhibited RRE-Rev complex formation *in vitro*, and blocked LTR-dependent gene expression and viral transcription in cellular assays. The best compound altered the splicing pattern of HIV-1 transcripts in a manner consistent with Rev inhibition. This mechanism of action is different from those used by current antiretroviral agents. The screening hits recognized the Rev binding site in the viral RNA, and the best compound did so with substantial selectivity, allowing the identification of a new RNA-binding scaffold. These results may be used for developing novel antiretroviral drugs.

**Keywords:** human immunodeficiency virus type 1; Rev; RNA; screen; transcription

Chemical compounds studied in this article

Ciprofloxacin (PubChem CID: 2764); Clomiphene (PubChem CID: 1548953); Cyproheptadine (PubChem CID: 2913); Homochlorcyclizine (PubChem CID: 3627); Mitoxantrone (PubChem CID: 4212); Neamine (PubChem CID: 72392); Neomycin B (PubChem CID: 8378)

## 1. Introduction

The current antiretroviral combination therapies imply life-long treatments with a strict adherence to dosage regimens to maintain viral suppression [1, 2]. In addition, the virus is capable of acquiring resistance to these treatments, and resistant strains spread through transmission  among individuals [3]. Consequently, there is a need to develop new antiretroviral agents with different inhibitory mechanisms relative to those used by existing HIV therapeutics, which act on all of the virus-encoded enzymatic functions as well as on virus entry into the cell [4]. One aspect of the virus life cycle that is not targeted by any of the compounds currently used in the market encompasses the gene expression processes of the virus, including transcription, RNA processing and transport.

Rev is a virally-encoded, 116-amino acid protein with a helix-turn-helix structure [5, 6] that uses an arginine-rich $\alpha$-helix ($Rev_{34-50}$) to enter the cell nucleus and recognize the Rev Recognition Element (RRE), a strongly conserved 350-nucleotide structure located in the second intron of the HIV-1 genomic RNA. $Rev_{34-50}$ forms a high-affinity complex with an internal loop of RRE subdomain IIB [7]. This interaction triggers the cooperative incorporation of additional Rev molecules to the complex through interactions with further sites on the RRE and protein-protein contacts [8, 9] (Figure 1). The subsequent tethering of this RRE-Rev ribonucleoprotein complex to the Crm1 host export factor allows transport of unspliced or incompletely spliced viral RNA molecules to the cytoplasm of the infected cell in the late phase of the virus cycle. In addition to this function, Rev has been reported to enhance RNA translation and packaging [10, 11] and to modulate the nucleocytoplasmatic shuttling of the HIV-1 integrase [12]. Indeed, this small regulatory protein is essential for virus viability. Selective Crm1 inhibitors that block Crm1-dependent nuclear export and exert anti-neoplastic and anti-HIV effects are currently being evaluated in clinical studies [13].

However, up to now there are no inhibitors acting on the RRE-Rev system under clinical development.

Here we report the identification of bioavailable HIV-1 inhibitors that interfere with gene expression processes of the virus. The compounds were detected by screening a small library of FDA-approved drugs with an assay based on measuring the displacement of Rev from its RNA binding site. The antiretroviral activity of two of the hits had not been previously reported and was based on interference with post-integration processes of the virus cycle. Both compounds inhibited RRE-Rev complex formation, and blocked HIV-1 LTR-dependent gene expression and viral transcription in cellular assays. The best hit altered the levels of multiple-spliced versus single-spliced and unspliced HIV-1 transcripts in a manner consistent with inhibition of Rev function. This mechanism of action is different from those used by currently marketed antiretroviral agents. On a broader perspective, all of the screening hits bound to RNA subdomain IIB with different degrees of affinity and specificity, and this study allowed us to identify a new RNA-binding scaffold. This is also important, as the search for RNA-binding therapeutics is relatively underdeveloped but shows increasing promise [14, 15]. The findings described in this report may be useful for the development of new antivirals with alternative mechanisms of action.

## 2. Materials and Methods

*2.1. Preparation of RNA, DNA, peptide and protein samples*. The $IIB_h$ RNA oligonucleotide (Figure 1D) used in NMR spectroscopy and fluorescence anisotropy experiments was purchased from Dharmacon (ThermoFisher, Wilmington USA). After removing the 2'-ACE protecting groups, this sequence was purified on denaturing 20% polyacrylamide gels containing 8 M urea, and subsequently electroeluted from the gel, ethanol-precipitated and desalted. For the fluorescence intensity experiments, two fluorescent $IIB_h$ constructs were purchased HPLC-purified from Microsynth AG and desalted. In the $IIB_h$-19ap system, 2-amino purine replaced adenine at unpaired loop nucleotide A19. Construct $IIB_h$-23fl contained a fluorescein (FITC) probe linked to the extra-helical loop nucleotide U23 (Figure 1D). Full-length RRE RNA (Figure 1B) was synthesized by run-off transcription from a pUC19 vector linearized with SmaI. This vector was generated by cloning a 231-nt portion of an RRE sequence obtained from plasmid pDM628 between the SmaI and EcoRI sites of pUC19. After transcription, the RRE RNA was purified using a gradient of NaCl in sodium phosphate buffer in a 26/60 anion exchange column (GE Healthcare, Little Chalfont UK), ethanol-precipitated and desalted. *Escherichia coli* $tRNA^{Cys}$ was transcribed *in vitro* from a BstNI-digested pUC19 plasmid, a generous gift from M. Eugenia Armengod (CIPF, Valencia Spain) and purified following a protocol based on phenol-chloroform extraction and anion-exchange chromatography [16]. The 26-nt d(GGCGGGACTTTCCGGAAAGTCCCGCC) self-complementary DNA oligonucleotide ($LTR_d$) containing NF-κB and Sp-1 binding sequences present in the LTR promoter of HIV-1 was purchased from Microsynth AG and purified as described for $IIB_h$.

An FITC-Ahx-GTRQARRNRRRRWRERQRAAAAR-am peptide (frevp) containing a fluorescein (FITC) moiety attached to the $N_t$ Gly residue was purchased HPLC-purified from Genscript (Piscataway USA) and used for the fluorescence anisotropy experiments. A similar but unlabeled succynyl-TRQARRNRRRRWRERQRAAAAR-am peptide (revp) was also purchased from Genscript. Both peptides contain the Arg-rich $Rev_{34-50}$ residue tract forming the RNA-binding α-helix of Rev, together with an additional $C_t$ AAAAR segment to stabilize the α-helix [17]. The full-length Rev protein was expressed in BL21 E. coli cells using a pET11d plasmid containing a Rev gene (HIV-1 BH10 strain) engineered to avoid usage of low abundance Arg codons in E. coli [18] (kindly provided by F. Blanco and R. Tycko). Purification of Rev was performed in the presence of 6 M urea essentially as described [18], passing the protein through anion and cation exchange columns followed by polishing on a 16/70 gel filtration column (GE Healthcare). Protein purity was assessed by SDS electrophoresis. To avoid aggregation, a purified stock dissolved in 6 M urea was microdialyzed at protein concentrations below 25 μM in an EMSA buffer containing 10 mM HEPES pH 7.5, 300 mM KCl, 1 mM $MgCl_2$ and 0.5 mM EDTA.

*2.2. Compounds*. The primary fluorescence anisotropy screen used a collection of 1,120 FDA-approved drugs dissolved in DMSO at a concentration of 5 mM, acquired from Prestwick Chemical (Illkirch France). According to the manufacturer, the compounds were selected for their high chemical and pharmacological diversity and accessible information on bioavailability and safety in humans. For subsequent experiments, neomycin B, clomiphene and ciprofloxacin were obtained from Sigma-Aldrich (St. Louis USA), neamine from Toronto Research (Toronto Canada), and cyproheptadine and mitoxantrone from Santa Cruz Biotechnology (Dallas USA). Additional amounts of other compounds were obtained from the Prestwick stocks. The antiretroviral drugs zidovudine and nelfinavir used as positive controls in the anti-HIV activity assay were obtained through the AIDS Reagent Program (NIAID, NIH USA).

*2.3. Fluorescence anisotropy*. These experiments were conducted at 25 $^{o}$C using 96-well plates and Victor X3 or Victor X5 (PerkinElmer, Waltham USA) plate readers set up with 480 and 535 nm excitation and emission filters, respectively. The fluorescence anisotropy buffer contained 30 mM HEPES (pH 6.8), 100 mM KCl, 10 mM sodium phosphate, 10 mM ammonium acetate, 10 mM guanidinium chloride, 2 mM $MgCl_2$, 20mM NaCl, 0.5 mM EDTA, and 0.001 % (v/v) Triton X-100 [19].

The primary screen used a Tecan robot to incubate frevp (at a concentration of 10 nM) and $IIB_h$ (2 nM) with each of the 1,120 drugs dissolved at a concentration of 100 μM in the 200 μL assay mixture. Each 96-well plate experiment had one positive (a mixture of $IIB_h$ and frevp, equivalent to 0% inhibition) and two negative (isolated frevp as well as a mixture of $IIB_h$, frevp and unlabeled revp) controls. In order to ensure that there was no spectral overlap with FITC, anisotropy data were collected for each of the compounds that inhibited more than 50% $IIB_h$-$Rev_{34-50}$ complex formation. We only accepted hits that did not fluoresce at the assay conditions.

Subsequent dose-response fluorescence anisotropy experiments used 10 nM frevp and 60 nM $IIB_{h.}$ As in the primary screen, each experiment had one positive and two negative controls, and we only accepted experiments were the anisotropy reached the expected minimum value at the highest concentrations of inhibitor. 50% inhibitory concentration ($IC_{50}$) values were calculated by fitting observed anisotropy (A) to the following equation with Prism (GraphPad Software, San Diego USA):

$$A = A_f + \frac{(A_b - A_f)}{1 + (\frac{C}{IC_{50}})^m}$$

where $A_f$ and $A_b$ are the anisotropy values measured for free and $IIB_h$-bound frevp, respectively, C is the total concentration of small molecule inhibitor and m is the slope of the linear portion of the sigmoidal curve. $IC_{50}$ and m were allowed to float during the fitting process. Each fluorescence anisotropy experiment was repeated at least two times. This assay had been previously validated in our laboratory by measuring the inhibitory activity of revp and neomycin B [20].

*2.4. Fluorescence intensity*. Depending on the absorbance spectra of the ligands, these experiments measured association to $IIB_h$-19ap or $IIB_h$-23fl RNA constructs labeled with 2-aminopurine and fluorescein at unpaired loop IIB residues A19 and U23, respectively (Figure 1D). The RNA (at 100 nM concentration) was pre-equilibrated for 5 minutes in a buffer containing 10 mM sodium phosphate pH 6.6 and 0.1 mM EDTA after ligand addition. For experiments involving $IIB_h$-19ap, fluorescence intensity was measured using a SPECTRA GEMINI XPS plate reader (Molecular Devices, Sunnyvale USA), with excitation and emission wavelengths of 310 and 385 nm, respectively. Experiments involving $IIB_h$-23fl were carried out in a Victor X5 plate reader (Perkin Elmer), using excitation and emission wavelengths of 485 and 520 nm, respectively. The equilibrium dissociation constants $K_d$ were determined by fitting the observed fluorescence intensities (*I*) to the following equation with Prism:

$$I = 1 + (I_{\min} - 1)\left( \frac{(K_d + C_{IIB} + C) - \sqrt{(K_d + C_{IIB} + C)^2 - 4C_{IIB}C}}{2C_{IIB}} \right)$$

where $C_{IIB}$ is $IIB_h$ concentration (100 nM), C ligand concentration, and $I_{min}$ fluorescence intensity in the absence of compound. The RNA and DNA specificity of the interactions was assessed by duplicating the experiments in the presence of a 100-fold molar excess (10 μM) of either $tRNA^{Cys}$ or DNA duplex $LTR_d$. These experiments were validated using the reference antibiotic neomycin B, for which we measured a $K_d$ of 0.84 ± 0.17 μM together with $K_d(IIB_h)/K_d(IIB_h+tRNA)$ and $K_d(IIB_h)/K_d(IIB_h+LTR_d)$ selectivity ratios of 0.01 and 0.02 (Table 2 and Figure 2D). Neomycin's $IIB_h$ affinity and limited specificity were consistent with our previous SPR observations [20] and other literature reports [21-23].

