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Benzamide-Based Thiolcarbamates: A New Class of HIV-1 NCp7 Inhibitors

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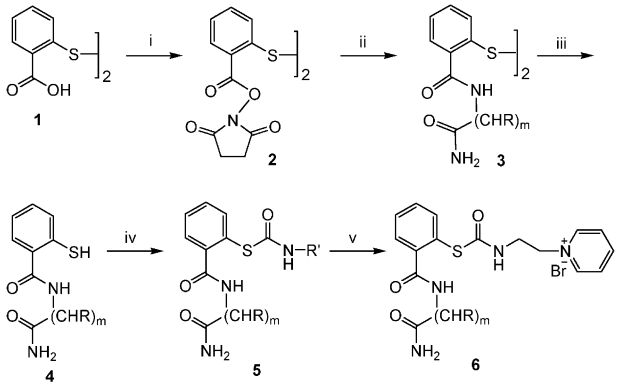
Abstract—The HIV-1 nucleocapsid protein NCp7, which contains two highly conserved zinc fingers, is being used as a novel target for AIDS therapy due to its pivotal role in viral replication and its mutationally intolerant nature. Herein we report a new class of NCp7 inhibitors that possess good antiviral activity with low cellular toxicity.

The nucleocapsid p7 protein (NCp7) zinc finger domains of human immunodeficiency virus type 1 (HIV-1) have been chosen and exploited as targets in the pursuit of alternative, novel anti-retroviral drugs.[1] NCp7, a basic 55 amino acid protein, results from specific post-translational processing of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ precursor polyproteins[2] and plays a pivotal role in both early (reverse transcription[3] and integration[4]) and late (protease processing[5] and packaging[6] of viral genomic RNA) stages of the viral replication cycle. The Cys-$(Xaa)_2$-Cys-$(Xaa)_4$-His-$(Xaa)_4$-Cys (CCHC) motifs[7] are present in both zinc fingers of NCp7 and are highly conserved and mutationally intolerant.[8] Mutational studies on virus infectivity indicated that modification in either zinc chelating, non-chelating and adjacent sequences of the zinc finger domains results in virions with defective RNA encapsidation and/or NCp7 function that renders them noninfectious.[6,9]

A variety of electrophilic agents have been identified that cause HIV-1 inhibition by chemical modification of Cys-sulfurs of the NCp7 zinc finger motifs. Studies on NCp7 inhibitors, such as 3-nitrosobenzamide (NOBA),[10] 2,2′-dithiobisbenzamides (DIBA)[1a,11] and their benzisothiazolone derivatives (BITA),[12] cyclic 2,2′-dithiobisbenzamides (SRR-SB3),[13] 1,2-dithiane-4,5-diol-1,1-dioxide (dithiane),[14] and azodicarbonamide (ADA)[15] showed that these compounds exhibited significant antiviral activity against laboratory and clinical isolates of HIV in several infected cell lines. Recently, we identified a novel chemotype, pyridinioalkanoyl thiolesters (PATEs),[16] that inhibits viral replication in both acutely and latently HIV-1 infected cells. PATEs possessed NCp7 zinc finger reactivity and virucidal activity. However, efforts to generate compounds with significantly improved antiviral potencies were not successful. The present study was focused on identifying a new class of NCp7 inhibitors, thiolcarbamates (TICAs), that possess improved anti-retroviral potency.



Scheme 1. Reagents and conditions: (i) *N*-Hydroxysuccinimide/DIC/THF-PrOH-2/25 °C; (ii) $H_2N(CHR)_mCONH_2.HCl/Et_3N/DMF/25\,°C$; (iii) TCEP.HCl/$Et_3N$/DMF–$H_2O$ (9:1)/25 °C; (iv) R′NCO/DMF/25 °C; (v) R′ = CH_2CH_2Br, pyridine/under N_2/25 °C.

