Cessation of HIV-1 Transcription by Inhibiting Regulatory Protein Rev-Mediated RNA Transport

Yuan Cao[1], Xinyong Liu[*,1] and Erik De Clercq[2]

[1]*Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, Jinan, P.R. China;* [2]*Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium*

Abstract: The HIV-1 Rev protein, which traffics through nucleolus and shuttles between nucleus and cytoplasm, facilitates export of unspliced and singly spliced viral transcripts containing RRE RNA by the CRM1 export pathway. Inhibitions of the various stages of Rev-mediated RNA transport can arrest HIV-1 transcriptional process. The current understanding to the mechanism of Rev function, Rev-RRE interaction, as well as inhibitors hereof is reviewed.

Keywords: AIDS, HIV-1, transcription, inhibitors, Rev, RRE, antiviral.

INTRODUCTION

The AIDS crisis continues to be a worldwide problem. Number of adult and child deaths due to AIDS was more than 2.0 million and number of people newly infected globally was 2.5 million in 2007. The introduction of highly active anti-retroviral therapy (HAART) has significantly decreased morbidity and mortality among patients infected with the human immunodeficiency virus type 1 (HIV-1) and prolonged the life of infected individuals. However, the continued increase of drug-resistant HIV-1 stains and cumulative toxicities limit the efficacy of currently available antiretroviral therapies targeting entry, reverse transcription and/or maturation of the virus. Therefore, continual endeavour should be make to develop new therapies and novel targets to increase the barrier to the evolution of drug resistant strains of HIV-1 [1].

For HIV-1, targeting the host cell factors might elicit fewer drug-resistant viruses. The HIV-1 transcription requires interactions with host cellular factors by vital viral proteins: Tat and Rev. The essential role of the two proteins in HIV-1 replication makes them to be attractive targets for the development of AIDS therapeutics [2, 3].

REV AND RRE RNA

Rev is a small protein of 116 amino acid residues with a molecular mass of 13 000, which is predominantly located in the nucleolus. Rev has a specified amino acid sequence to exert its biological functions, such as multimerization, RNA binding and interaction with host cellular proteins. Based on mutational studies, two distinct regions have been identified as essential for its biological activity *in vivo* [4].

Within the N-terminal of Rev is the arginine-rich motif (ARM) at aa positions 34 to 50 (TRQARRNRRRRWRER QR) which comprises both the nuclear localization signal (NLS) to mediate the nuclear and nucleolar localization of Rev, and the RNA-binding domain to mediate binding of Rev to Rev-binding element (RBE) within the Rev response element (RRE). Flanking the ARM are sequences involved in mediating Rev multimerization that appears to be critical for its biological role. Polymerized Rev that interacts with host cellular factors is prerequisite for RNA binding [4]. Within the C-terminal is a leucine-rich sequence at aa positions 73 to 83 (LQLPPLERLTL) which comprises the nuclear export signal (NES) of the protein (Fig. **1**) [5, 6].

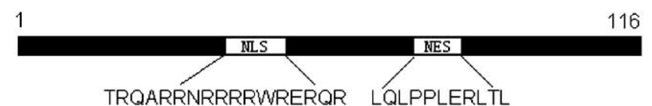

Fig. (1). HIV-1 Rev structure.

RRE is a 240-base region of complex RNA secondary structure which locates in the *env*, the second intron of HIV-1 and is present in all incompletely spliced transcripts. The RRE region can be folded in two different structures. In one structure, five short stem-loops protrude from an internal loop. In the second, three stem-loops (Stem IIC, the combined Stem III and IV, and Stem V) are located on the 3' side of a long stemcombining Stem IIB/Stem IIA/Stem I (Fig. **2**) [7, 8]. Biochemical experiments confirmed that all the stem-loops are important for RRE function in that they bind either Rev or cellular factors that mediate the mRNA transport to cytoplasm [9]. Studies of Rev-RRE complex *in vitro* indicated that the first Rev monomer binds to RBE, a high-affinity binding site which includes the purine-rich internal asymmetric loop near the base of Stem IIB [10].

