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Short Communication

Identification of low-molecular weight inhibitors of HIV-1 reverse transcriptase using a cell-based high-throughput screening system

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ABSTRACT

A cell-based drug screening system that utilizes a green fluorescent protein (GFP)-tagged recombinant lentiviral vector has been used to screen a chemical library of 34,000 small molecules for antiretroviral compounds. Thirty-three initial hits were analyzed and four compounds were selected based on their anti-human immunodeficiency virus type 1 (HIV-1) activity (EC_{50} values ranging from 0.17 to 1.9 μM) and low cellular toxicity (CC_{50} values >50 μM). The four compounds blocked reverse transcription and were able to inhibit the replication of a panel of different HIV-1 strains, including non-B subtype and viruses resistant to different drug classes. Serial in vitro passages of HIV-$1_{B\text{-}HXB2}$ in the presence of increasing drug concentrations selected for viruses with reduced susceptibility. Mutations previously associated with resistance to non-nucleoside reverse transcriptase (RT) inhibitors (L100I and Y181C for CBL-17 and CBL-21, respectively) or linked to nucleoside analogue resistance (A62V for CBL-4.0 and CBL-4.1) were identified. Viruses with reduced susceptibility to CBL-17 and CBL-21 but not the ones resistant to CBL-4.0 or CBL-4.1 showed a decrease in replicative fitness. Interestingly, two of the small molecules (CBL-4.0 and CBL-4.1) are indolopyridinones that were previously described as nucleotide-competing RT inhibitors.

1. Introduction

The ultimate goal of the present antiretroviral therapy is to suppress HIV-1 replication as much and as long as possible, however, treatment with combinations of antiretroviral drugs does not completely inhibit HIV-1 replication, eventually leading to treatment failure. Therefore, there is a substantial need for the identification and availability of not only new drugs against known and novel targets but also innovative antiretroviral strategies.

Historically, multiple different approaches have been used to identify and develop novel antiretroviral drugs, however, experimental screening of chemical libraries (or high throughput screening, HTS) to identify "primary hits" with potential antiretroviral activity continues to be a common approach (Westby et al., 2005). Among them, cell-based antiviral screening methods have been successfully used to identify novel drugs against HIV-1 (Blanco et al., 2005; Micheva-Viteva et al., 2005). This methodology is able to identify compounds capable to interfere with any event in the viral life cycle, which makes it crucial for the discovery of novel antiretroviral targets. Therefore, in this study we performed a cell-based HTS aimed to the identification of novel compounds inhibiting virus-specific or cellular (vital for virus replication) targets. Thirty-three initial hits were identified and four molecules with anti-HIV-1 activity in the sub-micromolar range were characterized. Virological and biochemical data, including in vitro selection of resistant viruses, suggested that all four molecules target HIV-1 reverse transcription.

A set of 34,000 small molecules, from ChemBridge's DIVERSet™ chemical library (ChemBridge Corporation, San Diego, CA) was screened for their ability to inhibit the replication of a recombinant