*2.5. NMR spectroscopy*. NMR spectra were acquired in a Bruker Avance III 500 MHz spectrometer, and analyzed using Topspin 1.3 (Bruker Biospin, Billerica USA) and Sparky 3.110 [24]. The $IIB_h$ RNA samples were previously microdialyzed in an aqueous solution containing 10 mM sodium phosphate (pH 6.0) and 0.1 mM EDTA. The interaction of 40-60 μM (5-8 ODs) $IIB_{h,}$ samples with the small molecule ligands was monitored at 27 $^{o}$C using two-dimensional (TOCSY) and/or one-dimensional experiments at increasing ligand:RNA molar ratios: 1:1, 2:1, 4:1 and, depending on the observed spectral changes, 6:1.

*2.6. Electrophoretic mobility shift assays (EMSA)*. These experiments utilized 78 nM full-length RRE and 1.32 μM full-length Rev dissolved in 10 mM HEPES pH 7.5, 300 mM KCl, 1 mM $MgCl_2$ and 0.5 mM EDTA binding buffer, and increasing concentrations of each compound [9]. The reactions were incubated at room temperature for 20 minutes and loaded onto 8% polyacrylamide gels with TB running buffer. Gels were run at 4ºC for 1–4 hours at 150 V, and the bands were stained with SYBR gold (Life Technologies, ThermoFisher) and quantified with Image J (W.S. Rasband, NIH Maryland, USA). $IC_{50}$ values were determined by fitting the data to a simple competitive binding model with Prism:

$$a - a_i = \frac{a_{\max} C}{IC_{50} + C}$$

where $a$ is the area of the band corresponding to unbound RRE species at compound concentration $C$, $a_{max}$ the best-fit value for the maximum area of each concentration–response curve, and $a_i$ the area of unbound RRE band in the absence of compound [25]. These experiments were repeated at least two times for each compound. The assay was validated by examining the inhibitory activity of neomycin B, for which we obtained an $IC_{50}$ of 7 ± 1 μM. This value was in very good agreement with previously reported data [25-27].

*2.7. Molecular modeling*. Three-dimensional models of complexes of loop IIB with the screening hits were built by docking the ligands into the subdomain IIB RNA structure (PDB code 1ETG) [7] using Gold 5.2 [28]. The ligand binding site was defined with a very large 60 Å radius around nucleotide C20. All calculations were unrestrained, employed the GoldScore fitness function [28] and generated 20 solutions for each ligand with maximum search efficiency. In all cases, the compounds were predicted to bind to the major groove side of loop IIB forming Rev's high-affinity site. For cyproheptadine and homochlorcyclizine, the docking runs resulted in converged sets of 11 (55%) and 12 (60%) solutions that had root-mean-square (rms) deviations lower than 1.4 and 0.7 Å, respectively, and included the best-scored poses. Clomiphene had higher scores but poorer convergence: 12 (60%) solutions including the best scored poses were distributed in five different sets with rms deviation lower than 1.1 Å.

*2.8. Plasmids, viruses and cells for ex vivo assays*. Vectors pNL4.3-Luc and pNL4.3-Ren were generated by cloning the luciferase and renilla genes, respectively, in the nef site of HIV-1 proviral clone pNL4.3 [29]. These constructs generate replication-competent viruses as previously shown [30]. pCMV-Rev expresses Rev, and pDM628 is a Rev-dependent luciferase-based reporter plasmid in which the RRE and a luciferase-coding sequence have been cloned. The LTR-luc plasmid carries a luciferase gene under the control of the HIV-1 LTR [31]. In the 3-enh-κB-ConA-luc plasmid, luciferase expression depends on three synthetic copies of the κB consensus of the immunoglobulin k-chain promoter cloned into the BamHI site located upstream from the conalbumin transcription start site [32]. pCMV-Rev and pDM628 were generous gifts from Dr. Cecilia Cabrera (IrsiCaixa Foundation, Barcelona Spain). MT-2 [33] cells (American Type Culture Collection, Rockville USA) were cultured in DMEM medium containing 10% (v/v) fetal bovine serum in standard conditions. 293T cells (American Type Culture Collection) were cultured in DMEM medium containing 10% (v/v) fetal bovine serum in standard conditions and were split twice a week.

*2.9. Evaluation of anti-HIV-1 activity and cellular toxicity*. Infectious supernatants were obtained from calcium phosphate transfection of plasmid pNL4.3-Ren on 293T cells. These supernatants were collected and titrated with two different methods: by infecting MT-2 cells ($10^6$ per well) with different concentrations of supernatants and measuring relative luminescence units (RLUs) 48 hours later, and by quantifying HIV-1 antigen p24 with an enzyme-like immunoassay (Innotest$^{TM}$ HIV Ag mAb; Innogenetics, Barcelona Spain). These supernatants were used to infect MT-2 cells ($10^6$ per well) with 100.000 RLUs per well, equivalent to 10 ng p24 per well, in the presence of the compounds to evaluate. Anti-HIV activity quantification was performed 48 h post-infection by determining luciferase activity in cell lysates using the Renilla-Luciferase Assay System Kit (Promega, Madison USA). RLUs were obtained in a luminometer (Berthold Detection Systems, Pforzheim Germany). Cellular viability was evaluated in mock infected cells similarly treated with the same concentrations of compounds using the CellTiter Glo (Promega) assay. 50% inhibitory ($EC_{50}$) and cytotoxic ($CC_{50}$) concentrations were calculated with Prism 6 using log(inhibitor) vs response non-linear regression analyses. The results represent the average of at least three independent experiments.

*2.10. Cellular transfection assays*. MT-2 cells were maintained in culture without stimuli and prior to assay were collected in 350 μL of RPMI without serum and antibiotics and pulsed at 320 V, 1500 μF and maximum resistance with a full-length proviral DNA plasmid containing a luciferase reporter gene whose expression is under the control of the HIV-1 LTR promoter (NL4.3-luc), or with luciferase plasmids under the control of the HIV-1 LTR (LTR-luc) or NF-κB (3-enh-κB-ConA-luc), at a concentration of 1 μg/$10^6$ cells, using an Easyject plus

Electroporator (Equibio, Middlesex UK). After transfection, cells were immediately cultured in RPMI with 10% fetal calf serum and antibiotics, treated or not with different concentrations of compound and harvested 48 h later. Luciferase activity (RLUs) was measured in a luminometer. The $EC_{50}$ values were calculated with Prism 6 as above.

*2.11. Rev-dependent RNA transport assay*. To analyze RRE-Rev inhibition, we employed pCMV-Rev and pDM628 plasmids, encoding Rev and a luciferase gene under the control of the RRE, respectively. Transcripts produced upon transcription of pDM628 contained the RRE and the luciferase gene, where both elements were situated between a splicing donor and a splicing acceptor. In the presence of Rev, the RRE-Rev interaction enables export of the transcript to the cytoplasm, resulting in luciferase expression. Experiments were performed with a constant concentration of pDM628 (500 ng/well) and 2-4 decreasing concentrations of pCMV-Rev. The $EC_{50}$ values were calculated with Prism 6 as explained above.

*2.12. Evaluation of HIV-1 RNA splicing*. MT-2 cells were transfected with a pNL4.3 plasmid and treated with two different concentrations of clomiphene (5 and 10 μM) or cyproheptadine (5 and 50 μM) for 72 or 96 hours. The compound concentrations were chosen on the basis of the observed RRE IIB $K_d$'s and cellular $EC_{50}$ and $CC_{50}$ values. Total cellular RNA was isolated with an RNeasy mini kit (Qiagen, Hilden Germany) and quantified with a Nanodrop-1000 spectrophotometer (ThermoFisher). After treatment with DNase I (Ambion, ThermoFisher), reverse transcription was performed on 500 ng of RNA with the GoScript cDNA synthesis kit (Promega). Unspliced, single-spliced and multiple-spliced HIV-1 RNA transcripts were quantified by qPCR (StepOne Plus, Applied Biosystems, ThermoFisher) using the primers and probes described by Mohammadi *et al*. [34]. Briefly, cDNA synthesis was carried out in a C1000 thermocycler (Bio-Rad, Hercules USA) for 60 minutes at 45ºC, immediately followed by qPCR with the following cycling conditions: 10 minutes at 95ºC, followed by 50 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. qPCR was performed by the comparative CT method relative to an untreated reference control. GAPDH was used as endogenous control.

## 3. Results

### 3.1. High-throughput screen and inhibition of RRE subdomain IIB-Rev$_{34\text{-}50}$ interaction

Aiming to identify bioavailable inhibitors of the RRE-Rev interaction, we screened a diverse library of 1,120 small-molecule drugs approved for clinical use. The assay was based on detecting the displacement of a FITC-labeled Rev$_{34\text{-}50}$ α-helix from its high-affinity site in RRE subdomain IIB by fluorescence anisotropy.

A single-concentration, high-throughput screen detected eleven hits that inhibited more than 90% complex formation at 100 μM without exhibiting fluorescence at the assay conditions. Further analyses of these hits with dose-response experiments allowed us to identify four compounds with low inhibitory IC$_{50}$ values (between 2.8 and 4.2 μM): mitoxantrone, clomiphene, ciprofloxacin and cyproheptadine (Table 1 and Figure 2). Homochlorcyclizine and the aminoglycoside antibiotics neomycin B and neamine were likewise among the eleven initial hits. Neomycin and neamine also had low IC$_{50}$'s (6.4 and 5.2 μM, respectively), whereas homochlorcyclizine exhibited weaker IIB-Rev$_{34\text{-}50}$ inhibitory activity (47.6 μM). Neomycin B did not inhibit HIV-1 replication in our cell infection assay at concentrations below 100 μM [20], but its RRE binding and RRE-Rev inhibition capacity are very well characterized [20-23, 25-27, 35, 36]. We therefore used both aminoglycoside antibiotics together with homochlorcyclizine as controls in subsequent experiments *in vitro*. The remaining hits had significantly higher IC$_{50}$ values.