Rev is highly prone to polymerization, the full molecular structures of Rev and Rev-RRE complexes are not clear [11]. NMR data revealed that ARM forms a α-helix which sits deeply within an opened major groove that is formed within a double-stranded RNA helix containing a non-Watson-Crick G-G base pair. Both the RBE region and ARM peptide undergo substantial changes upon complex formation [12,13]. In solution, according to the RBE model, the prolonged ''bubble'' at the triple junction of the Stems IIA/IIB/IIC is in equilibrium with purine-rich ''recognition bubble'' locked by two base pairs at the Stem IIB. The asymmetric bubble formation with G-G and G-A was observed in the crystal structure of the unbound RNA [14]. The binding of the ARM shifts the equilibrium from an ''open'' to a more structural conformation and facilitates the forma-

*Address correspondence to this author at the Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, No.44, Wenhuaxi Road, Jinan, 250012, P.R. China; Tel: (+86)-531-88382005; Fax: (+86)-531-88382264; E-mail: xinyongl@sdu.edu.cn

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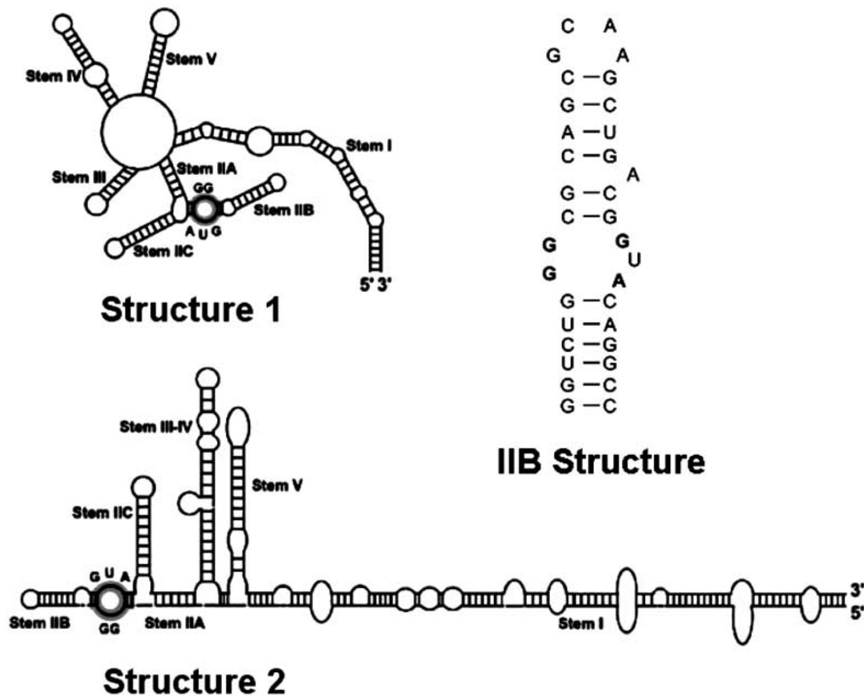


Fig. (2). RRE RNA structure and Stem IIB structure [8].

tion of the non-Watson-Crick base pairs G-G and G-A in the ''recognition bubble''. The formation of G-G and G-A base pairs is accompanied by substantial widening of the RNA major groove [8, 15].

REV FUNCTION

As one of the two transactivating nuclear proteins of HIV-1 responsible for differential regulation in the host cell, Rev regulates post-transcriptional processing of viral mRNA, which functions to ensure successful viral replication [16].

The HIV genes can be separated into two classes: the early genes and the late genes. The completely spliced messages that are exported to cytoplasm by the normal mRNA export pathway are Rev-independent, and are called the early genes. The intron-containing messages are expressed only when Rev is present and, therefore, they are Rev-dependent, and are called as the late genes. The mRNAs are classified as 3 types based on their size and extent of processing: the 9 kb unspliced RNA encoding Gag and Pol (Ⅰ), the 4 kb singly spliced RNA forming Vif, Vpr, Vpu and Env (Ⅱ) and the 2kb completely spliced RNA that code for Tat, Rev and Nef (Ⅲ) [17, 18].