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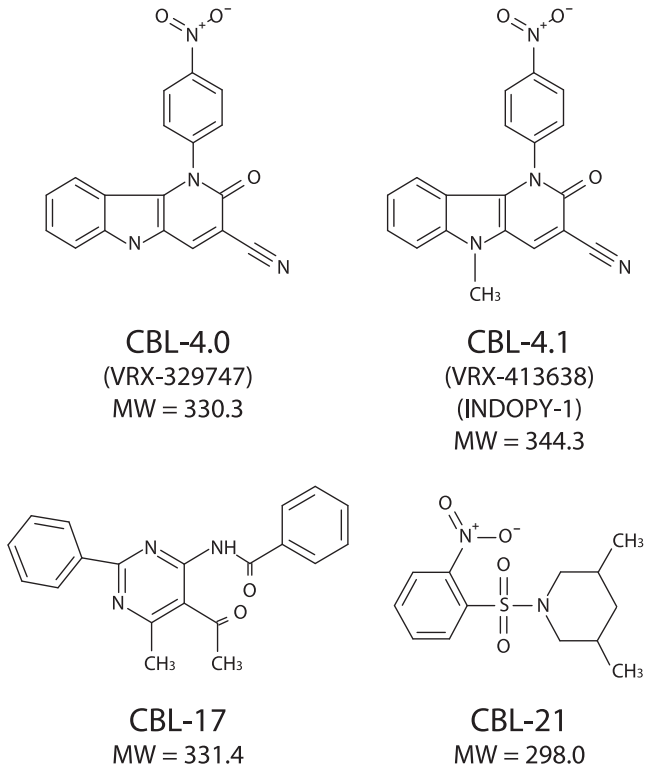
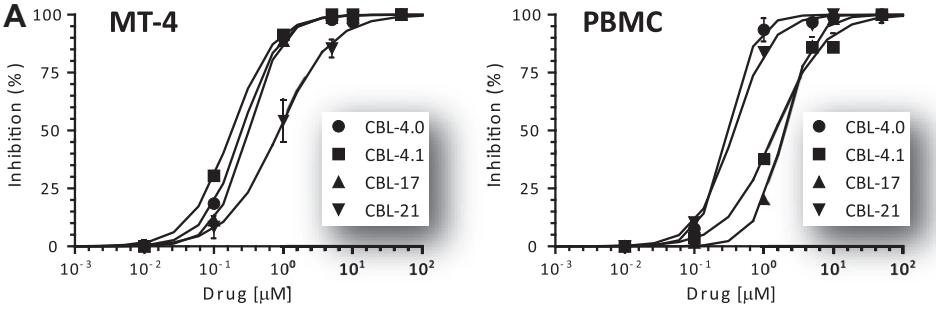


Fig. 1. Structure of the four CBL compounds characterized in this study. MW, molecular weight.

HIV-derived vector, pseudotyped with the G protein of the vesicular stomatitis virus (VSV) and expressing the green fluorescent protein (GFP) Naldini et al., 1996 (Supplementary Fig. 1). This assay allowed the screening of molecules potentially targeting all the steps in HIV-1 replication cycle with the exception of those related to virus entry and post-integration steps. The subset of ChemBridge's DIVERSet™ chemical library used in this study consisted of compounds with molecular weights in the range of 250 to 550 g/mol, with known structure and purity (>95%) validated by nuclear magnetic resonance (NMR). This small molecule library has previously been used for the identification of potential therapeutic agents againts the tumor suppressor protein 53 (p53) Gurova et al., 2005, interferon-induced ribonuclease RNase L (Thakur et al., 2007), human parainfluenza virus type 3 (Mao et al., 2008), and influenza virus (Vazquez and Quiñones-Mateu, submitted for publication).

The compounds were initially screened at concentrations ranging from 10 to 20 μM and only 33 (0.04%) molecules showing 100% inhibition of virus replication with non-detectable cellular toxicity were selected for further analyses. Cellular toxicity (CC_{50}) and antiretroviral activity (EC_{50}) values with HIV-$1_{B\text{-}HXB2}$ in MT-4 cells were determined for the chosen thirty-three initial hits and their selectivity index (SI) values calculated (data not shown). Based on their low cytotoxicity at the highest concentration tested (50 μM) and high SI (>50, which is considered as highly active) three compounds were selected for further characterization: CBL-4.0, CBL-17, and CBL-21 (Fig. 1). In addition, two compounds analogous to CBL-4.0 were obtained from a different subset of the ChemBridge's chemical library to elucidate the minimal pharmacophore needed for the antiretroviral activity of this molecule. Interestingly, while CBL-4.1 showed similar antiviral and cytotoxic characteristics than the parental CBL-4.0, not even 50 μM of the other molecule (CBL-4.2, Supplementary Fig. 2) was able to inhibit HIV-1 replication (data not shown).

