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Communication

Structure-based linker optimization of 6-(2-cyclohexyl-1-alkyl)-2-(2-oxo-2-phenylethylsulfanyl)pyrimidin-4(3*H*)-ones as potent non-nucleoside HIV-1 reverse transcriptase inhibitors

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ABSTRACT

In continuation of our efforts toward the discovery of potent HIV-1 NNRTIs with diverse structures, a series of novel *S*-DACO analogues of 6-(2-cyclohexyl-1-alkyl)-2-(2-oxo-2-phenyl-ethylsulfanyl)pyrimidin-4(3*H*)-ones were designed, synthesized and evaluated for their antiviral activities in MT-4 cells. Most of these new compounds showed moderate to good activities against wild type HIV-1 with IC_{50} values ranging from 7.55 μmol/L to 0.018 μmol/L. Among them, compound **5c** was identified as the most promising inhibitor against HIV-1 replication with an IC_{50} = 0.018 μmol/L, CC_{50} = 194 μmol/L, and SI = 12791, which was much more potent than the reference drugs NVP and DLV and comparable to AZT and EFV. In addition, **5c** also exhibited improved activity against double mutant HIV-1 strain RES056 compared to that of the reference drugs NVP/DLV and **DB02**. The preliminary structure-activity relationship (SAR) and molecular modeling studies were also discussed, which provides some useful indications for guiding the further rational design of new *S*-DACO analogues.

HIV-1 reverse transcriptase (HIV-1 RT), a key enzyme of the human immunodeficiency virus (HIV) catalyzing the RNA-dependent and DNA-dependent synthesis of double-strand vial DNA, is still a major target for developing new anti-HIV/AIDS drugs [1–3]. Based on the inhibitory mechanism, there are two types of HIV-1 RT inhibitors: (1) nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs) and (2) non-nucleoside reverse transcriptase inhibitors (NNRTIs) [4]. NNRTIs as an indispensable component of highly active antiretroviral therapy (HAART) are widely used in the clinical treatment of HIV-1-infected patients due to their unique antiviral potency and high selectivity [5,6]. Currently, six NNRTIs have been approved for clinical use: nevirapine (NVP), delavirdine (DLV), efavirenz (EFV), etravirine (ETR), rilpivirine (RPV) and doravirine (DOR) [7,8]. Nevertheless, therapeutic effectiveness of these available drugs has been limited to a certain extent by the emergence of drug-resistant viruses and potentially severe side effects in the long-term clinical use. As a consequence, discovery of novel NNRTI candidates, especially with better resistance profiles and improved safety and tolerability, is a continuous pursuit of drug development [9–11].

Up to now, more than 50 structurally diverse classes of compounds have been identified as NNRTIs with dihydro-alkyloxy-benzyl-oxopyrimidines (DABOs, Fig. 1) being one of them. Since DABOs were firstly disclosed in 1992 [12], a number of more potent and selective derivatives have been designed and synthesized [13,14], especially the *S*-DABOs with subnanomolar activity against both the HIV-1 wild type (WT) and clinically relevant HIV-1 mutants, where the *C*-2 alkyloxy is replaced by an alkylthio moiety [15–18]. Studies on *S*-DABOs suggested that an alkylthio (cycloalkylthio) substituent at the *C*-2 position and an aromatic ring linked through a methylene bridge to the *C*-6