Studies demonstrated that Rev acting as a traffic signal in the nucleus directs the viral mRNA away from splicing to form unspliced and singly spliced viral mRNA, which is essential for the transport of the 9 kb and 4 kb types of HIV-1 RNA to the cytoplasm (Fig. **3**) [19]. In case of the absence of Rev function, these RNA are retained in the nucleus and only the 2kb class RNA is transported into the cytoplasm. Consequently, Rev is a mediator of HIV-1 RNA transport from nucleus to the cytoplasm, circumventing the cellular control mechanisms that prevent export of incompletely processed host mRNA.

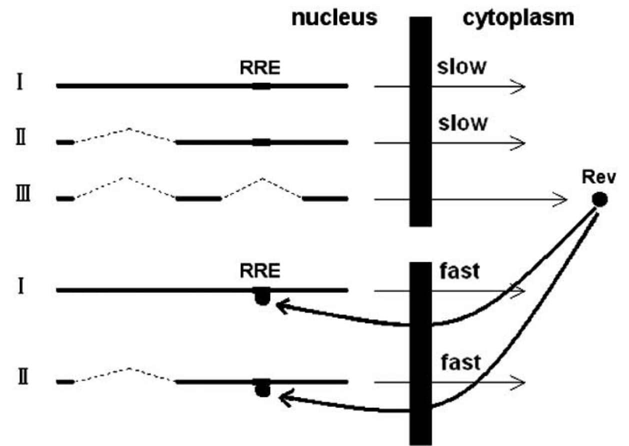


Fig. (3). Mechanism of Rev action.

Being a regulatory protein, Rev decreases the amount of viral spliced messages. It generates a negative feedback loop causing Rev expression levels to be strictly regulated, which is very important for balancing the products of viral genes expression. This regulation of viral gene expression results in increased levels of virion production. It has also been proposed that the need of Rev for the expression of the structural HIV proteins facilitates immune evasion in latently infected cells [20,21].

REV SHUTTLING

The HIV-1 Rev mediates the nuclear export of the intron-containing viral messages. This export is a consequence of the constant shuttling of Rev, and the shuttling is mediated

by NLS and NES contained in Rev, leading to the proteins continuous moving between the nucleus and cytoplasm [2]. The Rev RNA binding and NLS function ensure that Rev cannot be imported when binding to the RRE which could return HIV RNA to the nucleus. The receptor for the NES was identified as chromosomal region maintenance 1 (CRM1) which forms a complex with Ran-GTP and NES to mediate the export of the NES-containing protein from the nucleus [22,23].

A GTPase Ran plays a key role in both the nuclear import and export process of proteins. The GTP-bound form of Ran (Ran-GTP) is primarily found in the nucleus, while the GDP-bound form of Ran (Ran-GDP) predominates in the cytoplasm which is maintained by the localization of the Ran-specific exchange factor in the nucleus. Nuclear import of Rev is achieved through binding of the NLS of the ARM to the classic nuclear transport factor importin ß (IMP ß), while Ran-GDP is required for the NLS/IMP ß complex [24-26]. Nuclear export is mediated by binding of CRM1 to the NES in the Ran-GTP bound form. In the nucleus, the Ran-GTP bound form leads to the dissociation of Ran from the importin complex. The importin proteins are then recycled to the cytoplasm for subsequent action [20]. The association of Rev and RRE in the nucleus promotes the formation of the ternary complex (Rev/CRM1/Ran-GTP), subsequently, the complex interacts with nucleoporins and moves through the nuclear pore, eventually leading to the exit of Rev-associated RNA cargos from the nucleus [21]. Once into the cytoplasm, Ran-GTP is hydrolyzed to Ran-GDP by Ran GAP, subsequently making the ternary complex disassembled [27].