### 3.2. RRE subdomain IIB RNA binding

Since the screening hits inhibit the formation of the complex between internal loop IIB and $\mathrm{Rev}_{34\text{-}50}$, it is possible that they do so by binding to the RNA loop. To investigate RNA binding we measured subdomain IIB association with fluorescence intensity experiments, using $\mathrm{IIB_h}$-19ap or $\mathrm{IIB_h}$-23fl RNA hairpin constructs containing 2-amino purine and fluorescein probes at unpaired loop IIB residues A19 and U23, respectively (Figure 1D). The RNA specificity of the $\mathrm{IIB_h}$ interaction was assessed by duplicating the experiments in the presence of a 100-fold molar excess of $\mathrm{tRNA^{Cys}}$. We also evaluated specificity relative to double-stranded DNA by additionally carrying out the experiments with a 100-fold molar excess of a 26-base pair DNA duplex (hereafter identified as $\mathrm{LTR_d}$). This duplex contained binding sites of transcription factors NF-κB and Sp-1 in the HIV-1 LTR promoter. These two factors are essential for virus replication [37].

All of the compounds bound to the $\mathrm{IIB_h}$ RNA hairpin, but exhibited significant differences in affinity and specificity (Table 2 and Figures 2C and 2D). Mitoxantrone had the strongest affinity for $\mathrm{IIB_h}$ ($K_d$ 0.25 μM), but the interaction was unspecific relative to both $_{\mathrm{t}}\mathrm{RNA^{Cys}}$ and $\mathrm{LTR_d}$. Cyproheptadine and ciprofloxacin also exhibited low $K_d$ values (1.8 and 2.5 μM, respectively) but limited RNA and DNA specificity, the selectivity ratios being particularly low for ciprofloxacin. Clomiphene (with a $K_d$ of 12.4 μM) and the homochlorcyclizine control (30 μM), associated to $\mathrm{IIB_h}$ with less affinity, but the interaction was significantly more specific in both cases (Table 2 and Figures 2C and 2D). The reference antibiotics neomycin B and neamine exhibited $\mathrm{IIB_h}$ $K_d$ values of 0.84 and 27 μM, respectively, and were unspecific in relation to the RNA and DNA controls (Table 2 and Figure 2D). The binding parameters of neomycin B and neamine were in accordance with previous literature reports [21-23, 36] and with our SPR observations for neomycin [20]. In general, the $\mathrm{IIB_h}$ affinities approximately followed the trend observed for the $IIB_h$-$Rev_{34-50}$ inhibitory $IC_{50}$ values (Table 1).

In order to examine the location of the binding site(s) of each compound in the RNA hairpin we also monitored the titration of $IIB_h$ with the selected hits using one- and two-dimensional NMR spectroscopy. Figure 3A shows superpositions of two-dimensional TOCSY spectra allowing visual inspection of the effect of drug association on the RNA signals. Clomiphene and cyproheptadine induced broadening or chemical shift variations that mainly affected the resonances of internal loop nucleotides only (Figure 3A). This indicated that the interaction of these compounds was loop IIB-specific within the $IIB_h$ hairpin. In this respect, a model of a complex between loop IIB and clomiphene is shown in Figure 3B. In this model, built with unrestrained docking calculations and compatible with the NMR chemical shift displacements, the inhibitor is located in the major groove side of the loop recognized by the Rev protein. In contrast, mitoxantrone, and to a lesser extent ciprofloxacin, broadened or shifted the resonances of many $IIB_h$ nucleotides at low ligand:RNA molar ratios. This was indicative of strong but unspecific binding. The association of the homochlorcyclizine control was more specific, but affected nucleotides located both inside and outside the internal loop (data not shown). We showed before that neomycin B also induced chemical shift perturbations in $IIB_h$ stem nucleotides outside the internal loop [20]. Altogether, these NMR observations were approximately consistent with the affinities and specificities quantified by fluorescence experiments.

### 3.3. Interference with full-length RRE-Rev complex formation

In a process triggered by the high-affinity interaction between internal loop IIB and the $Rev_{34-50}$ α-helix, the RRE-Rev complex is formed by the incorporation of additional Rev monomers that bind to other sites in the RRE and to other Rev monomers through RNA-protein and protein-protein contacts mediated by α-helices [5, 6, 8] (Figure 1B). We have shown above that the screening hits bind to the high-affinity site formed by subdomain IIB and inhibit its interaction with $\text{Rev}_{34\text{-}50}$. Using an electrophoretic mobility shift assay (EMSA) involving full-length RRE and Rev, we next evaluated whether they were also capable of interfering with the formation of the complete RRE-Rev ribonucleoprotein.

The results indicated that all of the selected compounds inhibited the RRE-Rev interaction. They did so with $IC_{50}$ values that were in the same range as those measured for IIB-$\text{Rev}_{34\text{-}50}$ inhibition (Table 1 and Figure 4). The most potent compounds in this assay were cyproheptadine ($IC_{50}$ 3 μM) and clomiphene (5 μM). Mitoxantrone also blocked significantly the RRE-Rev interaction but could only be tested up to 10 μM probably because this agent interfered with the conformation of RRE at higher concentrations (Figure 4). Ciprofloxacin, in contrast, exhibited a higher $IC_{50}$ in this assay, 20 μM. Likewise, the homochlorcyclizine control, which had less $\text{IIB}_h$-$\text{Rev}_{34\text{-}50}$ inhibitory activity and less $\text{IIB}_h$ affinity, did not inhibit full-length RRE-Rev complex formation at the concentrations tested in the assay (up to 50 μM). The $IC_{50}$ values obtained in this assay for the reference antibiotics neomycin B and neamine (7 and 11 μM, respectively) agreed very well with previously reported values [25-27, 35].

### 3.4. Antiviral activity

We next checked whether the selected hits were able to block HIV-1 replication in cell cultures. All four compounds turned out to have antiviral activity in infection assays. The most potent inhibitor was mitoxantrone, with an $EC_{50}$ value of 54 nM, followed by ciprofloxacin and clomiphene (4.2 and 4.3 μM, respectively), and cyproheptadine (17.5 μM) (Table 3 and Figure 5A). As described in previous reports [20, 25], the neomycin B control did not exhibit antiviral activity at concentrations below 100 μM. A reverse transcriptase inhibitor (zidovudine) and a protease inhibitor (nelfinavir) were used as positive controls of inhibition of HIV-1 replication (Table 3).

## 3.5. Antiviral mechanism of action

In order to pinpoint the location of the main target, or set of targets, of each of the selected hits in the virus life cycle, we next carried out an assay based on transfecting a full-length competent HIV-1 clone. In this experiment, the entry, reverse transcription and DNA integration steps of the virus cycle are bypassed, so that only transcriptional or post-transcriptional processes occur and can be blocked by the inhibitor being evaluated.

The $EC_{50}$ values obtained with this transfection assay for clomiphene (4.3 μM) and cyproheptadine (25.6 μM) were similar relative to those obtained with the infection experiment (Table 3 and Figure 5). This indicates that the main target (or set of targets) for these molecules is likely contained in transcriptional or post-transcriptional steps of the virus infectious cycle. In contrast, the post-integration $EC_{50}$'s of mitoxantrone and ciprofloxacin were 570-fold and more than 25-fold higher, respectively, relative to the $EC_{50}$ values measured in the infection assay, implying that these two drugs mainly acted on pre-transcriptional processes. Mitoxantrone, which had high activity in the infection assay ($EC_{50}$ 54 nM) still retained significant post-integration activity (30.8 μM). On the contrary, the post-integration effect of ciprofloxacin was not detected at the highest concentration tested of 100 μM (Table 3 and Figure 5B).

## 3.6. Inhibition of LTR- and NF-κB-dependent gene expression

To further characterize the mechanism of action of the screening hits, we used a cellular assay based on transfecting a plasmid encoding a luciferase gene whose expression depends on the LTR promoter of the virus [31] (Table 4 and Figure 6A). Clomiphene and cyproheptadine inhibited LTR-dependent gene expression with $IC_{50}$ values that were very similar to those obtained in the infection and post-integration experiments (see Tables 3 and 4, and Figures 5 and 6A). The LTR inhibitory activity of mitoxantrone was in the same range as the value registered in the post-integration test, confirming that this agent was acting on post-integration events of the virus cycle at higher concentrations than it did in pre-transcriptional steps. Ciprofloxacin did not inhibit LTR-dependent expression as expected, since this compound was inactive in the post-integration experiments.

The $LTR_d$ DNA duplex used as a control in fluorescence experiments contained binding sites of transcription factors NF-κB and Sp-1, which are essential for LTR-dependent transcription and virus replication [37]. To test whether the screening hits specifically interfered with the activity of NF-κB, we also evaluated inhibitory activity in cells transfected with a luciferase construct under the control of this transcription factor (Table 4 and Figure 6A). Clomiphene, cyproheptadine and mitoxantrone were able to decrease luciferase expression through inhibition of NF-κB activity with $IC_{50}$ values that were approximately similar relative to those obtained with the LTR reporter assay. As observed with the LTR system, ciprofloxacin was inactive.

### 3.7. Inhibition of RRE-Rev function

The RRE-Rev interaction allows transport of unspliced or partially spliced viral transcripts to the cytoplasm, a post-transcriptional process absolutely needed by the virus to complete its replication cycle. Although with different degrees of selectivity, we have shown that all of the screening hits bound to loop IIB and inhibited the high-affinity interaction between subdomain IIB and $Rev_{34\text{-}50}$ (Tables 1 and 2). Using EMSA's, we also found that all four selected hits hampered the formation of the full-length RRE-Rev complex *in vitro*, although the inhibitory activity of ciprofloxacin was significantly weaker (Table 1 and Figure 4). To test whether the compounds acted on this system in a cellular context, we first used an assay based on transfecting plasmids encoding Rev and an RRE-luciferase reporter system (Table 5 and Figure 6B). Once again, ciprofloxacin had no activity, in agreement with the absence of post-integration activity and the weaker EMSA effect exhibited by this agent. Clomiphene and cyproheptadine were active in this assay, and their inhibitory concentrations were similar to those measured in the infection and post-integration assays involving full-length viruses. However, they also paralleled the $IC_{50}$ values registered in the LTR and NFκ-B experiments (see Tables 3, 4 and 5). Since the expression of the RRE-luciferase and Rev genes used in this assay depends on transcription, the RRE-Rev activities registered by this assay may be dominated by a transcriptional effect. Mitoxantrone also inhibited RRE-Rev function. In this case, the $IC_{50}$ values were below the post-integration and LTR inhibitory concentrations, suggesting that RRE-Rev inhibition may contribute to the post-integration activity of this agent. The RRE-Rev $IC_{50}$ values of all hits were only slightly affected by differences in the concentration of the Rev-encoding plasmid used in the assays (Table 5 and Figure 6B).

To clarify whether clomiphene and cyproheptadine inhibited Rev function, we subsequently transfected cells with a full-length proviral DNA plasmid and quantified the amount of unspliced, single-spliced and multiple-spliced HIV-1 RNA transcripts present in the cells with RT-qPCR. The results obtained with an untreated control were compared with those observed upon exposing the cells to the compounds at two different concentrations and incubation time periods after transfection. Since Rev transports unspliced or partially spliced viral transcripts to the cytoplasm where they cannot be spliced, inhibition of Rev function should result in lower levels of unspliced and single-spliced transcripts. At a concentration of 5 μM, clomiphene similarly reduced the amount of all types of HIV-1 RNA transcripts by approximately half relative to the untreated control (0.4 vs. 1). This result was fully consistent with the $EC_{50}$ values obtained for this compound in infection and transfection assays (between

3 and 6 μM; Tables 3 and 4) and confirmed that this agent blocked HIV-1 transcription, but did not suggest Rev inhibition. At 10 μM, however, clomiphene diminished the levels of unspliced and single-spliced HIV-1 RNA transcripts to a higher degree relative to multiple-spliced species. This result suggests that in these conditions this agent blocked Rev function in addition to viral transcription. In contrast, cyproheptadine did not induce significant differences in the levels of HIV-1 RNA splice variants at either concentration, although a small effect suggestive of Rev inhibition was observed at 5 μM and 96 hours of incubation (Figure 7). This agent only reduced the levels of all HIV-1 transcripts at 50 μM, in agreement with the $EC_{50}$ values determined in infection and transfection experiments (Tables 3 and 4). This confirmed that cyproheptadine had an effect on HIV-1 transcription. Mitoxantrone's impact on HIV-1 splicing could not be evaluated because of the toxic effect of this agent (see below) at the assay concentrations.