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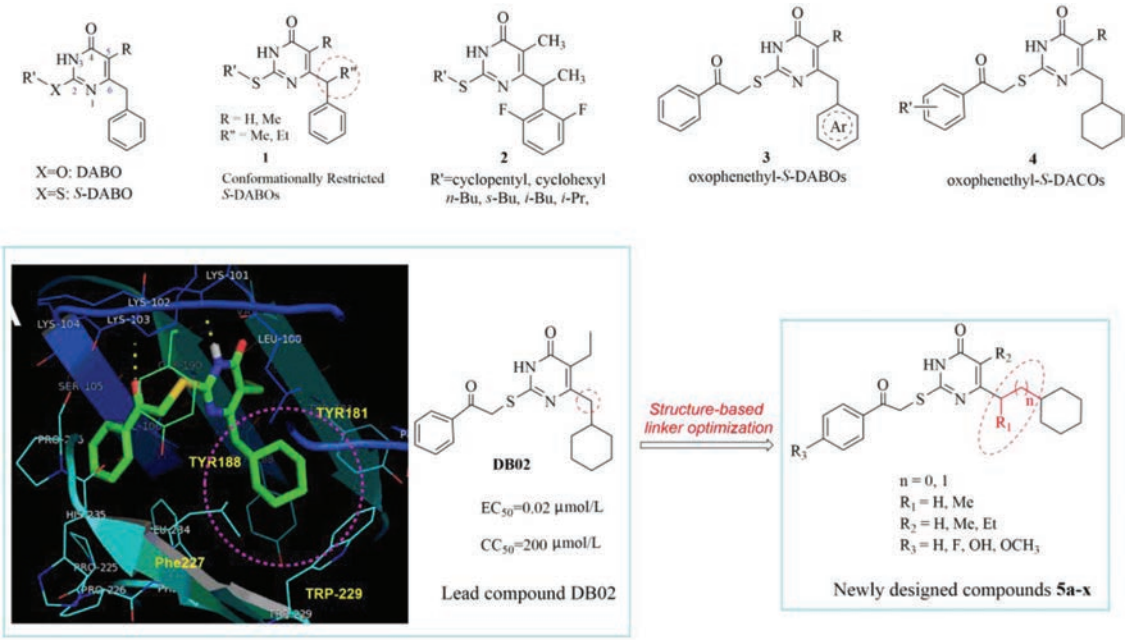


Fig. 1. Structures of DABOs and newly designed compounds **5a-x**.

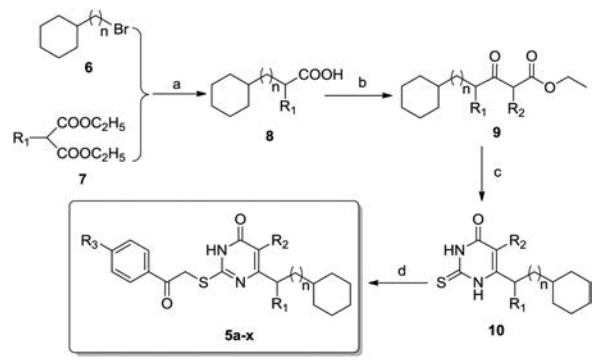
position, as well as the unmodified NHCO fragment representing the *N*-3 and *C*-4 positions of the pyrimidine ring, are structural determinants for the antiviral activity of these compounds [19].

Moreover, a series of conformationally restricted *S*-DABOs (**1**, Fig. 1) featuring a methyl or ethyl at the benzylic carbon of *C*-6 position was reported by A. Mai *et al*. [20,21]. Among these compounds, the 2,6-difluorobenzyl-α-methylthymidine derivatives (**2**, Fig. 1) were identified as the most active compounds to exert inhibitory activity against RT in the nanomolar range. The SAR analysis indicated that significant improvement of potency associated with two methyl groups, one at the benzylic carbon and the other at the pyrimidine 5-position, was related to an intramolecular steric effect.

In arduous efforts to discover more potent *S*-DABOs, a novel series of oxophenethyl-*S*-DABOs (**3**, Fig. 1) compounds was reported by He *et al*. as unique NNRTIs [22–25]. To follow up on this work, a further series of oxophenethyl-*S*-DACOs (**4**, Fig. 1) was designed and synthesized subsequently by our group, the most significant characteristic of which being the replacement of the *C*-6 arylring with a *C*-6 cyclohexylmethyl moiety [26,27]. In comparison with a planar aromatic ring, this replacement would result in better conformational flexibility to the mutated drug-binding site, where a better binding efficiency could be achieved from optimized van der Waals contacts. It should highlight that compound **DB02** (Fig. 1) possessed outstanding anti-HIV-1 activity in cell culture and displayed an improved activity against K103 N or Y181C compared to most *S*-DABOs [28].

The molecular modeling indicated that the cyclohexyl group of compound **DB02** is positioned in the top hydrophobic pocket formed by the residues of Tyr181, Tyr188, Phe227, and Trp229, and forms hydrophobic contact with these hydrophobic residues [28]. Furthermore, it seems that there still is extra space to accommodate a larger group to make interactions with surrounding residues (Fig. 1). Based on these findings, together with the above conformationally restricted strategy, we have recently investigated novel *S*-DACOs of general formula **5a-x** designed to further obtain the chemically diverse space and preliminary SAR information of these oxophenethyl-*S*-DACOs. Most of these investigations mainly focused on the *C*-6 position where a cyclohexyl moiety was connected to the thiopyrimidinone scaffold *via* spacers of different alkyl lengths. We postulated that the newly constructed *S*-DACOs, retaining *C*-2 preferential active groups and introducing novel *C*-6 side chain linker, might permit the terminal cyclohexyl to extend farther into the depth of the hydrophobic sub-pocket and make tighter interactions with surrounding residues, especially the conserved residue Trp229. Herein we describe the synthesis, anti-HIV activity evaluation *in vitro*, and preliminary SAR studies of these new compounds.