In Rev nuclear export, CRM1 accumulates in the nucleolus and eukaryotic initiation factor 5A (eIF-5A), located at the nucleoplasmic face of the nuclear pore, interacts with nucleoporins to facilitate NES to bind to CRM1. Additional required host factors such as Sam68 (initially identified as a 68 kD src-associated protein in mitosis) and human Rev-interacting protein (hRIP), also appear to promote the RNA nuclear export of Rev [2,22]. The nucleoporins Nup98 and Nup214 are major downstream cofactors required for CRM1-mediated export and Rev function in HIV-1 biology (Fig. **4**) [20].

CELL-BASED BIOCHEMICAL SCREENING APPROACHES FOR REV TARGETED INHIBITORS

Cell-based screening approaches offer some advantages for novel inhibitors because in a single screen they contain multiple targets and in some cases reveal targets and structures not determined by biochemical screening approaches [28]. Cell-based assays for HIV-1 Rev have been designed to monitor Rev-mediated nuclear export of RNA transcripts.

High throughput screening (HTS) assays reported by Tang and Su described that in mammalian cells Rev is coexpressed with a Rev-dependent secreted alkaline phosphatase (SEAP) reporter construct. Inhibition of Rev-RRE complex in these cells resulted in a decreased alkaline phosphatase signal [29]. Arrigo showed another mammalian cell-based assay which relies on the expression of a replication-deficient HIV-1 construct, in which the *nef* open reading frame has been substituted with ß-Gal. As an early gene, *nef* relies on completely spliced mRNA; therefore, the Rev inhibition causes increased reporter signal [30]. Peled-Zehavi's bacterial anti-termination assay [31] was used to screen specifically for peptide sequences binding to Rev-RRE complex. The peptide library that was fused to the N-protein was

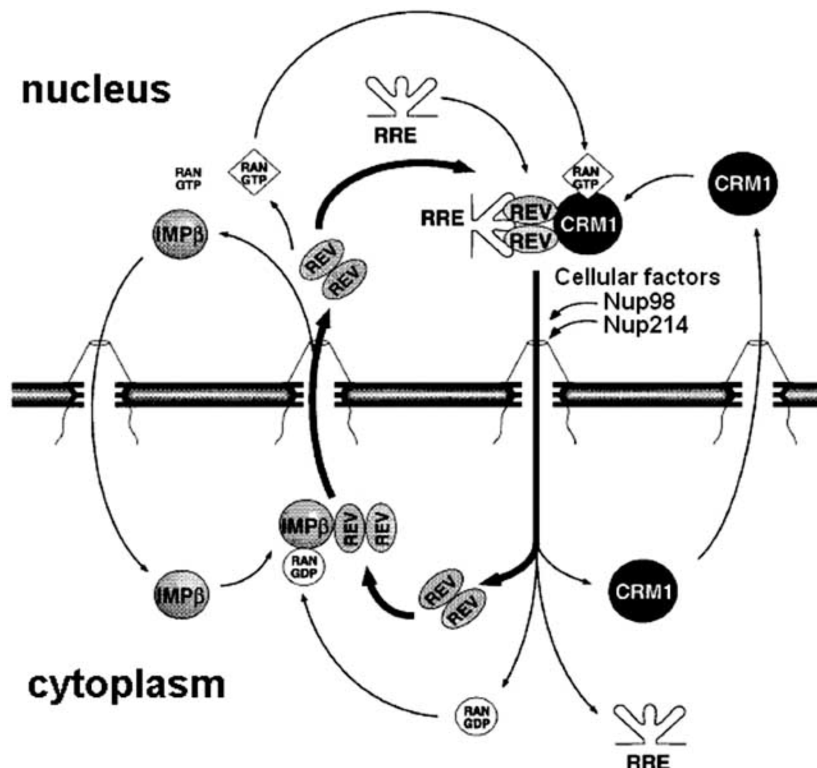


Fig. (4). The cycle of Rev shuttling [20].

