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Synthesis and biological evaluation of novel dihydro-aryl/alkylsulfanyl-cyclohexylmethyl-oxopyrimidines (*S*-DACOs) as high active anti-HIV agents

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ABSTRACT

A novel dihydro-aryl/alkylsulfanyl-cyclohexylmethyl-oxopyrimidines (*S*-DACOs) combinatory library was synthesized and evaluated with C8166 cells infected by the HIV-1_{IIIB} in vitro, using Nevirapine (NVP) and Zidovudine (AZT) as positive control. The anti-HIV screening results revealed that *C*-6-cyclohexylmethyl substituted pyrimidinones possessed higher selective index than its 6-arylmethyl counterparts. Compounds **1g**, **1c**, **1e** and **1b** showed potent anti-HIV activities with EC_{50} values of 0.012, 0.025, 0.088 and 0.162 nM, respectively.

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are a class of antiretroviral drugs which play an important role in treatment of HIV-1 infection. NNRTIs deactivate HIV-1 reverse transcriptase by inducing conformation change of the enzyme via binding to a hydrophobic site close (approximately 10 Å) to its catalytic site.[1] Since these compounds do not need intracellular metabolic activation, have relatively low cytotoxicity, act on a nano molar scale and are not active against other retroviruses, they are highly specific HIV-1 inhibitors. Currently, four NNRTIs, namely nevirapine, delavirdine, efavirenz, and etravirine, have been approved by the FDA for clinical use.[2] NNRTIs are highly hydrophobic and chemically diverse compounds that comprise over 50 different members. Among them, 1-[(2-hydroxyethoxy)-methyl]-6-(phenylthio)thymine (HEPT) was the first NNRTIs beiidentified.[3,4] Dihydro-alkoxy-benzyl-oxopyrimidines (DABOs) are structurally modified HEPT with improved inhibitory activity and pharmacokinetic properties.[5–7]

The rapid development of HEPTs structural modification has led to a series of potent DABO derivatives, such as MKC-442,[8] TNK-651,[9] MTM-*S*-DABOs,[10] and 6-(1-naphthylmethyl)-2β-carbonyl-*S*-DABOs[11] (Fig. 1). Both HEPTs and DABOs are the derivatives of 4-pyrimidinone series and have an aromatic ring at *C*-6 position of the pyrimidine ring, which played an important role in their anti-HIV activities.[12–15] Molecular modeling studies have suggested that the *C*-2 side chain of DABOs, a major contributor to the anti-HIV-1 activity, was locked in the same region of the binding site of the N1-substituted HEPTs.[10,16]

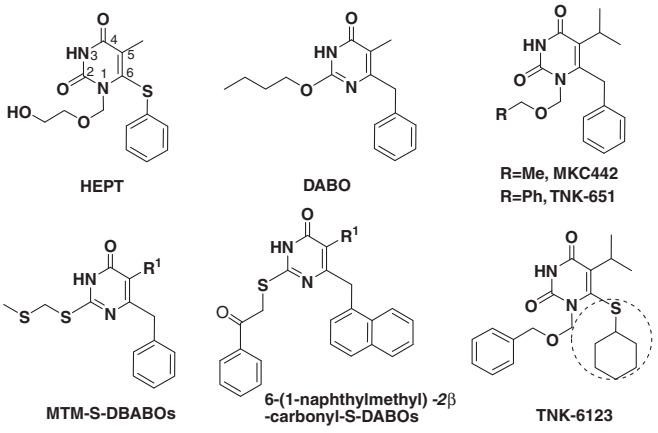

Figure 1. Structures of HEPTs and DABOs.

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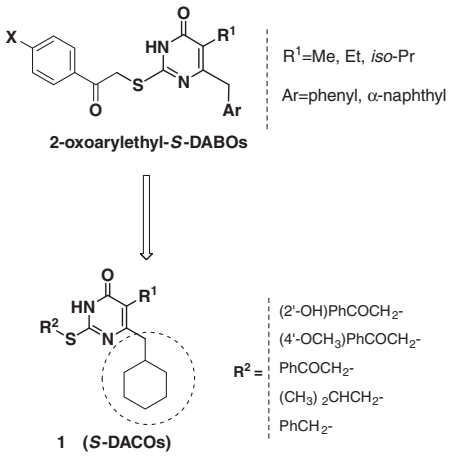


Figure 2. Structures of 2-oxoarylethyl-*S*-DABOs and DACOs.