The general synthetic route utilized to obtain desired compounds **5a-x** is outlined in Scheme 1. Detailed procedures and compound characterizations can be found in supporting information. The commercially available bromides **6** were converted to cyclohexyl substituted acids **8** by the reaction with proper malonic esters **7** in the presence of sodium ethoxide followed by alkaline hydrolysis and decarboxylation. The latter key intermediate β-ketoesters **9** were synthesized by exposure of cyclohexyl substituted acids **8** to *N,N′*-carbonyl-diimidazole (CDI) followed by treatment with different ethyl potassium malonates in the magnesium chloride/triethylamine system [29]. Condensation of



Scheme 1. Synthesis of compounds **5a–x**. Reagents and conditions: (a) (i) Na, EtOH, reflux, 2-4 h; (ii) NaOH, EtOH, H_2O, reflux, 2 h; (iii) heat at 160-170 °C, 4 h. (b) (i) CDI, CH_3CN, r.t., 30 min; (ii) $R_2CH(CO_2Et)(COOK)$, $MgCl_2$, Et_3N, CH_3CN, r.t., overnight, reflux, 2 h; (iii) 13% HCl, r.t. (c) thiourea, EtONa, reflux, 6-8 h. (d) $R_3PhCOCH_2Br$, K_2CO_3, DMF, r.t., 8-24 h.

β-ketoesters **9** with thiourea in alkaline medium afforded the substituted thiouracils **10**, which were subsequently treated in anhydrous *N,N*-dimethylformamide (DMF) with the appropriate halide ($R_3PhCOCH_2Br$) in the presence of potassium carbonate to yield the corresponding target compounds **5a–x**. Both analytical and spectral data of all the newly synthesized compounds are in full agreement with the proposed structures.

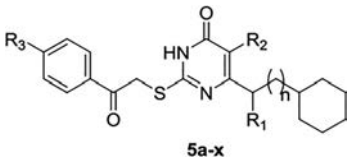
According the MTT method [30,31], the newly synthesized oxophenethyl-*S*-DACO derivatives (compounds **5a–x**) were evaluated for their biological activity in MT-4 cells infected with WT HIV-1_{IIIB} strain, HIV-1 mutant strain RES056 (K103N + Y181C double RT mutant) and HIV-2 ROD strain in comparison with nevirapine (NVP), dideoxycytidine (DDC), efavirenz (EFV) and azidothymidine (AZT) used as reference drugs. The results, expressed as inhibitory concentration (IC_{50}), cytotoxic concentration (CC_{50}) and selective index (SI, given by the CC_{50}/IC_{50} ratio), are depicted in Table 1. In addition, **DB02** was also included in our assays for comparison.

As shown in Table 1, the majority of tested compounds, with the exception of five compounds **5h**, **5i, 5n**, **5p** and **5q**, exhibited moderate to good activities against HIV-1_{IIIB} with IC_{50} values in the range of 7.55–0.018 μmol/L and SI values in the variable range of 22–12791. Compound **5c** was found to be the most active and selectivity inhibitor against HIV-1_{IIIB} replication with an IC_{50} = 0.018 μmol/L, CC_{50} = 194 μmol/L, and SI = 12791. Taking into account the results of the cytotoxicity assessment, **5c** was much more potent than the reference drug NVP (IC_{50} = 0.06 μmol/L, SI > 63), DLV (IC_{50} = 0.021 μmol/L, SI > 942) and EFV (IC_{50} = 0.001 μmol/L, SI > 1982), and comparable to AZT (IC_{50} = 0.002 μmol/L, SI = 13293) and **DB02** (IC_{50} = 0.02 μmol/L, SI = 14050). In addition, some other compounds **5e, 5 j, 5k** and **5l**, also displayed higher anti-HIV-1 activities (IC_{50} = 0.08, 0.10, 0.14 and 0.05 μmol/L, respectively) and better selectivity indices (SI = 3354, 2334, 1168 and 2843, respectively) than those of NVP, DDC and DLV.