## 3.8. Cellular toxicity

We also evaluated the cellular toxicity of the compounds (Table 3 and Figure 5A) and compared this parameter with the HIV inhibitory activities (Tables 3, 4 and 5). Cyproheptadine and ciprofloxacin were not toxic at the concentrations tested in the assays (up to 100 μM). In contrast, clomiphene and mitoxantrone did exhibit some cellular toxicity. The $CC_{50}$ of clomiphene (17.4 μM) was approximately 2- to 6-fold above the inhibitory concentrations registered for this compound in the infection experiment and all of the transfection assays (between 3.3 and 8.7 μM). The $CC_{50}$ value of mitoxantrone (25-50 μM) was much higher than its activity against HIV-1 infection (54 nM), but similar to its post-integration and LTR $IC_{50}$'s (30.8 and 42.2 μM, respectively). This suggested that these effects may be related with the unspecific cellular toxicity of this agent.

## 4. Discussion

This study had a double objective: first, identify bioavailable inhibitors of HIV-1 acting on a viral target not used by current antiretroviral agents and second, find new RNA-binding scaffolds and explore their biological effect in a cellular context. To achieve these goals, we screened a diverse set of 1,120 bioavailable drugs approved for clinical use with an assay based on detecting the displacement the $\text{Rev}_{34\text{-}50}$ $\alpha$-helix from its RRE subdomain IIB high-affinity site, and selected four inhibitor hits. The RRE-Rev inhibition properties and RNA subdomain IIB binding affinity and selectivity of these compounds were subsequently analyzed, together with their antiviral effect, cellular toxicity and mechanism of action.

All four hits selected on the basis of the RRE IIB-$\text{Rev}_{34\text{-}50}$ screen turned out to have anti-HIV-1 activity. Ciprofloxacin, however, was a false positive in the sense that it had no post-integration activity in cellular assays (Table 3 and Figure 5). This compound inhibited the $\text{IIB}_h$-$\text{Rev}_{34\text{-}50}$ interaction and bound to the $\text{IIB}_h$ RNA hairpin with a low $K_d$ (2.5 μM) but limited RNA and DNA specificity (Tables 1 and 2 and Figure 2). The full-length RRE-Rev inhibition capacity of this compound detected by EMSA (20 μM) was weaker than that measured for the rest of the hits (Table 1 and Figure 4). Ciprofloxacin belongs to the quinolone family of compounds widely used as antibacterials, and is in fact employed to treat opportunistic infections affecting AIDS patients. The quinolone antibiotics inhibit the action of prokaryotic topoisomerases by binding to the complexes formed by these enzymes and DNA. Several quinolone compounds have more recently been reported to have antiretroviral activity, with a mechanism of action proposed to be based on integrase inhibition or transcriptional blockage [38, 39]. Our results support the former mechanism for ciprofloxacin, since this compound inhibited HIV-1 replication in cell infection assays but lacked activity in post-integration experiments. Actually we demonstrate that ciprofloxacin has no detectable effect on LTR-dependent transcription, NF-κB activity or RRE-Rev function at concentrations up to 100 μM (Tables 3, 4 and 5).

Mitoxantrone inhibited subdomain $IIB_h$-$Rev_{34-50}$ and full-length RRE-Rev interactions and, like ciprofloxacin, bound to $IIB_h$ with high affinity (0.35 μM) but low DNA and RNA specificity (Tables 1 and 2 and Figure 2). Not surprisingly, mitoxantrone has also been described to associate with several other RNA targets [40-43]. This compound intercalates into double-helical DNA, disrupting DNA and RNA synthesis and topoisomerase II function, and is used as an anticancer and immunosuppressive drug [44]. It has also been reported to inhibit HIV-1 integrase activity at low micromolar concentrations [45]. Like in the ciprofloxacin case, the interference with the integration step may be related to the DNA-binding properties of the compound. In our experiments, mitoxantrone inhibited HIV-1 replication at nanomolar concentrations in the cell infection assay, and had an $EC_{50}$ of 30.8 μM in the post-integration experiment (Table 3 and Figure 5). This indicated that the main target of the drug was contained in integration and/or pre-integration steps of the virus cycle, as observed for ciprofloxacin. The post-integration effect of mitoxantrone, however, was still significant. The compound interfered with HIV-1 post-integration processes and LTR-dependent expression at concentrations close to its $CC_{50}$ value (Tables 3 and 4). This suggests that these effects are related with the cytotoxicity of the compound, and are likely connected to transcriptional blockage in accordance with previous studies [44]. The NF-κB and cellular RRE-Rev inhibitory concentrations were below the $CC_{50}$ and the LTR and post-integration $EC_{50}$´s (Tables 3, 4 and 5). DNA binding probably contributed to the observed effect on NF-κB-dependent expression, since we indirectly detected mitoxantrone association to an $LTR_d$ duplex containing NF-κB binding sites (Table 2 and Figure 2). Likewise, RRE-Rev inhibition may participate in the post-integration activity of mitoxantrone. Nevertheless, the combined $EC_{50}$ and $CC_{50}$ data of this agent favor its usage for inhibiting pre-integration or integration steps of the viral cycle.

In contrast to ciprofloxacin and mitoxantrone, no nucleic acid binding and no antiretroviral activity had been reported to date for the two remaining hits. Clomiphene, the most potent compound of the pair ($EC_{50}$ 4.4 μM in the infection assay), is a selective estrogen receptor modulator used to treat infertility [46], whereas cyproheptadine is an $H_1$ antihistamine agent [47]. In contrast to the previous drugs, these agents exhibited similar activities in the HIV-1 infection and post-integration assays (Table 3 and Figure 5). This indicated that their main target was likely contained in transcriptional or post-transcriptional steps of the virus infectious cycle. *In vitro*, both drugs inhibited $IIB_h$-$Rev_{34-50}$ and RRE-Rev complex formation with similar $IC_{50}$'s (between 3 and 5 μM; Table 1 and Figures 2 and 4), and associated to RRE subdomain IIB with low μM affinities (12.4 and 1.8 μM; Table 2 and Figures 2C and 2D). Subdomain IIB recognition by clomiphene was substantially specific. This was demonstrated by fluorescence control experiments employing a 100-fold molar excess of $tRNA^{Cys}$ or $LTR_d$ DNA duplex, as well as by $IIB_h$ titration experiments monitored by NMR, which indicated specific recognition of loop IIB nucleotides by this ligand (Figures 2 and 3). Cyproheptadine associated to $IIB_h$ with more affinity but less specificity (Table 2 and Figure 2). The specificity factors, however, were above those observed for ciprofloxacin, mitoxantrone or the aminoglycoside antibiotics, and the NMR analyses indicated specific binding to loop IIB nucleotides (Figure 3A).

Docking calculations revealed that clomiphene fitted well in the widened major groove of RNA loop IIB (Figure 3B). Moreover, the non-planar triphenylethylene and dibenzocycloheptene scaffolds of clomiphene and cyproheptadine, respectively, superposed surprisingly well, exhibiting approximately similar orientations of the aromatic rings and positively-charged groups (Figure 3C). Homochlorcyclizine, the weaker $IIB_h$-$Rev_{34-50}$ inhibitor hit used as a control in experiments *in vitro,* also superposed well with the other two ligands. This compound, another $H_1$ antihistamine agent related to cyproheptadine, recognized

$IIB_h$ with less affinity but significant selectivity (Table 2 and Figure 2D). The less specific ciprofloxacin and mitoxantrone hits, in contrast, have a more planar structure and a likely different RNA-binding mode based on stacking and/or intercalation [40]. Interestingly, clomiphene and the two antihistamine hits share homologies regarding the nature of their binding sites in their preferred estrogen and histamine $H_1$ receptor targets. In both cases, the drug binding sites are quite rich in $\alpha$-helices and relatively hydrophobic [48, 49].

When the mechanism of antiretroviral action of clomiphene and cyproheptadine was evaluated, we found that both hits inhibited luciferase expression mediated by the HIV-1 LTR promoter and NF-κB. For each compound, the LTR and NF-κB inhibitory concentrations had similar values, which were close to the $EC_{50}$'s determined in the infection and post-integration experiments (see Tables 3 and 4). The RRE-Rev inhibitory concentrations of both hits followed a similar trend (Table 5). However, since the expression of the RRE-luciferase and Rev genes used in the RNA transport assay depended on transcription, it is possible that the RRE-Rev activities obtained in this assay were dominated by transcriptional blockage. In this respect, varying the concentration of Rev-encoding plasmid affected only slightly the RRE-Rev $IC_{50}$ values (Figure 6B), in contrast to what was observed with recently designed Rev mimics [20]. To investigate whether clomiphene and cyproheptadine had an effect on Rev function, we conducted experiments based on monitoring the levels of unspliced, single-spliced and multiple-spliced HIV-1 RNA transcripts. Both compounds reduced the amount of all viral transcripts at concentrations close to their infection and transfection $EC_{50}$'s (Figure 7 and Tables 3 and 4), confirming an effect on HIV-1 transcription. At a concentration of 10 μM, clomiphene reduced the levels of unspliced and incompletely spliced HIV-1 transcripts to a greater extent relative to multiple-spliced RNA species (Figure 7). This suggests that at least at this concentration, the antiviral activity of this agent is based on RRE-Rev inhibition in addition to transcriptional blockage. In contrast, cyproheptadine did not induce significant differences in the patterns of HIV-1 RNA splicing, although at the higher concentration transcriptional blockage might have hidden the effect on splicing patterns (Figure 7).

It is unlikely that the HIV-1 transcriptional inhibitory activity of clomiphene has a DNA-based mechanism, since this agent showed very weak LTR DNA association (Table 2 and Figure 2C). Rather, this effect may be due to RNA binding or to interference with the estrogen receptor or other nuclear receptor factors involved in LTR transcription [50, 51]. Interference with Rev function likely contributes to the antiviral action of this agent, as suggested by the effect on RRE-Rev association detected *in vitro* and by the changes in HIV-1 splicing patterns observed in cellular assays. It is relevant to note in this respect that the processes of transcription and RNA export are tightly coupled[52, 53]. Interestingly, clomiphene and other estrogen receptor modulators have been recently reported to inhibit Ebola virus replication [54, 55]. In this case, however, the mechanism was reported to be based on blockage of virus entry [54].