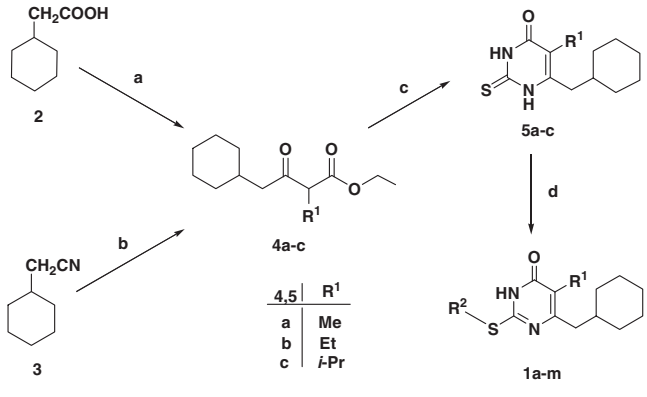
While doing MKC-442 analogue modification, Hopkins et al.[9] reported synthesis of compound TNK-6123, which had an improved activity against drug-resistant HIV mutants. We noticed that this compound had a *C-6*-aromatic benzyl moiety replaced by a thiocyclohexyl group. In comparison with a planar aromatic 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.

Encouraged by these findings, we looked to obtain more potent anti-HIV agents by exploring the effect of the 6-cyclohexylmethyl moiety in 4-pyrimidinone series on the activity against HIV-1. We prepared a dihydro-aryl/alkylsulfanyl-cyclohexylmethyl-oxopyrimidines (*S*-DACOs **1**, Fig. 2) inhibitor combinatory library, with a series of selective R^2 (2-phenyl-2-oxoethylsulfanyl, 2-(4-methoxyphenyl)-2-oxoethylsulfanyl, 2-(2-hydroxyl-phenyl-2-oxoethylsulfanyl, *iso*-butysulfanyl and benzylsulfanyl) group at *C-2*, and R^1 (methyl, ethyl or *iso*-propyl) group at *C-5* of the pyrimidine ring (Fig. 2). In this study, we report the synthesis, anti-HIV-1 activity evaluation and preliminary SAR studies of these new compounds.

The synthesis of the new *S*-DACO derivatives was accomplished according the route outlined in Scheme 1. The key intermediate, β-ketoesters **4a**–**c**, was synthesized by two alternative routes: **4a** and **4b** were prepared by exposure of 2-cyclohexylacetic acid **2** to 1,1′-carbonyl-diimidazole (CDI) followed by treatment with different ethyl potassium malonates in the presence of anhydrous $MgCl_2$ and Et_3N.[17] The β-ketoester **4c**, on the other hand, was obtained via a modified Blaise procedure[18,19] involving the reaction between the commercially available 2-cyclohexylacetonitrile and ethyl 2-bromo-3-methylbutanoate in the presence of freshly prepared zinc turnings, followed by acidic hydrolysis of the enamine intermediate (Scheme 1, route b). Condensation of **4a**–**c** with thiourea in the presence of EtONa in refluxing EtOH led to substituted uracil **5a**–**c**. Subsequent treatment of **5a**–**c** with appropriate halide (R^2X) in the presence of K_2CO_3 in anhydrous DMF afforded the corresponding target compounds **1a**–**m** with 36%–68% yield. Both analytical and spectral data of all the synthesized compounds are in full agreement with the proposed structures.[20]

The novel *S*-DACO derivatives (**1a**–**m**) were tested for their cytotoxicities and anti-HIV-1 activities in C8166 cells infected by the HIV-1_{IIIB}, and compared with Nevirapine (NVP) and Zidovudine (AZT). The activity data were interpreted in CC_{50} values (cytotoxicities), EC_{50} (anti-HIV-1 activities) and SI (selectivity, given by the CC_{50}/EC_{50} ratio) (Table 1). As another control, previously reported compounds[21] (**6a**–**d**) were included in our test assays for comparison.