The anti-HIV-1 activity and cytotoxicity properties of the four compounds were tested against a laboratory adapted strain (HIV-$1_{B\text{-}HXB2}$) in MT-4 cells and a primary isolate (HIV-$1_{B\text{-}92US026}$) in PBMC. All four molecules inhibited HIV-$1_{B\text{-}HXB2}$ with similar potency, however CBL-4.1 and CBL-17 were approximately 8-fold less potent in PBMC (Fig. 2). The four compounds showed a favorable toxicity profile, measured as cell viability by the trypan blue exclusion assay (Fig. 2B) with similar data obtained measuring cell



B

	MT-4 EC50	MT-4 CC50	MT-4 SI	PBMC EC50	PBMC CC50	PBMC SI
● CBL-4.0	0.25	50	200	0.29	80	276
■ CBL-4.1	0.17	>50	>300	1.42	>50	>35
▲ CBL-17	0.24	>50	>210	1.90	>50	>26
▼ CBL-21	0.84	>50	>60	0.37	>50	>135

Fig. 2. Activity of the CBL compounds against a laboratory strain (HIV-$1_{B\text{-}HXB2}$) and a primary isolate (HIV-$1_{B\text{-}92US026}$) in MT-4 cells and peripheral blood mononuclear cells (PBMC), respectively. (A) Drug susceptibility curves. Error bars indicate the ranges of values obtained from three independent experiments. (B) Antiviral activity (EC_{50}) and cellular toxicity (CC_{50}) was used to calculate the selectivity index (SI) for each drug in each condition. Values represent the mean of three independent experiments.