In view of the hydrophobic nature of NNRTI binding site, the common characteristic of NNRTIs is poor water solubility, which often leads to low bioavailability and difficulties in formulation. As can be seen from Table 1, the cLog*P* values of most compounds range from 4.40 to 5.90, indicating that water solubility of these compounds is still not ideal enough. Experimentally, the water solubility of **5c**, **5e** and **DB02** at pH 6.0 was 216 ng/mL, 213 ng/mL and 407 ng/mL respectively, which was higher than RPV (20 ng/mL) [32].

These analogues were also assayed against the frequently encountered HIV-1 double mutant strain K103 N/Y181C (RES056).

Table 1
Anti-HIV activities, cytotoxicities and selectivity indices of newly designed oxophenethyl-*S*-DACO derivatives (**5a-x**) and reference drugs.



5a-x

Compd.	R_1	R_2	R_3	n	IC_{50} (μmol/L)[a]		CC_{50} (μmol/L)[b]	SI[c]	cLog*P*[d]
					HIV-1 III_B	HIV-2			
5a	CH_3	H	H	0	2.17 ± 0.15	266	267 ± 20.05	123	4.40
5b	CH_3	H	F	0	7.55 ± 0.21	165	165 ± 9.72	22	4.61
5c	CH_3	Et	H	0	0.018 ± 0.001	194	194 ± 13.64	12791	5.37
5d	CH_3	Et	OCH_3	0	0.03 ± 0.004	30	30 ± 0.58	925	5.59
5e	CH_3	CH_3	H	0	0.08 ± 0.004	259	259 ± 4.93	3354	4.84
5f	CH_3	CH_3	OH	0	0.05 ± 0.03	13	13 ± 0.58	260	4.71
5g	CH_3	CH_3	OCH_3	0	0.06 ± 0.02	28	28 ± 0.40	439	5.06
5 h	H	H	F	1	79.9 ± 3.20	80	80 ± 6.64	1	4.74
5i	H	H	OCH_3	1	35.7 ± 5.40	156	156 ± 6.93	4	4.74
5 j	H	Et	H	1	0.10 ± 0.01	241	241 ± 12.45	2334	5.50
5k	H	Et	F	1	0.14 ± 0.02	164	164 ± 7.99	1168	5.72
5 L	H	Et	OH	1	0.05 ± 0.00	132	132 ± 8.05	2843	5.37
5 m	H	CH_3	H	1	1.72 ± 0.06	124	124 ± 20.78	72	4.97
5n	H	CH_3	F	1	7.32 ± 0.08	7.3	7.3 ± 0.30	1	5.19
5o	H	CH_3	OH	1	0.24 ± 0.01	8.2	8.2 ± 0.67	34	4.84
5p	CH_3	H	H	1	9.58 ± 0.18	151	151 ± 7.97	16	4.92
5q	CH_3	H	F	1	10.9 ± 0.53	108	108 ± 5.18	10	5.14
5 r	CH_3	H	OH	1	1.44 ± 0.07	31	31 ± 0.59	22	4.79
5 s	CH_3	Et	H	1	0.09 ± 0.002	32	32 ± 0.66	359	5.90
5 t	CH_3	Et	OCH_3	1	0.07 ± 0.01	31	31 ± 0.34	469	6.12
5 u	CH_3	CH_3	H	1	0.35 ± 0.00	179	179 ± 28.83	509	5.37
5v	CH_3	CH_3	F	1	1.01 ± 0.004	115	115 ± 26.80	114	5.59
5 w	CH_3	CH_3	OH	1	0.13 ± 0.01	24	24 ± 0.97	179	5.24
5x	CH_3	CH_3	OCH_3	1	0.93 ± 0.36	43	43 ± 18.40	46	5.59
DB02	H	Et	H	0	0.02 ± 0.002	263	263 ± 8.70	14050	4.97
NVP					0.06 ± 0.15	–	>4	>63	–
EFV					0.001 ± 0.15	–	>2	>1982	–
DLV					0.021 ± 0.15	–	>20	>942	–
AZT					0.0019 ± 0.15		>25	>13293	–

[a] IC_{50}: concentration of compound required to achieve 50% protection of MT-4 cells against HIV-induced cytopathicity, as determined by the MTT method.
[b] CC_{50}: concentration required to reduce the viability of mock-infected cells by 50%, as determined by the MTT method.
[c] SI: selectivity index (CC_{50}/IC_{50}).
[d] cLog*P*: predicted by using software ChemBioDraw Ultra 12.0.