As shown in Table 1, most of the *S*-DACOs, with the exception of compounds **1j**, **1l** and **1m**, exhibited significant improvement on anti-HIV-1 activities (EC_{50} = 0.012–101.0 nM) with reduced cytotoxicity (CC_{50} = 69.81–412.81 μM) and enhanced selectivity index (SI values of 691–6,315,000). Compound **1g** exhibited the highest potent inhibitory activity against HIV-1 replication with an EC_{50} value of 0.012 nM, CC_{50} value of 75.78 μM and the viral selectivity index of 6,315,000. In addition to this compound, compounds **1a**,

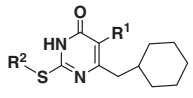


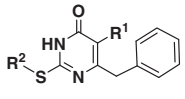
1	R^1	R^2
a	i-Pr-	(2'-OH)PhCOCH$_2$-
b	i-Pr-	(4'-OCH$_3$)PhCOCH$_2$-
c	i-Pr-	PhCOCH$_2$-
d	i-Pr-	(CH$_3$)$_2$CHCH$_2$-
e	i-Pr-	PhCH$_2$-
f	Et-	(4'-OCH$_3$)PhCOCH$_2$-
g	Et-	PhCOCH$_2$-

1	R^1	R^2
h	Et-	PhCH$_2$-
i	Et-	(CH$_3$)$_2$CHCH$_2$-
j	Me-	(4'-OCH$_3$)PhCOCH$_2$-
k	Me-	PhCOCH$_2$-
l	Me-	PhCH$_2$-
m	Me-	(CH$_3$)$_2$CHCH$_2$-

Scheme 1. Synthesis of compounds **1a**–**m**. Reagents and conditions: (a) (i) CDI, CH_3CN, rt, 30 min; (ii) $R^1CH(CO_2Et)(CO_2K)$, Et_3N, anhydrous $MgCl_2$, rt, overnight, then refluxed for 2 h; (iii) 13% HCl; (b) (i) $(CH_3)_2CHCHBrCOOEt$, Zn, THF, refluxed for 4 h; (ii) 50% K_2CO_3; (iii) 13% HCl; (c) thiourea, EtONa, EtOH, refluxed for 8 h; (d) R^2X (X = Br or Cl), K_2CO_3, DMF, 25–80 °C, 12–24 h.

Table 1
Antiviral activity of target compounds against HIV-1 in C8166 cells[a]


1a-m


6a-d

No.	R^1	R^2	CC_{50}[c] (μM)	EC_{50}[b] (nM)	SI[d] (CC_{50}/IC_{50})
1a	*i*-Pr	(2′-OH)PhCOCH$_2$–	228.12	1.950	116,974
1b	*i*-Pr	(4′-OCH$_3$)PhCOCH$_2$–	225.51	0.162	1,391,975
1c	*i*-Pr	PhCOCH$_2$–	90.60	0.025	3,624,000
1d	*i*-Pr	(CH$_3$)$_2$CHCH$_2$–	158.62	92.92	1706
1e	*i*-Pr	PhCH$_2$–	223.40	0.088	2,538,636
1f	Et	(4′-OCH$_3$)PhCOCH$_2$–	111.91	0.217	515,668
1g	Et	PhCOCH$_2$–	75.78	0.012	6,315,000
1h	Et	PhCH$_2$–	412.81	531.6	776
1i	Et	(CH$_3$)$_2$CHCH$_2$–	69.81	101.0	691
1j	Me	(4′-OCH$_3$)PhCOCH$_2$–	420.40	1060	396
1k	Me	PhCOCH$_2$–	162.81	75.80	2147
1l	Me	PhCH$_2$–	60.88	1254	48
1m	Me	(CH$_3$)$_2$CHCH$_2$–	59.91	1734	34
6a	Et	PhCOCH$_2$–	>549	80.15	>6849
6b	Et	(4′-OCH$_3$)PhCOCH$_2$–	>507	48.28	>10,501
6c	*i*-Pr	PhCOCH$_2$–	>528	14.01	>37,687
6d	*i*-Pr	(4′-OCH$_3$)PhCOCH$_2$–	>489	144.1	>3393
AZT			5041	11.35	444,140
NVP			>749.1	49.43	>15,154

[a] All data represent mean values for three separate experiments.
[b] Effective concentration required to protect C8166 cell against the cytopathogenicity of HIV by 50%.[21]
[c] Cytostatic concentration required to reduce C8166 cell proliferation by 50% tested by MTT method.[21]
[d] Selectivity index: ratio CC_{50}/IC_{50}, a higher SI means a more selective compound.

1b, **1c**, **1e** and **1f** were also displayed higher anti-HIV-1 potency (EC_{50} = 1.950, 0.162, 0.025, 0.088 and 0.217 nM, respectively) and better selectivity index (SI = 116,974, 1,391,975, 3,624,000, 2,538,636 and 515,668, respectively) than those found for NVP and AZT.