coexpressed with a reporter plasmid, in which the RRE is upstream of a transcription termination sequence. Binding of the peptide-N-protein fusion induced antitermination of reporter expression and kanamycin resistance to mark peptides. Over 500 000 compounds have been screened by a high throughput scintillation proximity assay (SPA) for potential Rev inhibitors by Chapman *et al.* [32]. Before the addition of streptavidin coated SPA beads and scintillaton counting, the recombinant Rev was biotinylated and incubated with radiolabeled RRE and the inhibition of Rev-RRE interaction caused a decrease in the signal. Qian-Cutrone described a filter-binding assay, which has been used to screen a natural product library, and also described a new inhibition of HIV Rev-RRE [33].

REV RELATED INHIBITORS

Due to its essential role in the HIV-1 life cycle, the regulatory protein Rev has become an attractive target for the discovery and development of new antiviral agents. Here we summarize some strategies which have been employed to suppress HIV-1 replication through inhibition of one or more stages of Rev function.

A) Inhibitors Targeted at Nuclear Export Factor CRM1

Targeting at Rev nuclear export has yielded great success [34]. One of the first demonstrations consumed the *Streptomyces* cytotoxin, leptomycin B (LMB) (**1**), which was discovered to be a potent antifungal antibiotic blocking the eukaryotic cell cycle [35]. Treatment with actinomycin D results in a shift in Rev subcellular distribution from predominately nuclear/nucleolar to being predominantly cytoplasmic. Treatment with nanomolar concentrations of LMB prevents this redistribution and blocks Rev function. LMB has been confirmed to bind to CRM1, disrupting the formation of the CRM1/NES/Ran-GTP ternary complex, thereby blocking the NES-mediated nuclear export [36]. Subsequent studies revealed that this effect is induced because LMB inactivates CRM1 by covalent modification at Cys-539 [37]. However, due to the variability of the quality of LMB production lots in Streptomyces cultures, the use of LMB in the study of nuclear export pathways has been hampered.

A low molecular weight compound, PKF050-638 (**2**), identified by further chemical screens is capable to block Rev function at position Cys-539 of CRM1 with a similar inhibition mechanism as LMB [38]. In an alternative assay to inhibit Rev-CRM1 interaction, RNA aptamers were also studied. LMB dose-dependently disrupts the Rev-dependent mRNA expression. LMB is active in the nanomolar range with a greatly varying toxicity, resulting from variable quality of the LMB lots. PKF050-638 acts in the micromolar range with a selectivity index of 76, whereas its enantiomer, PKF050-637 (**3**), is 50 times less active against Rev-mediated nuclear export. The data indicated that PKF050-638 and PKF050-637 have the same chemical properties but different conformations, suggesting that PKF050-638 is optimal fitting in the NES-binding pocket of CRM1. The spatial conformation of the ethylester group seems to be crucial for its activity [39].