Several groups have studied the RRE-binding and RRE-Rev inhibition properties of aminoglycoside antibiotics and conjugates [19, 21-23, 26, 35, 36], diphenylfurans [56], acridines [57], metal complexes [19] and peptide boronic acids [58], but either these molecules lacked antiviral activity, or their activity and/or mechanism was not reported. To our knowledge, there is only one precedent of a small-molecule, high-throughput RRE-Rev screen based on the usage of purified RRE and Rev species: a Glaxo team tested over 500,000 compounds with an scintillation proximity assay [25]. Several classes of RRE-Rev inhibitors with low μM potencies were identified, but most of them lacked antiviral activity. On the other hand, Shuck-Lee *et al*. conducted an RRE-Rev screen of 40,000 compounds based on a cellular assay [27]. These authors identified three HIV post-integration inhibitors that interfered with RRE-Rev function. However, these molecules did not inhibit RRE-Rev binding *in vitro*, and thus may act on some other step of the Rev pathways [27]. The lack of antiviral activity of the compounds identified by Glaxo might have been influenced by their cellular penetration properties: 40% of those hits were negatively charged, and all of the tested molecules associated to Rev rather than RRE. In our case, the positively-charged RRE-Rev inhibitor hits associated to RRE, and the post-integration $EC_{50}$'s of the hits were similar to the $K_d$'s of nucleic acid association. This suggests that these compounds have better cell and nucleus penetration properties.

In conclusion, by screening one thousand FDA-approved drugs according to their ability to displace $\text{Rev}_{34\text{-}50}$ from its RRE subdomain IIB site, we have identified two bioavailable drugs, clomiphene and cyproheptadine, that are capable of inhibiting the post-integration stage of HIV-1. Both compounds bound to RRE subdomain IIB and blocked RRE-Rev complex formation at low μM concentrations similar to their cellular $EC_{50}$ values, and RNA loop IIB recognition by clomiphene was substantially specific. Remarkably, no antiretroviral activity and no nucleic acid binding had been previously reported for these two agents. Although the anti-HIV activities of both drugs were apparently dominated by inhibition of LTR-dependent transcription, the observed blockage of RRE-Rev binding may also contribute to their antiviral effect. For clomiphene, this mechanism was supported by the detection of changes of HIV-1 splicing patterns consistent with Rev inhibition. In this regard, these hits have defined a new RNA-binding and RRE-Rev inhibition motif that may serve as a starting point for the development of HIV-1 gene-regulation inhibitors.

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## Figure Legends

**Figure 1.** The RRE-Rev interaction of HIV-1. (A) Three-dimensional structure of a Rev monomer [5, 6]. The arginine-rich α-helix ($\text{Rev}_{34\text{-}50}$) is shown in red. (B) Model of full-length RRE-Rev complex. The location of the main Rev binding site in subdomain IIB is indicated.

(C) Three-dimensional view of the high-affinity complex formed by an internal loop within subdomain IIB of the RRE (grey) and $\text{Rev}_{34\text{-}50}$ (red) [7]. (D) Secondary structure of hairpin $\text{IIB}_h$, containing the internal loop forming the high-affinity Rev binding site. This construct was used for fluorescence anisotropy and NMR experiments. For fluorescence intensity assays we employed two labeled $\text{IIB}_h$ constructs: $\text{IIB}_h$-19ap, where 2-aminopurine replaced adenine at unpaired loop residue A19 (marked with an asterisk); and $\text{IIB}_h$-23fl, containing a fluorescein probe linked to the extra-helical loop residue U23 (marked with two asterisks).

**Figure 2.** Inhibition of the $\text{IIB}_h$-$\text{Rev}_{34\text{-}50}$ interaction and recognition of RNA hairpin $\text{IIB}_h$ by the screening hits. (A) Chemical structure of the small-molecule inhibitors identified in the fluorescence anisotropy screen. The weaker inhibitor homochlorcyclizine was used as a control in experiments *in vitro*, together with the aminoglycoside antibiotics neomycin B and neamine (not shown). (B) Representative curves of $\text{IIB}_h$-$\text{Rev}_{34\text{-}50}$ inhibition by the best hits, clomiphene and cyproheptadine, obtained with fluorescence anisotropy experiments. (C) $\text{IIB}_h$ binding curves of clomiphene, cyproheptadine and mitoxantrone obtained with fluorescence intensity experiments in the absence (light blue) and presence of a 100-fold molar excess of unlabeled competitor RNA ($\text{tRNA}^{\text{Cys}}$; dark blue) or unlabeled competitor double-helical DNA ($\text{LTR}_d$; green). (D) Comparison of the $\text{IIB}_h$ affinities (left) and specificities (relative to $\text{tRNA}^{\text{Cys}}$ and $\text{LTR}_d$; right) of all screening hits. Note that the $K_d$ and specificity scales are logarithmic. In (B) and (C), the error bars represent standard deviations of at least two independent experiments.

**Figure 3**. Internal loop IIB recognition by screening hits. (A) RNA hairpin $\text{IIB}_h$ interaction with the best hits, clomiphene and cyproheptadine, monitored by NMR spectroscopy. The H5-H6 region of the TOCSY spectrum (60 ms mixing time, 27 $^{o}$C) of hairpin $\text{IIB}_h$ (blue) is superposed on the spectra of complexes with increasing RNA:drug molar ratios, color-coded as indicated in the graph. On the right, the location of the ligand binding site in the $IIB_h$ hairpin is indicated by highlighting in red the nucleotides whose aromatic protons underwent broadening or chemical shift variations greater than 0.1 ppm upon the addition of one or two equivalents of drug. Nucleotides with overlapped aromatic resonances are black-colored, and residues whose aromatic signals were not affected by ligand binding are colored grey. The chemical shift variations were monitored with one-dimensional and TOCSY experiments. (B) Best-scored model of a complex between internal loop IIB (grey) and clomiphene (green carbon atoms), built by docking the ligand into the structure of subdomain IIB (PDB code 1ETG) [7]. (C) Superposition of the chemical structures of clomiphene, cyproheptadine and homochlorcyclizine. The drugs are depicted with green, orange and yellow carbon atoms, respectively.

**Figure 4.** Inhibition of full-length RRE-Rev complex formation by the screening hits analyzed by EMSA. (A) EMSA results. All lanes contained 78 nM RRE, 1.32 μM Rev and the indicated concentration of compound. We did not detect inhibition by homochlorcyclizine at the assay concentrations. (B) Plots of unbound RRE band area as a function of compound concentration. The error bars represent the standard deviation of at least two independent EMSA experiments.

**Figure 5.** Cellular assays of selected screening hits. (A) Antiviral activity in HIV-1 cell infection experiments and cellular toxicity. (B) Inhibition of HIV-1 post-integration steps: the cells were transfected with a full-length proviral DNA plasmid containing a luciferase reporter gene whose expression is under the control of the HIV-LTR promoter. In all cases, results are expressed as percentage of relative luminescence units (RLUs), where 100% is the level of viral replication obtained in the presence of the vehicle used to dissolve the compounds.

**Figure 6.** Mechanism of antiviral action of selected screening hits. (A) Inhibition of LTR- and NF-κB-dependent luciferase expression. (B) Inhibition of Rev-mediated transport of RRE-containing RNA into the cytoplasm, evaluated at different concentrations of the plasmid encoding Rev (200, 100, 20 and 10 ng/well). In (A) and (B), results are expressed as percentage of RLUs, where 100% is the level of viral replication obtained in the presence of the vehicle used to dissolve the compounds.

**Figure 7**. Effect of clomiphene and cyproheptadine on HIV-1 RNA splicing. Unspliced, single-spliced and multiple-spliced HIV-1 RNA transcripts were quantified by RT-qPCR after RNA isolation from treated or untreated MT-2 cells transfected with a wild-type HIV-1 plasmid (pNL4.3). Results are expressed as RNA relative quantity (RQ), using untreated cells as a reference control (RQ=1) and GADPH as endogenous control.

**Table 1.** RRE subdomain IIB-Rev$_{34-50}$ and full-length RRE-Rev 50% inhibitory concentrations of the screening hits.

| Compound | *IC$_{50}$* IIB$_h$-Rev$_{34-50}$ ($M \cdot 10^6$) | *IC$_{50}$* RRE-Rev ($M \cdot 10^6$) |
|---|---|---|
| Clomiphene | 3.7 ± 1.3 | 5 ± 1 |
| Cyproheptadine | 4.2 ± 2.2 | 3 ± 1 |
| Mitoxantrone | 2.8 ± 1.3 | ~8[b] |
| Ciprofloxacin | 4.1 ± 1.2 | 20 ± 7 |
| Neomycin B | 6.4 ± 0.8[a] | 7 ± 1 |
| Neamine | 5.2 ± 1.6 | 11 ± 6 |
| Homochlorcyclizine | 47.6 ± 1.4 | >50[c] |

The RRE IIB-Rev$_{34-50}$ IC$_{50}$ values were obtained with fluorescence anisotropy experiments using 60 nM IIB$_h$ and 10 nM frevp. The full-length RRE-Rev IC$_{50}$'s were measured by EMSA with 78 nM RRE and 1.32 μM Rev. All experiments were repeated at least two times, and the table shows the average and standard deviation of independent experiments.

[a] This value was taken from a previous study in our laboratory [20], where it was measured under identical experimental conditions.

[b] The RRE-Rev IC$_{50}$ of mitoxantrone could not be reliably determined because this compound likely caused a RRE conformational change at concentrations >10 μM that affected all bound and unbound RRE electrophoretic bands.

[c] We did not detect RRE-Rev inhibition by homochlorcyclizine at the concentrations tested by the assay (up to 50 μM).

**Table 2**

**Table 2.** $IIB_h$ interaction parameters of screening hits, measured by fluorescence intensity experiments at 25 °C.

| compound[a] | $K_d\,(IIB_h)$ (M·10$^6$) | $K_d\,(IIB_h + {}_tRNA)$ (M·10$^6$) | IIB/RNA specificity $\frac{K_d\,(IIB_h)}{K_d\,(IIB_h + {}_tRNA)}$ | $K_d\,(IIB_h + LTR_d)$ (M·10$^6$) | IIB/DNA specificity $\frac{K_d\,(IIB_h)}{K_d\,(IIB_h + LTR_d)}$ |
|---|---|---|---|---|---|
| Clomiphene | 12.4 ± 1.2 | 6.0 ± 1.1 | 2.1 | 16.1 ± 1.5 | 0.77 |
| Cyproheptadine | 1.8 ± 0.7 | 11.2 ± 4.7 | 0.16 | 9.2 ± 5.1 | 0.20 |
| Mitoxantrone | 0.35 ± 0.05 | 4.0 ± 0.8 | 0.088 | 11.4 ± 4.0 | 0.030 |
| Ciprofloxacin | 2.5 ± 0.9 | 22 ± 11 | 0.11 | 47 ± 43 | 0.053 |
| Neomycin B | 0.84 ± 0.17 | 87 ± 24 | 0.0097 | 47 ± 14 | 0.018 |
| Neamine | 27.0 ± 8.5 | >120 | <0.006 | >120 | <0.006 |
| Homochlorcyclizine | 30 ± 12 | 74 ± 23 | 0.41 | 48 ± 15 | 0.63 |

[a] $IIB_h$ equilibrium dissociation constants ($K_d$) in the absence ($IIB_h$) and presence of a 100-fold molar excess of ${}_tRNA^{cys}$ ($IIB_h$+${}_tRNA$) or DNA duplex $LTR_d$ ($IIB_h$+$LTR_d$). The IIB/RNA and IIB/DNA specificities of the interaction were quantified with the $K_d(IIB_h)/K_d(IIB_h + {}_tRNA)$ and $K_d(IIB_h)/K_d(IIB_h + LTR_d)$ ratios, respectively. Interactions with specificity ratios close to 1 are specific, whereas those with ratios << 1 are unspecific. Clomiphene, neomycin B and homochlorcyclizine were studied with $IIB_h$-19ap, and ciprofloxacin, cyproheptadine, mitoxantrone and neamine were analyzed with $IIB_h$-23fl. All experiments were repeated at least two times, and the table shows the average and standard deviation of independent experiments.