In the 2-oxoarylethylsulfanyl series, replacing the *C-6* benzyl at the pyrimidine ring by a cyclohexylmethyl caused a dramatic selectivity index increase. As seen, *C-6* cyclohexylmethyl substituted derivatives (**1b**, **1c**, **1f**, and **1g**) were 49–410-folds (SI ratio) more active than their benzyl substituted counterparts (**6d**, **6c**, **6b**, and **6a**). This is in agreement with the results reported by Hopkins et al.[9] These results provided further evidence that compared with aromatic electronic interaction, the *C-6* cyclohexylmethyl group could provide better conformational flexibility and hydrophobic interaction with the binding pocket of the HIV-1 reverse transcriptase which in turn enhanced the anti-HIV activity of the 2-oxoarylethyl-4-pyrimidinone derivatives.

The role of the *C-5* substituent in the anti-HIV activity of the compounds has also been studied. As with the other DABO series, this *S*-DACOs unambiguously showed that the inhibitory activity increased with the modification of the *C-5* substituent in the order of *i*-Pr > Et > Me, as indicated in the 2-benyl-sulfanyl (**1e**, **1h**, and **1l**) and 2-*iso*-butyl-sulfanyl substituted DACOs (**1d**, **1i**, and **1m**) series. This *C-5* hydrophobic contact increase effect has been indicated by D'cruzl and Uckun[5] in a computational modeling study. Our results with this library, as well as the other DABO series, clearly support it. The only exception occurred in 2-phenyloxoethylsulfanyl DACOs (**1c**, **1g**, and **1k**). In this group, the influence of the ethyl substituent was slightly higher than its isopropyl counterpart. For example, **1g** (EC_{50} = 0.012 nM) was twofold more potent than its 5-*i*-Pr counterpart **1c** (EC_{50} = 0.025 nM). Additional computational modeling and SAR study are needed to explain this observation.

In terms of the role of alkyl group substitution at the *C-2* side chain, one thing we noticed was that the $(CH_3)_2CHCH_2$– substitute (Table 1) reduced the anti-HIV-1 activity of compounds **1d**, **1i**, and **1m**. This result implied that certain aromaticity was required at this position in order to get a good affinity for this drug to bind to the binding pocket. In our previous report, we have indicated the importance of the C2-β-carbonyl to the anti-HIV-1 catalytic activity.[22,23] However, this is not true in this novel *S*-DACOs library. As indicated in Table 1, compound **1e** still had a high activity (EC_{50} = 0.088 nM) after the β-carbonyl of compound **1c** was removed. This information will provide us better guidance in future structural designs on the *S*-DACOs development.

We have also chosen active compound **1c** to evaluate its anti-HIV-1 activity in human peripheral blood mononuclear cells (PBMCs) and the NNRT inhibitor resistant strain A17, which contains two mutations (Y181C and K103N).[24] Results are presented in Table 2. Interestingly, it was found that **1c** showed a favorable anti-HIV-1 activity in both human PBMCs and A17 with the EC_{50} 0.031 and 0.046 μM, respectively. Further anti-activity evaluation of these new DACOs is currently undergoing and will be reported upon completion.

In summary, substitution of a cyclohexylmethyl group to aromatic group at the *C-6* position of the thymine ring and modification its *C-2* and *C-5* positions have led to a series of novel dihydroaryl/alkylsulfanyl-cyclohexylmethyl-oxopyrimidines (*S*-DACOs). Among those compounds obtained, **1g**/**1c**/**1e**/**1b** appear to be the promising candidates for further anti-HIV-1 agent development. Further modification and optimization of R^1 and R^2 groups on 6-cyclohexylmethyl pyrimidone analogs will lead to more potent compounds for active anti-HIV agents.