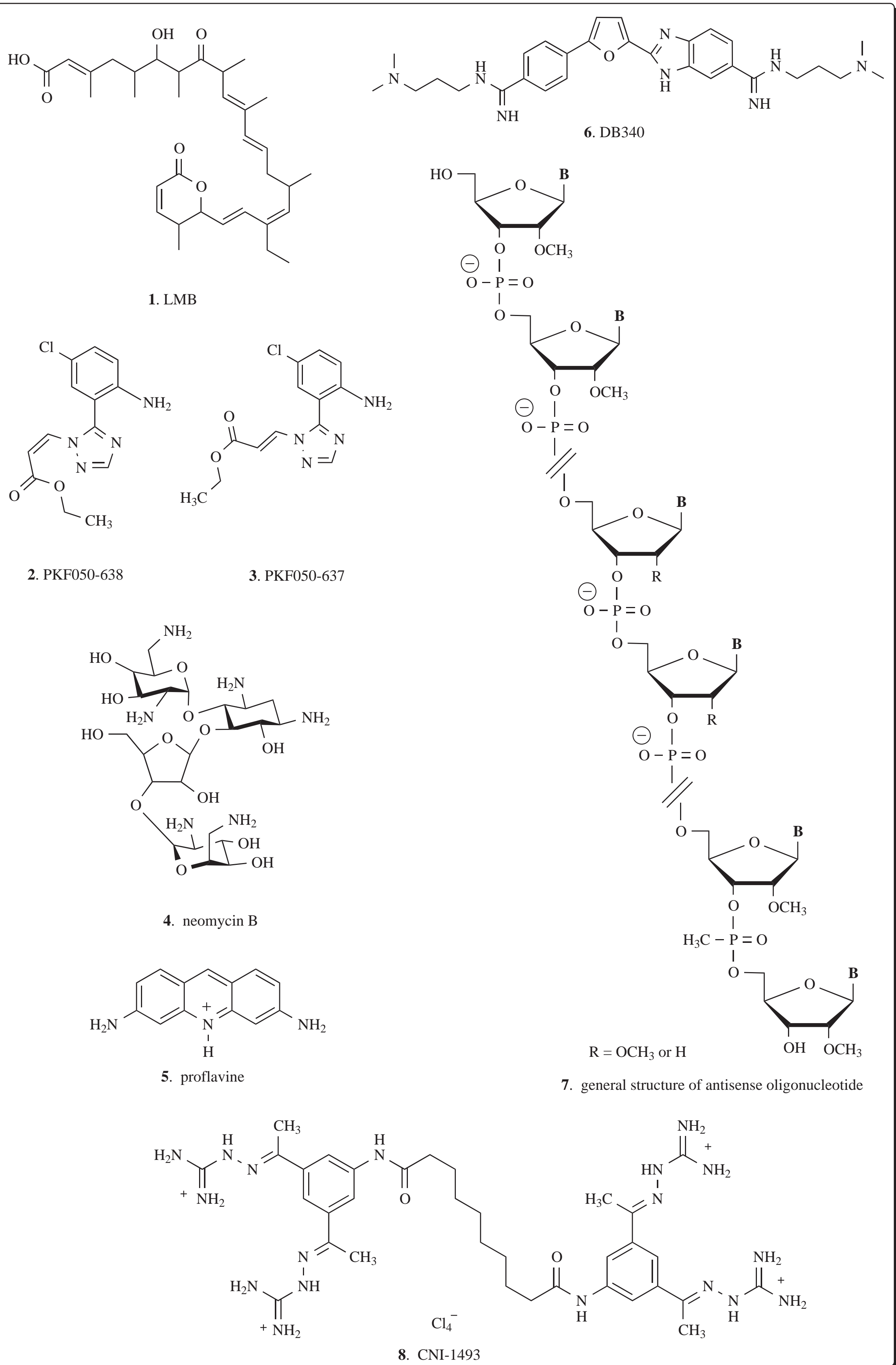
As a chemically synthesized compound, PKF050-638 would not suffer from variability of the quality and its effect is more readily reversible than the effect of LMB. Because its effects on CRM1, a general nucleocytoplasmic export factor of proteins, the compound exhibits cellular toxicity which results in failure to pursue its potential as a therapeutic. Despite its toxicity, this compound is very useful in exploring CRM1-mediated export mechanisms and import rates of proteins by the CRM1-dependent export pathway [39].