**Table 3**

**Table 3.** Anti-HIV activity in infection ($EC_{50}$, infection) and transfection ($EC_{50}$, post-integration) experiments, and cellular toxicity ($CC_{50}$), of selected screening hits.

| compound[a] | $EC_{50}$ *(infection)*[b] ($M \cdot 10^6$) | $EC_{50}$ *(post-integration)* ($M \cdot 10^6$) | $CC_{50}$ ($M \cdot 10^6$) |
|---|---|---|---|
| Clomiphene | 4.3<br>(1.1-10.2, 0.9486) | 4.3<br>(2.8-6.6, 0.8373) | 17.4<br>(6.6-45.7, 0.7475) |
| Cyproheptadine | 17.5<br>(7.9-38.5, 0.9315) | 25.6<br>(16.5-39.7, 0.8527) | >100 |
| Mitoxantrone | 0.054<br>(0.031-0.092, 0.8018) | 30.8<br>(16.3-58.3, 0.6967) | >25<50 |
| Ciprofloxacin | 4.2<br>(2.0-8.9, 0.592) | >100 | >100 |

[a] Confidence intervals and $R^2$ values are shown in parentheses when applicable.

[b] The $EC_{50}$ (infection) values obtained for the control antiretrovirals zidovudine and nelfinavir in the infection assay were 0.0083 µM (0.0056-0.0123 µM, $R^2 = 0.9677$) and 0.0021 µM (0.0010-0.0044 µM, $R^2 = 0.9056$), respectively.

**Table 4**

**Table 4.** Effect of selected screening hits on LTR- and NF-κB-dependent luciferase expression.

| compound[a] | $EC_{50}$ *(LTR)*<br>(M·$10^6$) | $EC_{50}$ *(NF-κB)*<br>(M·$10^6$) |
|---|---|---|
| Clomiphene | 3.3<br>(2.1-5.0, 0.8772) | 5.9<br>(3.1-11.2, 0.8032) |
| Cyproheptadine | 20.7<br>(4.7-92.3, 0.8779) | 22.5<br>(5.4-93.9, 0.903) |
| Mitoxantrone | 42.2<br>(7.3-242.4, 0.5792) | 14.7<br>(4.4-49.4, 0.7719) |
| Ciprofloxacin | >100 | >100 |

[a] Confidence intervals and $R^2$ values are shown in parentheses when applicable

**Table 5.** Cellular inhibition of RRE-Rev function by selected screening hits.

| compound[a] | *$EC_{50}$ (RRE-Rev)* *10 ng pCMV-Rev* ($M \cdot 10^6$) | *$EC_{50}$ (RRE-Rev)* *20 ng pCMV-Rev* ($M \cdot 10^6$) | *$EC_{50}$ (RRE-Rev)* *100 ng pCMV-Rev* ($M \cdot 10^6$) | *$EC_{50}$ (RRE-Rev)* *200 ng pCMV-Rev* ($M \cdot 10^6$) |
|---|---|---|---|---|
| Clomiphene | 7.1<br>(3.0-16.8, 0.9322) | 8.7<br>(1.5-50.2, 0.6827) | 5.1<br>(3.2-8.3, 0.9827) | 5.6<br>(0.9-34.7, 0.702) |
| Cyproheptadine | 29.3<br>(17.5-49.2, 0.7837) | 34.0<br>(18.24-63.35, 0.6791) | 27.4<br>(14.2-52.8, 0.7235) | 23.9<br>(14.7-39.1, 0.7181) |
| Mitoxantrone | - | 22.3<br>(15.5-32.1, 0.8666) | - | 18.3<br>(14.4-23.1, 0.9034) |
| Ciprofloxacin | - | >100 | - | >100 |

[a] Confidence intervals and $R^2$ values are shown in parentheses when applicable

Figure 1

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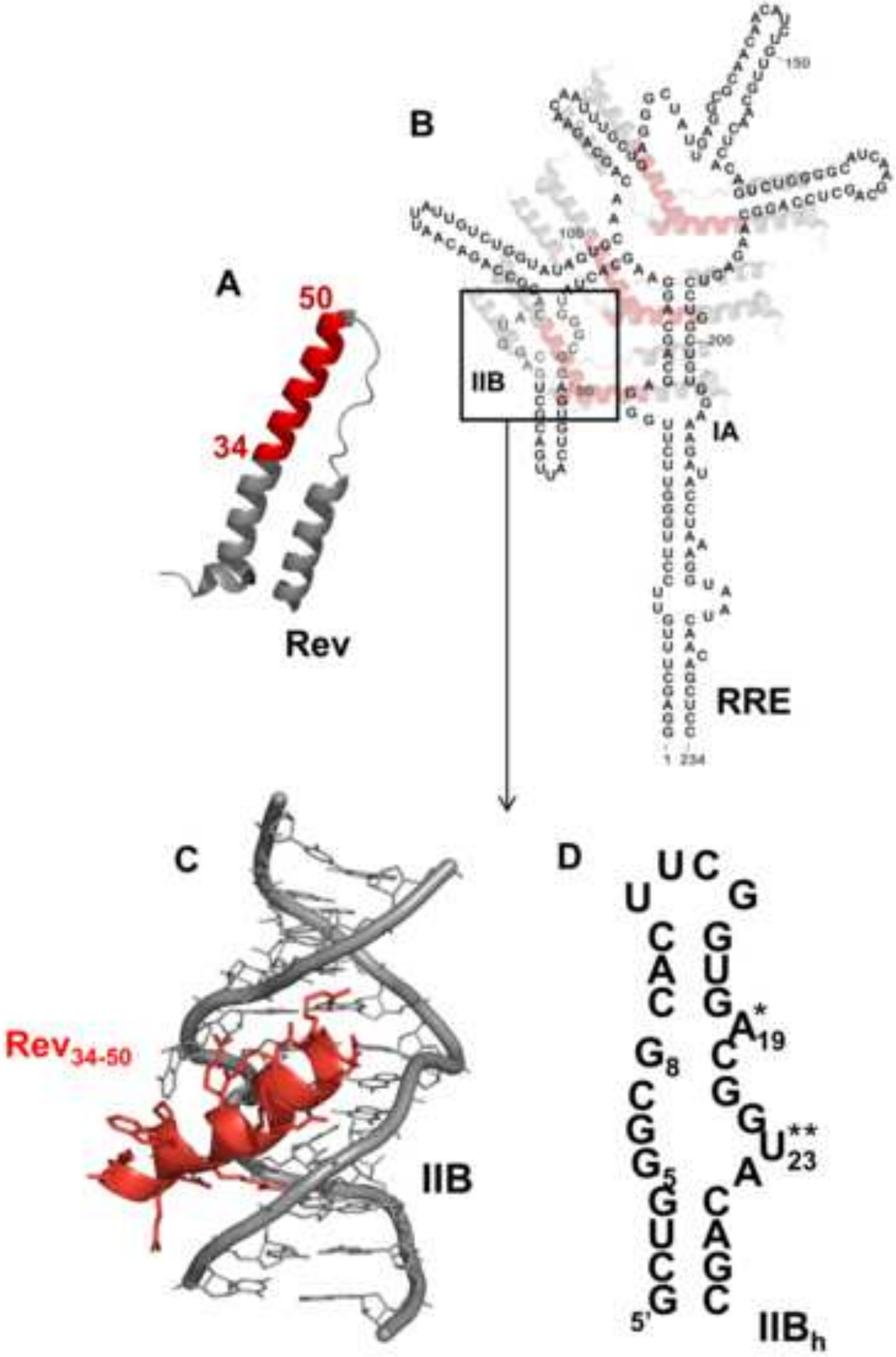
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Fig. 1

Figure 2
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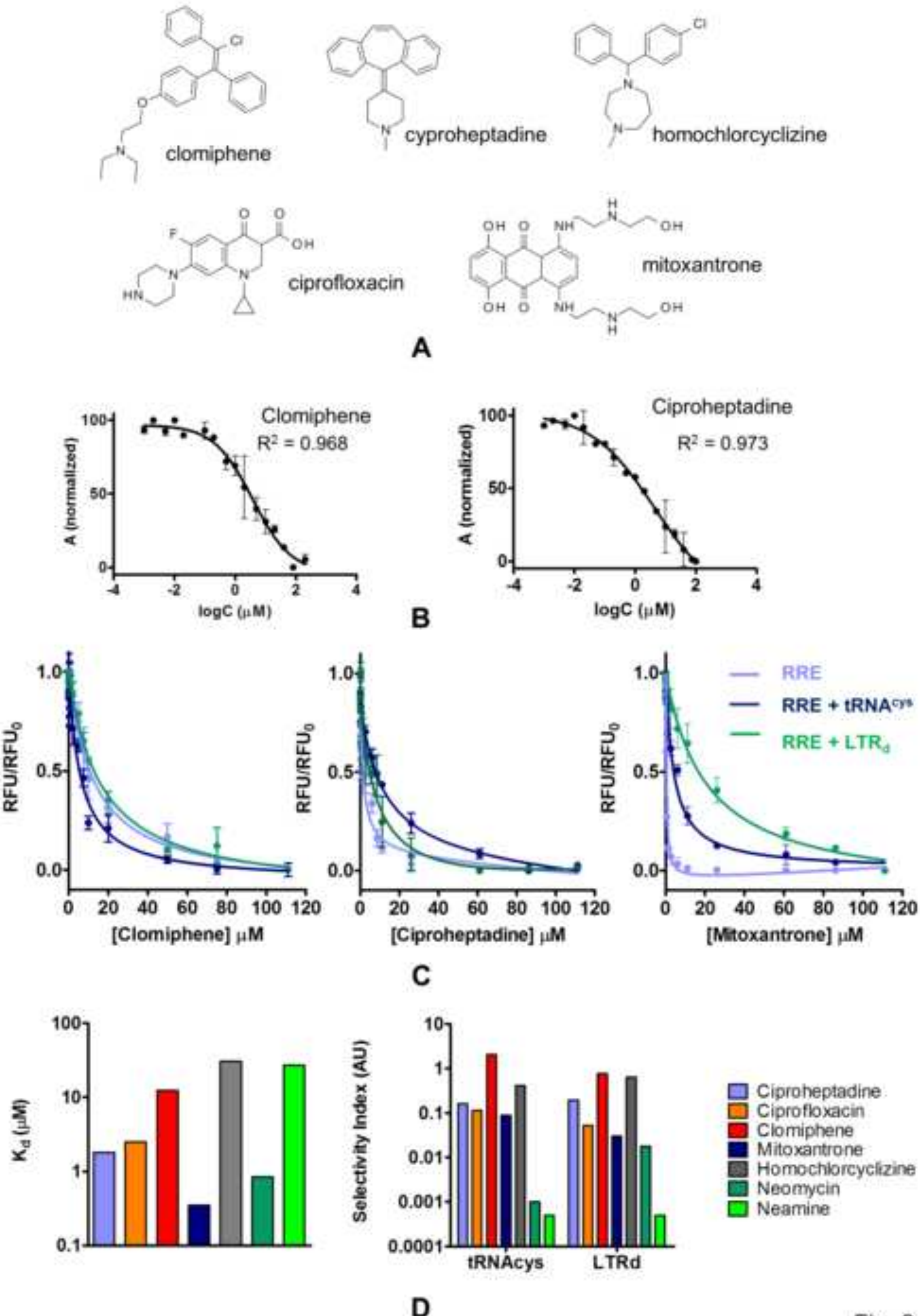