Both the absolute activity against the HIV-1 mutant (IC_{50} value) and the relative activity (fold-resistance) were used to define the resistance profile of the tested compounds. The results indicated that most derivatives, including the lead compound **DB02**, lost activity against the double mutant strain RES056, while four compounds (**5c**, **5d**, **5s**, **5t**) were more potent than the reference drug DLV against the resistant mutant strain with the IC_{50} value of 11.68, 7.89, 9.84 and 4.39 μmol/L respectively (versus > 20 μmol/L, DLV) and a fold-resistance ratio of 770, 243, 108 and 66, respectively (*versus* > 942, DLV). In this work, all compounds were also screened for their inhibition against the replication of the HIV-2 strain (ROD) in MT-4 cells, but none of them exhibited inhibitory activity at sub-toxic concentrations, indicating that the novel series of *S*-DACO derivatives were specific to HIV-1. Preliminary structure-activity relationship (SAR) derived from these results was analyzed as follows.

First, we focused our attention on the linker optimization at the *C*-6 position of the pyrimidine ring. As we can see, methylation of *C*-6 secondary carbon of the lead compound **DB02** led to compound **5c** with similar potency (IC_{50} = 0.018 μmol/L) to **DB02** (IC_{50} = 0.020 μmol/L), while the insertion of a carbon into *C*-6 CH_2-cyclohexyl substituted moiety of **DB02** yielded compound **5j**, the increased length of the carbochain linker reduced the activity about 5-fold (IC_{50} = 0.10 μmol/L). Subsequently, methylation of the secondary carbon of the $\underline{C}H_2CH_2$-cyclohexyl of the **5j** gave the inhibitor **5s** (IC_{50} = 0.09 μmol/L) , which is as potent as **5j** but the cytotoxicity was increased about 8-fold. The above results of the C_6-linker optimization provided some support for our hypothesis that introduction of a methyl group to the C_6-CH_2 linker would be feasible and effective.

Just as SAR studies on *S*-DABOs, the *para* substituents (-F, −OCH_3, −OH) of the terminal phenyl ring at the *C*-2 side chain also had significant effects on the antiviral activity of these novel *S*-DACOs. As shown in Table 1, introduction of a 4′-hydroxyl group at the phenyl ring led to compounds **5f, 5l**, **5o, 5r** and **5w** with slightly better anti-HIV-1 activities than their unsubstituted counterparts **5e, 5j, 5m, 5p** and **5u**. On the other hand, all of the 4'-fluoro-substituted compounds displayed decreased activities comparing to their 4'−OCH_3/OH/H-substituted counterparts. Generally, the sequence of beneficial effects of the *para* substituents at the *C*-2 terminal phenyl ring on activity is as follows decreasing order: OH > H ∼ OCH_3 > F. At the same time, it is worth noting that the introduction of 4'−OH, 4'−OCH_3 or 4'-F at the *C-2ω*-phenyl ring led to increased cytotoxicity.

When tested against wt HIV-1, the compounds **5b**, **5p**, **5q** and **5r** displayed low inhibitory activity andparticularly compounds **5h** and **5i** almost lost their activity. With the insertion of a methyl or ethyl group at the *C*-5 position of the pyrimidine, a marked increase of anti-HIV-1 activity and selectivity index was observed for all of the substituted compounds. Moreover, the influence on activity of an ethyl substituent was obviously better than its methyl counterpart. For instance, compounds **5c, 5l**, **5j**, **5k**, **5t** and **5s** (IC_{50} = 0.018, 0.05, 0.10, 0.14, 0.07 and 0.09 μmol/L, respectively) were more potent than their methyl counterparts **5e**, **5o**, **5m**, **5n**, **5x** and **5u** (IC_{50} = 0.08, 0.24, 1.72, 7.32, 0.93 and 0.35 μmol/L, respectively). So, the order of activity of the *C*-5 substituents can be summarized as follows: Et > Me > H, which is in agreement with our previously reported results on the *S*-DACO series [26,27].