B) Inhibitors Interfering Rev-RRE Interaction

A few organic compounds with small molecules have been screened and evaluated for disrupting the Rev-RRE interaction *in vitro* with inhibition constants in micromolar range [32]. A group of aminoglycoside antibiotics effectively competed with Rev for binding to RRE RNA through *in vitro* binding assays [40]. The first reported compound neomycin B (**4**) is capable of interfering with the Rev-RRE interaction, thereby inhibiting HIV-1 replication in a cell culture system. However, 85% inhibition of virus replication requires 2.5 mM of neomycin B, a relatively high dose. Intercalating agents such as pyronin Y and diphenylfuran derivatives were found to enhance the affinity for RRE [41, 42]. The plant metabolite niruside [33] and the fungal metabolites harziphilne and fleephilone which are able to block Rev-RRE interaction at low micromolar concentrations were identified by random screen as inhibitory agents of Rev function [43]. However, these compounds failed to show any activity in virus challenge cell assays. Nevertheless, random screening is continued. The value of compounds such as 1,8-diaminooctane as potential therapeutic agents remains to be further researched [44]. Although the inhibitory effect of neomycin B on the Rev-RRE complex has focused much attention on the discovery of novel antiviral agents, the molecular complexity, toxicity, poor oral absorption and low specificity of neomycin B hamper its further development as a clinically useful antiviral drug [45].

A second group of small compounds which interfere with the Rev-RRE interaction *in vitro* are the diphenylfuran cations [41]. Some of these aromatic cationic compounds tightly bind to RRE at low concentrations of 0.1 μM. A detailed biochemical and structural analysis of a high-affinity RRE binding compound from the diphenylfuran cations, a tetracation heterocycle that contains a phenylfuranbenzimidazole aromatic system, has disclosed that this molecule binds to the minor groove of IIB motif as a dimer with pronounced selectivity [46]. The RRE-IIB contains a purine-rich internal bulge structure which has been identified as a high-affinity Rev binding site. Fluorescence and NMR experiments indicated that proflavine (**5**) was identified as a specific and high-affinity binder of IIB through the screening of a selected set of small molecular weight heterocyclic compounds. Proflavine is also shown competing with the ARM when binding to IIB with a 2:1 (proflavine: IIB) stoichiometry that is two molecules bound in a specific conformation and close to each other at a high-affinity binding site. The proflavine-IIB 2:1 formation results in a stabilization of the base pairing and stacking in the purine-rich internal bulge of IIB [47].

DB340 (**6**) binds to RRE with high affinity and forms the DB340-RRE complex at a 2:1 ratio. NMR spectra, footprinting results and studies with mutant RRE sequences indicated that the purine-rich internal bulge of RRE-IIB is required for


1. LMB
2. PKF050-638
3. PKF050-637
4. neomycin B
5. proflavine
6. DB340
R = OCH3 or H
7. general structure of antisense oligonucleotide
8. CNI-1493

the binding of DB340 at the Rev protein. A tetracationic molecule of DB340 with positive charges symmetrically distributed around the furan center of the molecule will cause dimerization and may be neutralization of the tetracationic charge of the first molecule upon binding to RNA. DB340 binds to RRE at a high-affinity binding site, while the tetracationic charge of the molecule promotes a high degree of nonspecific binding which complicates the molecule's therapeutic development. However, the binding model of compound DB340 provides knowledge about the mechanism of the drug's binding to RNA and facilitates rational drug design [48, 49].

Recently, the interaction of antisense oligonucleotides (**7**) with RRE-IIB was investigated [50,51]. The oligonucleotides were found to target at the 5' or 3' side of the stem IIB. Thermal denaturation experiments indicated that chimeric oligonucleotides form highly stable duplexes with complementary single-stranded RNA, and gel electrophoretic mobility shift assays (EMSA) showed that the oligonucleotides bound with specificity and high-affinity to RRE-IIB with apparent dissociation constants, K (D), in the nanomolar range.

C) Inhibitors Targeting the Rev Protein

There is one strategy deriving from the observation that mutations in the NES of Rev can generate proteins capable of interfering with the wild type protein. In one of these mutants, M10, Leu is changed into Asp at aa position 78 and Glu altered to Leu at position 79 by site-specific mutagenesis. They either compete with the binding to RRE with wild type Rev or are incorporated into Rev multimers to generate an inactive complex. High level expression of the mutant is required to ensure effective competition with the wild type [52, 53]. However, RevM10-resistant HIV-1 variants with RRE and vpu reading frame mutations have been identified [54]. Gene-targeting strategies playing a part in disease outcome are being tested in ongoing and planned clinical development. There are still many barriers to overcome for this particular technology. But gene therapy inhibits progressive HIV-1 infection by interference with viral replication and has the potential to prevent HIV-1 in the patients not responding to traditional antiviral therapy.