Fig. 2

Figure 3
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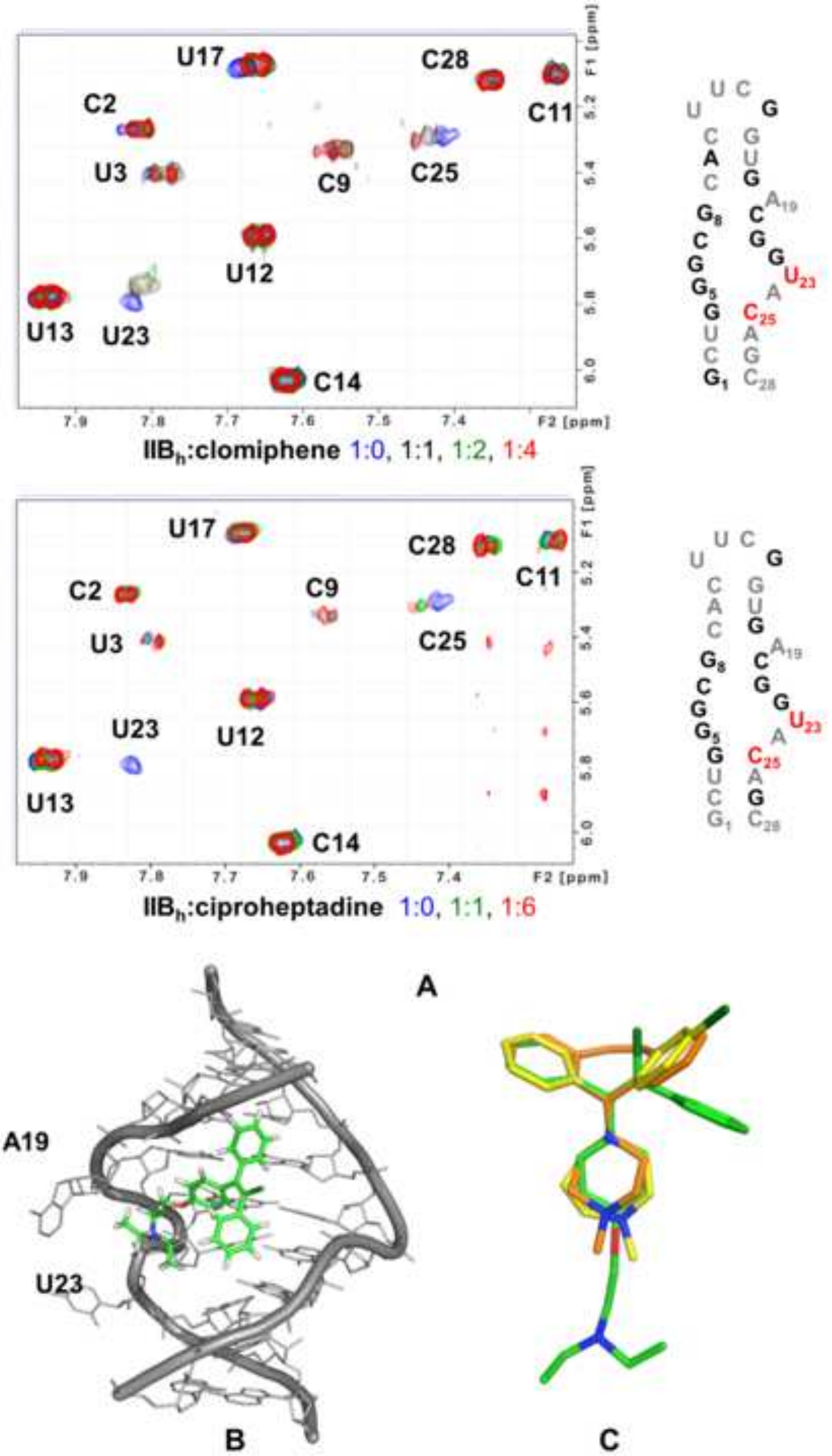

Fig. 3

Figure 4

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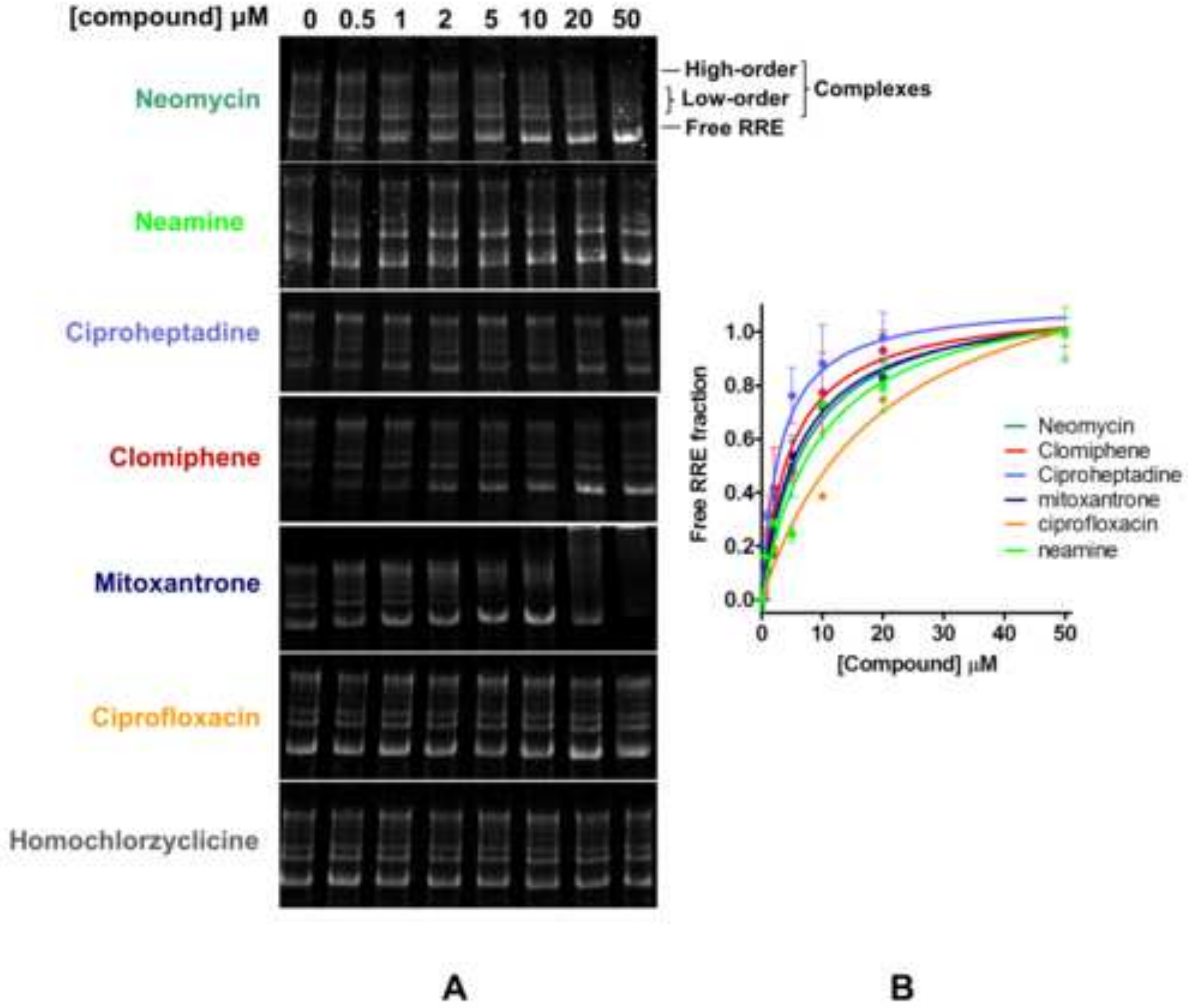

Fig. 4

Figure 5

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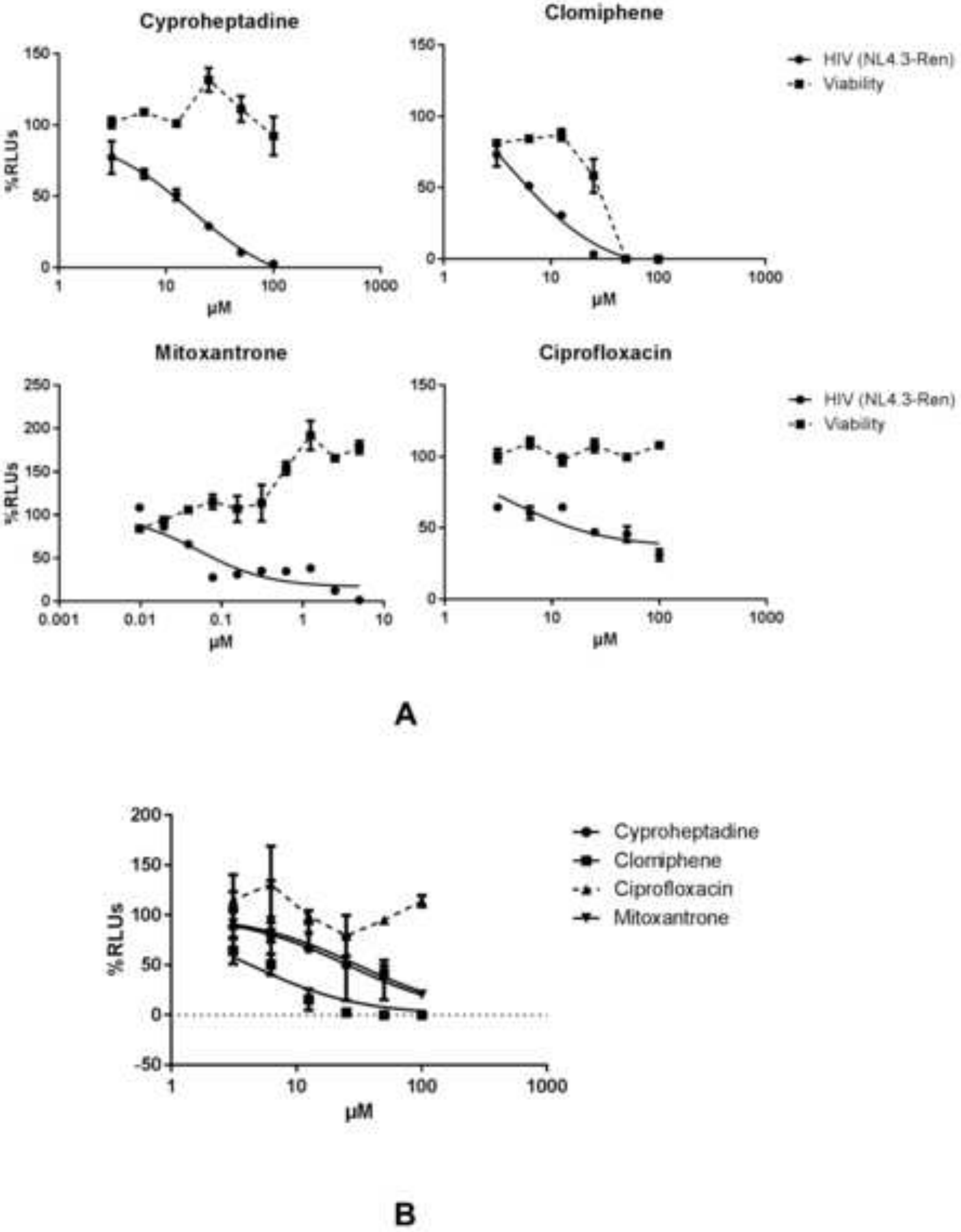