Table 2
Anti-HIV activity of selected title compounds **1C** in PBMC and A17

Compd	EC_{50} values (μM)	
	PBMC	A17 (Y181C and K103N)
1c	0.031	0.046

Acknowledgments

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20. *General procedure for the preparation of compounds* **1a**–**m**: Sodium metal (8 g, 0.348 mol) was dissolved in 180 mL of absolute ethanol, and thiourea (19.2 g, 0.252 mol) and β-ketoesters **4a**–**c** (0.084 mol), were added to the clear solution. The reaction mixture was refluxed for 6–8 h. After cooling, the solvent was removed under reduced pressure, and the residue was dissolved in H_2O (100 mL) and acidified with 13% HCl. The resulting precipitate was filtered, washed sequentially with H_2O, EtOH, and Et_2O, then dried to give **5a**–**c** as a pure solid. To a solution of 2-thiouracil **5a**–**c** (2 mmol) in anhydrous DMF (8 mL) were added K_2CO_3 (2.2 mmol) and halide (R^2X) (2.2 mmol). The mixture was stirred at room temperature for 8–24 h. After TLC (EtOAc/PE) revealed the disappearance of the starting material, the reaction mixture was filtered. The suspension was then diluted with cold water and extracted with ethyl acetate. The combined organic extract was washed with brine, dried with Na_2SO_4, and evaporated to furnish crude product, which was purified by flash chromatography or by crystallization to give the pure target compounds **1a**–**m**. As an example, spectroscopic data for compound **1b** are reported. ^{1}H NMR (DMSO-d_6): δ (ppm) 0.77–0.78 (m, 2H, cyclohexyl), 1.17–1.19 (m, 2H, cyclohexyl), 1.26–1.30 (m, 1H, cyclohexyl), 1.29–1.30 (d, 6H, J = 6.9 Hz, $2CH_3$), 1.58–1.71 (m, 6H, cyclohexyl), 2.26–2.28 (d, 2H, J = 6.6 Hz, CH_2–cyclohexyl), 2.89–2.95 (m, 1H, $CHMe_2$), 3.88 (s, 3H, OCH_3), 4.53 (s, 2H, CH_2S), 6.97–8.04 (d, 4H, phenyl), 12.6 (s, br s, 1H, NH); ^{13}C NMR (DMSO-d_6): δ (ppm) 20.23 ($2CH_3$), 26.63 ($2CH_2$, cyclohexyl), 26.74 (CH), 28.12 ($2CH_2$, cyclohexyl), 37.4 (CH_2), 37.89 (CH_2S), 42.50 (CH_2, cyclohexyl), 55.8 (OCH_3), 114.29–132.5

(5C, phenyl), 129.50 (C-5), 154.97 (C-6), 162.51 (C-2), 164.32 (C-4), 164.53 (Ar-C4), 192.1 (C=O). IR (KBr, cm^{-1}): 3421 (ν_{NH}), 1673 ($\nu_{C=O}$), 1647 ($\nu_{C=O}$), 1578–1445 (ν_{Ar}).

21. Human T-cell lines (C8166) and HIV-1IIIB were kindly donated by Medical Research Council, AIDS Regent Project. The cells were maintained at 37 °C in 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivating fetal calf serum (Gibco). HIV-1IIIB was prepared from the supernatants of H9/HIV-1IIIB cells. The 50% HIV-1 tissue culture infectious dose (TCID50) in C8166 cells was determined and calculated by Reed and Muench method. Virus stocks were stored in small aliquots at −70 °C. The titer of virus stock was 6.0×10^5 TCID50 per cm. *Cytotoxicity assay*. The cellular toxicity of compounds on C8166 cells was assessed by MTT colorimetric assay as described previously. Briefly, 100 mm^3 of 4×10^5 cells·cm^3 were plated into 96-well microtiter plates, 100 mm^3 of various concentrations of compounds was added and incubated at 37 °C in a humidifed atmosphere of 5% CO_2 for 72 h. Discard 100 mm^3 supernatant, MTT reagent was added and incubated for 4 h, 100mm^3 50% DMF–10% SDS was added. After the formazum was dissolved completely, the plates were read on a BioTek ELx 800 ELISA reader at 595 nm/630 nm. The results were shown by absorbance values. The minimum toxic concentration that caused the reduction of viable cells by 50% (CC_{50}) was determined from dose–response curve.
Inhibition of syncytium formation C8166 cells (4×10^5 cells·cm^3) were treated with different concentrations of the compounds and HIV-1IIIB (M.O.I. = 0.02), and incubated in a humidified incubator at 37 °C in final volume of 200 mm^3. AZT was used for positive drug control. After 3 days incubation of culture, the cytopathic effect (CPE) was measured by counting the number of syncytium (multinucleated giant cell) in each well under an inverted microscope. The Percentage inhibition of syncytial cell formation (EC_{50}) was calculated by the percentage of syncytial cell number in compounds treated culture to that in infected control culture.
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