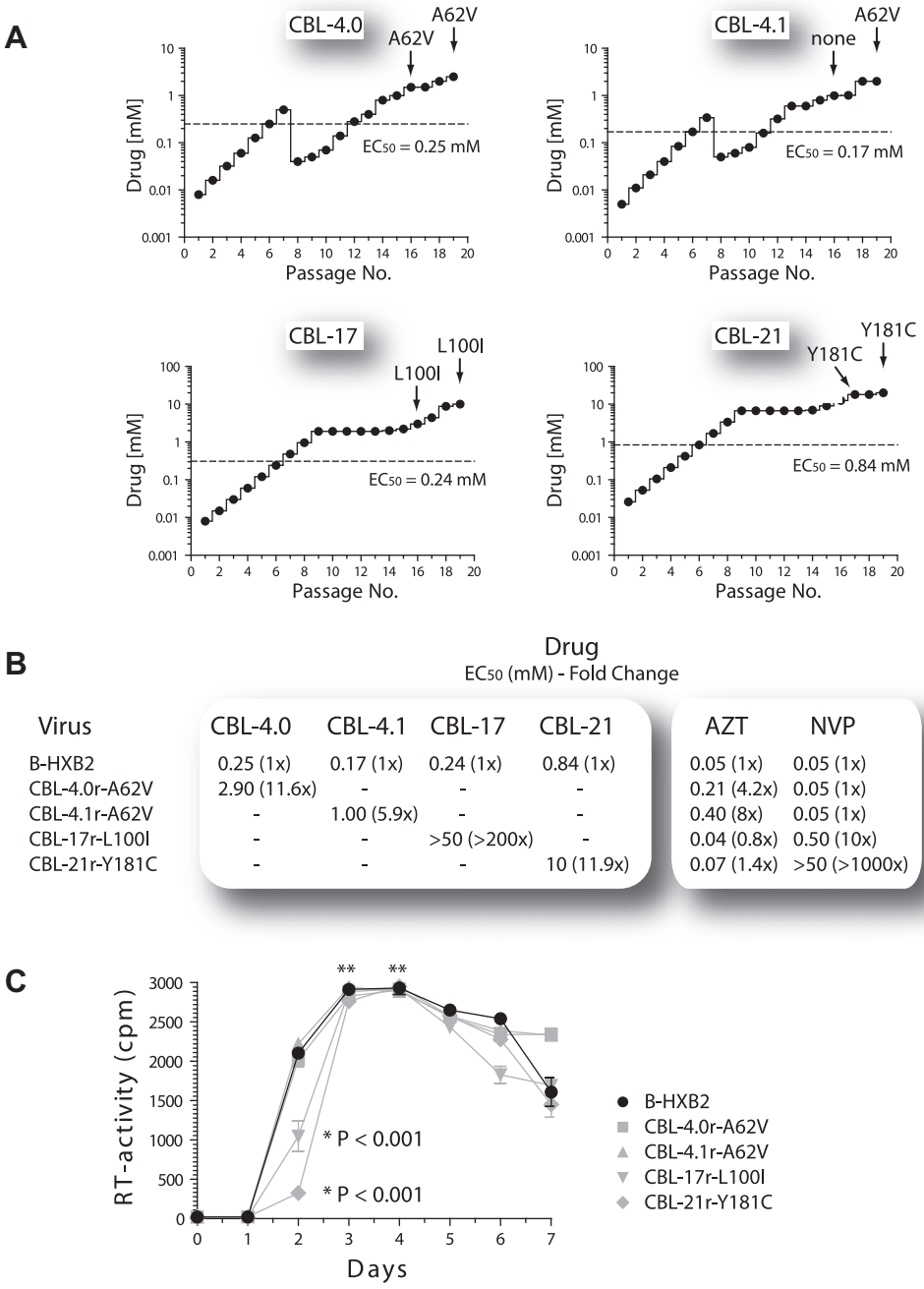


Fig. 3. In vitro selection and characterization of viruses with reduced susceptibility to CBL-4.0, CBL-4.1, CBL-17, and CBL-21. (A) HIV-$1_{B\text{-}HXB2}$ was propagated in C8166-45 cells in the presence of increasing concentrations of the respective drug, starting with sub-inhibitory concentrations (i.e., 1/30th of the calculated EC_{50} value as indicated). Each passage corresponds to seven days. Amino acid substitutions (genotype) compared to the original virus are indicated for passages 16 and 19. (B) EC_{50} values of the resistant viruses and fold changes (between parentheses) compared to the wild-type HIV-$1_{B\text{-}HXB2}$. AZT and NVP were used as controls. Values represent the mean of three independent experiments. (C) Viral growth kinetics of the four CBL resistant viruses compared to HIV-$1_{B\text{-}HXB2}$. Cultures were monitored daily using an in-house RT-assay. Error bars indicate the ranges of values obtained from three independent experiments. The replication kinetics of viruses indicated by a single asterisk (*) were significantly different than the reference virus (HIV-$1_{B\text{-}HXB2}$) at day two post-infection ($p < 0.001$, 95% CI, t test). No significant difference in replication kinetics was observed at days 3 and 4 post-infection (**). cpm, counts per minute.

proliferation by the MTT assay (Pauwels et al., 1988) (data not shown). More important, selectivity indices for all four compounds were above 50 (Fig. 2B), which warranted further characterization of these molecules.

At this point in the study it was evident that the chemical structures of two of the small molecules, i.e., CBL-4.0 and CBL-4.1, were similar to that described for the prototype (INDOPY-1) of a novel series of RT inhibitors called indolopyridones (Jochmans et al., 2006a). In fact, the structures of both molecules were identical to two compounds (i.e., VRX-329747 and VRX-413638) originally described by Zhang et al. (2006) as novel NNRTIs that selected for NRTI resistance mutations. These nucleotide-competing reverse transcriptase inhibitors (NcRTI) are able to block HIV-1 replication by binding to the RT active site in competition with the next incoming nucleotide (Ehteshami et al., 2008; Jochmans et al., 2006a). As a consequence, viruses carrying mutations associated with NNRTI or multidrug NRTI resistance remain susceptible to indolopyridones, while the M184V and Y115F substitutions are

associated with decreased susceptibility to this drug (Ehteshami et al., 2008; Jochmans et al., 2006a; Zhang et al., 2006). On the other hand, and to our knowledge, the anti-HIV activity of molecules with the chemical structure of compounds CBL-17 and CBL-21 have not been described (http://chemdb2.niaid.nih.gov). Thus, we decided to continue with the characterization of not only CBL-17 and CBL-21 but with the indolopyridones CBL-4.0- and CBL-4.1 with the goal to contribute to the study of this novel class of antiretroviral drugs.

A time of drug addition assay showed that the four CBL compounds, similar to NRTIs and NNRTIs and unlike entry inhibitors, integrase inhibitors, and protease inhibitors, potently inhibited HIV-1 replication when added within 4 h post-infection (Supplementary Fig. 3). This first evidence, suggesting that the CBL molecules block HIV-1 reverse transcription, was confirmed using cell-free assays based on homopolymeric poly(rA)•poly(dT) template/primer extension by wild-type reverse transcriptase supplied from virions or purified enzyme (Quinones-Mateu et al., 1997) (Supplementary Fig. 4). Additional experiments confirmed the sole mechanism of action of the CBL compounds, discarding the possibility that they could also inhibit HIV-1 replication by blocking viral entry, integration or interfere with the activity of the viral protease (data not shown).