In the Rev/MS-C fusion protein, the deletion of aa residues 18-24 of Rev inactivates the potential of both RRE and MS2 targets, although this deletion mutant is still effective in specific RNA binding, protein multimerization, and nuclear localization. This indicates that the N-terminal sequence of RRE RNA is non-specific [55]. Rev mutant lacking residues 18-24 of the Rev/MS-C fusion protein fails to activate RRE and MS2 targets, acting as a novel trans-dominant inhibitor of Rev function.

The use of backbone cyclic peptides (BCPs) which belong to conformationally constrained peptides presents an absolute advantage among the respective linear forms because they may be engineered to mimic the structure of the corresponding active site in the protein. Tat-BCPs, BCPs mimic the ARM of Tat, have demonstrated the inhibition of Tat function *in vitro* [56]. A series of BCP analogs bearing a conformationally constrained ARM of Rev simplified as Rev-BCPs were tested for inhibition of HIV-1 replication in chronically infected T lymphocytic cells, a potent depression of HIV-1 replication was observed. Rev-BCPs slightly interfere with the Rev-dependent nuclear import and are very active in the disruption of the mechanism controlling Pr55*gag* and gp160*env* expression. The use of Rev-BCPs offers a promising strategy for the development of novel peptide-based agents in HIV-1 therapeutics [57].

D) Inhibitors Targeted at Other Cellular Factors

Semapimod hydrochloride, guanylhydrazone CNI-1493 (**8**) is an efficient inhibitor of deoxyhypusine synthase (DHS) which is essential for the synthesis of eIF-5A. CNI-1493 suppresses activation of eIF-5A, thereby, inhibits replication of HIV-1 and does not have measurable drug-induced adverse effects, apoptosis, and cytotoxicity [58].

A C-terminal deletion mutant of Sam68, Sam68ΔC is able to inhibit HIV-1 replication by prohibiting translation of viral RNA after the nuclear export [59].

The hRIP mutant, RIPΔN360, with the first 360 residues deleted from N-terminal inhibits the Rev function by mislocalizing RRE-containing RNA in the perinuclear space [60].

CONCLUSIONS

As an essential HIV-1 regulatory protein that binds to RRE RNA, Rev is involved in transport of unspliced and singly spliced viral transcripts from the cell nucleus to the cytoplasm. Any intervention with the various stages of Rev-mediated RNA transport can arrest HIV-1 transcriptional process. The toxicity and drug resistance of traditional HIV-1 reverse transcriptase and protease inhibitors result in their failure in clinical trials. In this viewpoint, Rev-RRE interaction which targets to transcription machinery seems to be a good candidate as a target for inhibition of HIV-1 replication and Rev inhibitors may become a new therapeutic modality when added onto current anti-HIV chemotherapy.

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ABBREVIATIONS

HAART	=	Highly active anti-retroviral therapy
HIV-1	=	Human immunodeficiency virus type 1
ARM	=	Arginine-rich motif
NLS	=	Nuclear localization signal
RBE	=	Rev-binding element
RRE	=	Rev response element
NES	=	Nuclear export signal
CRM1	=	Chromosomal region maintenance 1
eIF-5A	=	Eukaryotic initiation factor 5A
hRIP	=	Human Rev-interacting protein
IMP ß	=	Importin ß
HTS	=	High throughput screening

SEAP	=	Secreted alkaline phosphatase
SPA	=	Scintillation proximity assay
LMB	=	Leptomycin B
EMSA	=	Electrophoretic mobility shift assays
BCPs	=	Backbone cyclic peptides
DHS	=	Deoxyhypusine synthase

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