Since the lead compound **DB02** showed an IC_{50} of 0.28 μmol/L against the RT enzyme and a similar dose-dependent pattern in inhibiting HIV-1 RT activity with NVP [33], we inferred that these newly synthesized compounds also acted as classical NNRTIs. To better understand the activity and the interaction mechanism between these compounds and RT, the selected compounds **5c** and **5j** were docked into the NNRTIs binding pocket (NNIBP) compared with **DB02** using the AutoDock4.2 program (Supporting information). Binding modes of compounds **5c** and **5 j** in the allosteric site of HIV-1 WT RT in comparison with **DB02** were present at Fig. 2. The location of compound **5c** was different due to the presence of *C*-6 chiral carbon (Figs. 2C and D). Results showed that compound **5c** and **5j** had similar binding pattern with **DB02** in the NNIBP that the pyrimidine ring was stabilized by the hydrogen bond between the 3-NH of the pyrimidine ring with the carbonyl oxygen of Lys101. While the *C*-2 side chain extend in the same direction, forming hydrogen bonds between the carbonyl oxygen of the *C*-2 side chain and the NH group of the Lys103 backbone. The interactions between **5c** and RT were almost identical to that of **DB02** with RT, explains the fact that **5c** and **DB02** have the same anti-HIV activity. On the other hand, the hydrogen bond between compound **5j** and RT is weaker than that of **DB02**, so the activity of compound **5j** is decreased by 5 times compared with **DB02**. The C_6-cyclohexyl group of the inhibitors is positioned in a hydrophobic sub-pocket formed by Try181, Try188, Phe227 and Trp229 and develops additional interactions with the hydrophobic pocket. Moreover, the C_6-cyclohexyl group of **5c** and **5j** is closer to Trp229 than that of **DB02**, because Trp229 is highly conserved, this interaction is expected to be retained despite mutations in the binding pocket, and this may be the reason why **5c** retains potency against the mutant strain compared to **DB02**. In particular, it can be seen from Figs. 2C and D, that the hydrogen bond between compound (*R*)-**5c** and RT is stronger than that between compound (*S*)-**5c** and RT (distances 1.66 Å and 2.10 Å *vs.* 1.76 Å and 2.18 Å), and the cyclohexyl group of compound (*R*)-**5c** is closer to Trp229 than compound (*S*)-**5c** (distance 3.33 Å *vs.* 3.86 Å). Therefore, we inferred that compound (*R*)-**5c** had better anti-HIV activity than compound (*S*)-**5c**.

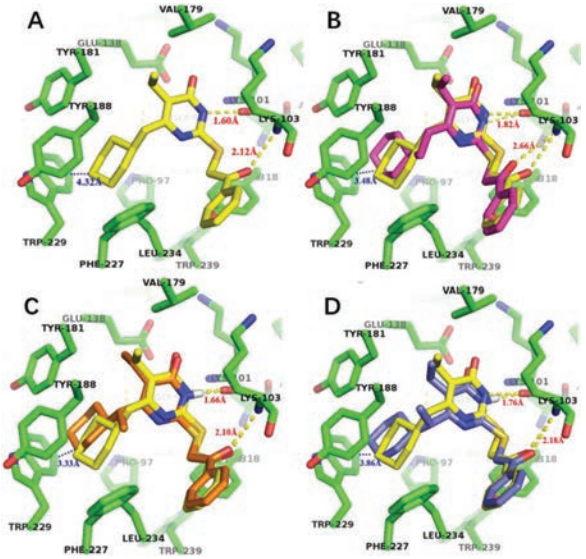


Fig. 2. Predicted binding modes of selected compounds with RT (PDB: 1RT2). (A) **DB02** (carbons in yellow) with RT; (B) **5j** (carbons in magenta) with **DB02**; (C) (*R*)-**5c** (carbons in orange) with **DB02**; (D) (*S*)-**5c** (carbons in slate) with **DB02**. Residues involved in interactions are shown as green sticks. Dotted lines show the interactions between HIV-1 RT and inhibitors.

In conclusion, to extend the range of NNRTIs chemical structures and overcome the issue of resistance, we designed a series of oxophenethyl-*S*-DACO derivatives as potent new HIV-1 NNRTIs based on the molecular modeling of lead compound **DB02** and using conformationally restricted strategy. The experimental data indicated that most of the compounds showed moderate to good anti-HIV activity with IC_{50} values in the range of 7.55–0.018 μmol/L. Among them, the most potent HIV-1 inhibitor was **5c** (SI = 12791), which was much more potent than the reference

drugs NVP, DLV and EFV and comparable to AZT and **DB02**. In addition, **5c** also exhibited improved activity against double mutant HIV-1 strain RES056 compared to that of the reference drugs NVP/DLV and **DB02**. The preliminary SARs were discussed and the molecular simulation was performed, providing insights for discovery of more active and selective *S*-DACOs with diverse structures. At last, since some active derivatives contain one stereogenic center, therefore, exist as racemic or stereoisomeric mixtures. Further systematic investigation on their potential enantio-/diastereoselective anti-HIV-1 activity is still ongoing in our lab.

Declaration of competing interest

The authors report no declarations of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cclet.2020.09.035.

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