The antiretroviral specificity of the CBL compounds was tested against a panel of primary HIV-1 strains from different subtypes, group O and HIV-2. CBL-4.0, CBL-4.1, and CBL-21 retained their activity against all HIV-1 strains but showed reduced susceptibility against HIV-2_{CBL-20} (Supplementary Table 1). Interestingly, INDOPY-1 was shown to be active against a different strain of HIV-2 (HIV-2_{ROD}, EC_{50} 0.18 μM) (Jochmans et al., 2006a). The antiviral profile of the CBL compounds was further evaluated using a panel of viruses resistant to PI, NRTI, NNRTI and/or enfuvirtide (Supplementary Table 2). Viruses carrying NNRTI-associated mutations such as K103N or Y181C showed reduced susceptibility to CBL-17 and CBL-21. On the other hand, both compounds were able to inhibit the replication of viruses with multiple mutations associate with resistance to NRTI, suggesting that the mechanism of action of CBL-17 and CBL-21 resembles that of other NNRTI drugs (de Bethune, 2010), although with a possible difference in the binding and overall anti-HIV potency of these molecules. In the case of CBL-4.0 and CBL-4.1, both compounds were equally active against most of the drug resistant HIV-1 strains as previously described (Jochmans et al., 2006a; Zhang et al., 2006).

Definitive evidence of specific anti-HIV activity is usually achieved upon in vitro selection of viruses with reduced susceptibility to a given compound (De Meyer et al., 2005). Here we used C8166-45 cells to propagate HIV-1_{B-HXB2} in the presence of increasing concentrations of the CBL compounds until the drug concentration was at least 10-fold of the original EC_{50} value (Fig. 3A). Amino acid substitutions L100I and Y181C emerged in viruses grown under the selective pressure of CBL-17 or CBL-21, respectively, mutations that are associated with resistance to NNRTIs (Johnson et al., 2010). In the case of CBL-4.0 and CBL-4.1, the only mutation detected at passage 19 (i.e., 10- to 12-fold the EC_{50} values for each drug) was A62V. This amino acid substitution has been linked to multi-NRTI resistance as part of two different groups of mutations, i.e., the 69 insertion and the 151 complexes (Johnson et al., 2010; Menendez-Arias, 2008), however, it is not considered a primary NRTI mutation by itself (Shirasaka et al., 1995). Interestingly, in vitro selection of viruses resistant to a given antiretroviral compound seems to depend on the genetic background of the virus and the target cells since previous studies with indolopyridones selected for viruses with different or additional mutations, i.e., Y115F + M184V mutations in a HIV-1_{B-IIIB} background (Jochmans et al., 2006b) and M41L + A62V + S68N + G112S + V118I + M184V in a HIV-1_{NL4-3} backbone (Zhang et al., 2006). Thus, it is possible that a longer period of in vitro passages with CBL-4.0 or CBL-4.1 could have selected for viruses carrying additional mutations that may (i) confer additional resistance to these compounds and/or (ii) compensate for any potential impairment in replicative fitness (Quinones-Mateu and Arts, 2006). Finally, and as expected, all four viruses showed reduced susceptibility to the respective CBL compound (Fig. 3B) but only the viruses resistant to CBL-17 and CBL-21 exhibited a decrease in replicative fitness (Fig. 3C). The A62V substitution alone does not seem to have a major effect on the replicative fitness of the virus (Maeda et al., 1998; Quinones-Mateu and Arts, 2001).

In summary, here we used a cell-based drug screening system to interrogate a chemical library of 34,000 small molecules and identified four compounds with anti-HIV-1 activity in the low micromolar range. Two compounds (CBL-17 and CBL-21) were characterized as NNRTIs while two molecules (CBL-4.0 and CBL-4.1) have been described as indolopyridones, members of a new class of RT inhibitors (Jochmans et al., 2006a; Zhang et al., 2006). Additional studies will be aimed to (i) evaluate the potential antiretroviral utility of molecules related to CBL-17 and CBL-21 and (ii) use our cell-based drug screening system with additional small-molecule libraries.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.05.004.

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