Fig. 5

Figure 6

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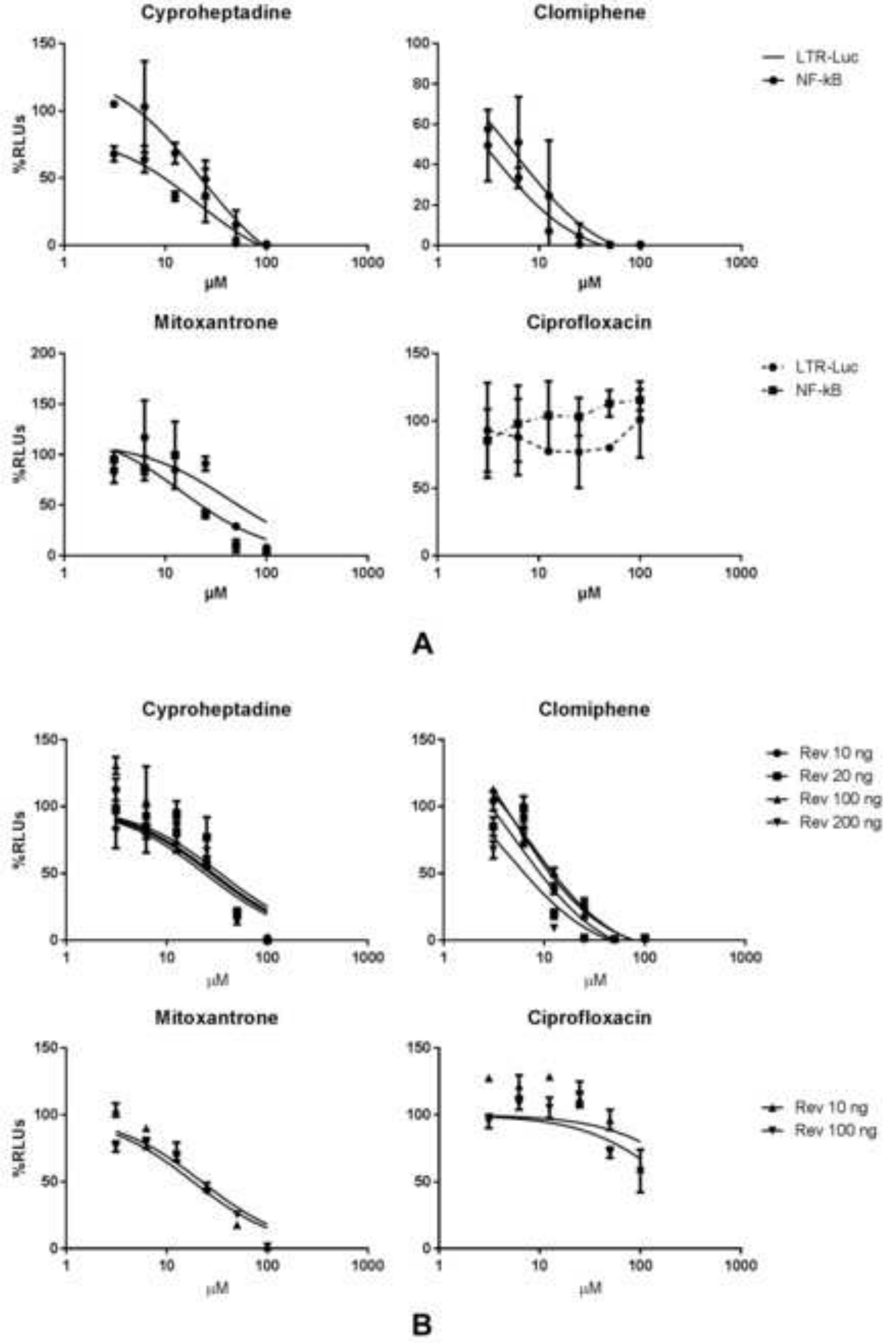

Fig. 6

Figure 7

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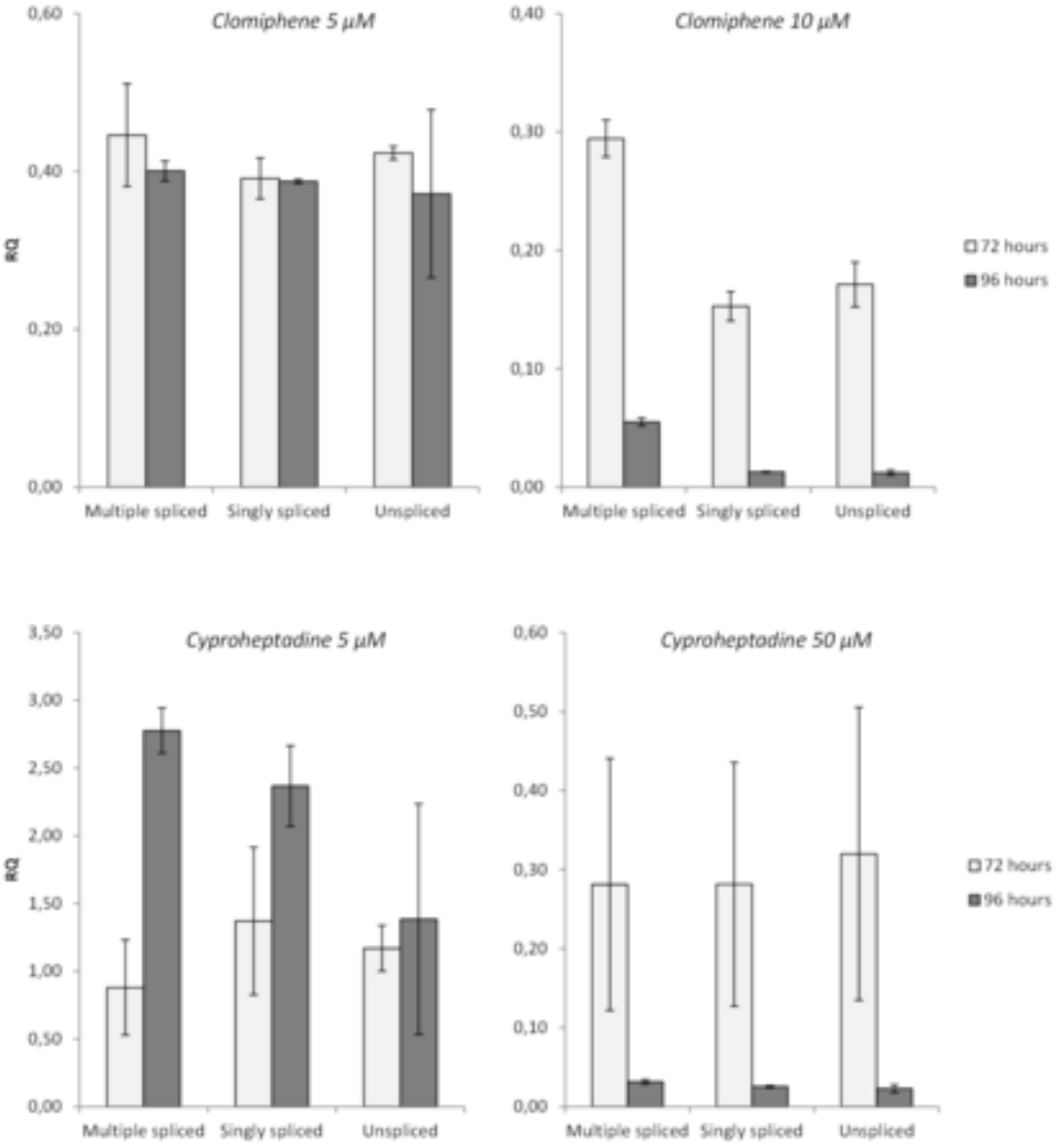

Fig.7

graphical abstract
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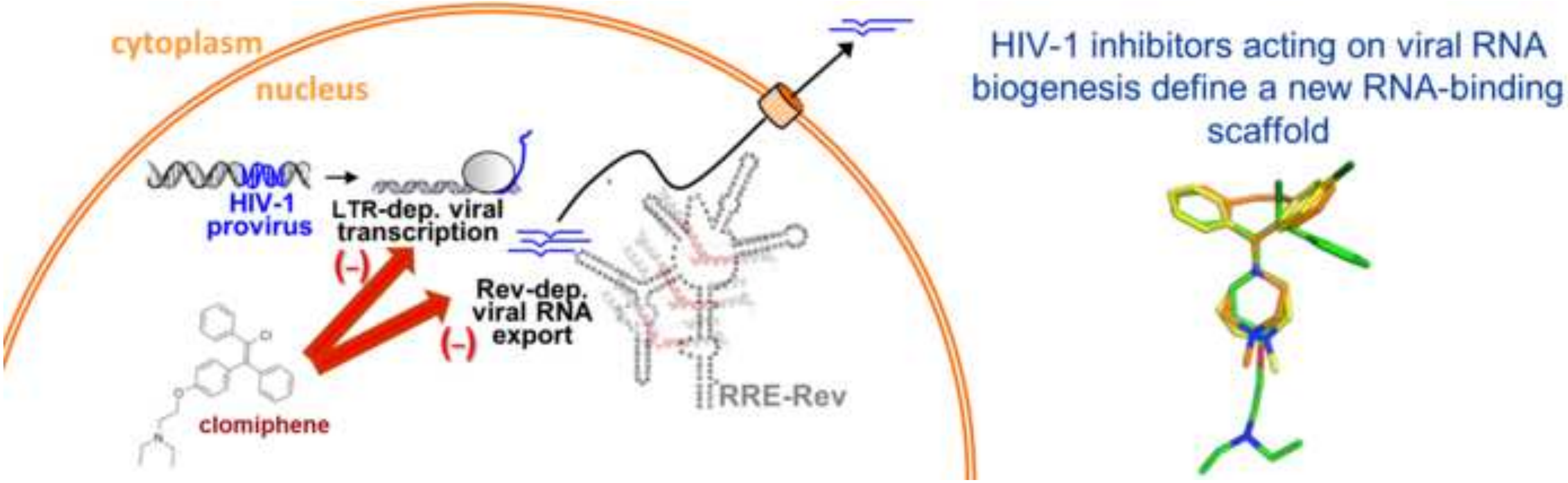
cytoplasm
nucleus
HIV-1 provirus
LTR-dep. viral transcription
(-)
Rev-dep. viral RNA export
(-)
clomiphene
RRE-Rev
HIV-1 inhibitors acting on viral RNA biogenesis define a new RNA-binding scaffold