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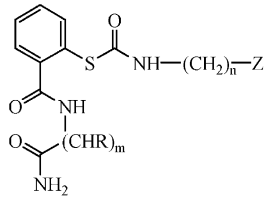
The existing methodologies[17] for thiolcarbamate formation are limited by the need for specialized reagents such as transition metal complexes, and the use of strong bases, high temperatures and/or cumbersome reagents such as phosgene. However, our synthetic approach to preparing benzamide-based thiolcarbamates through the use of commercially available reagents is straightforward (Scheme 1). The synthesis of the 2,2′-dithiobis(benzamides) (**3**) was accomplished via coupling of *N,N*′-disuccinimidyl-2,2′-dithiosalicylate (**2**, prepared from the 2,2′-dithiosalicylic acid (**1**), *N*-hydroxysuccinimide and 1,3-diisopropylcarbodiimide) with the desired amino acid amides. These disulfide benzamides (**3**) were reduced using tris(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl) in 90% DMF in water under nitrogen atmosphere. The desired thiolcarbamates (**5**) were prepared by condensation of **4** with the corresponding isocyanates at room temperature. All bromo derivatives (**5n**,**o**) were converted to their respective pyridinium salts (**6**) by stirring in pyridine at room temperature under nitrogen. All the synthesized compounds were characterized by spectroscopic analyses, and the composition analysis data for one of the representative compounds is given in the reference section.[18]

Compounds **5a**–**o** and **6a**–**c** were evaluated for their antiviral activity (Table 1) with CEM-SS cells and HIV-1_{RF} virus using the HIV-1 cytoprotection assay.[10b,14] Our initial efforts were focused on the structure–activity relationships (SARs) associated with substitution on the nitrogen atom of the carbamoylthio moiety of the TICAs as shown in Table 1. All the compounds (**5a**–**o** and **6a**–**c**) of this set showed similar EC_{50} values (0.16–1.19 μM), but exhibited a wide range of cellular toxicity (IC_{50}) values lying between 4 and 200 μM (highest concentration tested). Changing the hydrophobicity by preparing linear alkyl homologues (**5a**–**d**) did not impact on their antiviral potencies. On the other hand, increasing the steric bulk on the carbamoyl nitrogen atom by substitution with isopropyl (**5e**,**f**) or tertiary butyl (**5g**,**h**) groups reduced the cellular toxicity, resulting in good therapeutic indices. Compounds comprised of a saturated ring (**5i**,**j**) at the carbamoyl nitrogen, showed high cellular toxicities and correspondingly poor therapeutic indices, while substitution with aromatic groups (**5k**–**m**) gave rise to less toxic compounds, except for **5l**. The effect of the electron withdrawing bromo group at the terminus of the *N*-ethylcarbamoylthio group (**5n**,**o**) resulted in increased cellular toxicity. The replacement of the bromo group by a pyridinio moiety (**6a**–**c**), yielding pyridinium salts, reduced cellular toxicity while maintaining submicromolar EC_{50}s and therapeutic indices >140. Thus, the combination of the carbamoylthio moiety and certain substitutents like tertiary butyl and pyridinium moiety at the carbamoylthio nitrogen with the carboxamide terminal yields antiviral agents with submicromolar EC_{50}s and reduced cytotoxicity. These branched or cationic structures may confer lower toxicity by slowing the reverse of reaction *iv* (Scheme 1), which results in the release of isocyanate.

We next determined if the observed advantages in antiviral activity were associated with Zn Finger reactivity.

Table 1. Antiviral activity of **5a**–**o** and **6a**–**c** was measured by the HIV-1 cytoprotection assay[a]



S.N.	m	n	R	Z	Antiviral activity EC_{50}(μM)	Cellular toxicity IC_{50}(μM)	TI
5a	2	4	H	CH_3	0.50	41.2	82
5b	2	5	H	CH_3	0.43	39.4	92
5c	1	7	D-CH_3	CH_3	0.23	38.3	167
5d	2	7	H	CH_3	0.16	40.2	251
5e	1	0	L-CH_3	$CH(CH_3)_2$	0.77	76	99
5f	2	0	H	$CH(CH_3)_2$	0.33	55.1	167
5g	1	0	D-CH_3	$C(CH_3)_3$	0.36	125	347[b]
5h	2	0	H	$C(CH_3)_3$	0.37	142	384[b]
5i	2	0	H	Cyc-Pentyl	0.81	17.3	21
5j	2	0	H	Cyc-Hexyl	0.55	11.9	22
5k	1	0	D-CH_3	4-FC_6H_4	0.47	130	277
5l	1	0	D-CH_3	4-$NO_2C_6H_4$	1.19	23.1	19
5m	2	0	H	4-$CF_3C_6H_4$	0.45	128	284
5n	2	2	H	Br	0.48	4.9	10
5o	1	2	D-CH_3	Br	0.92	8.0	9
6a	1	2	H	Py^+Br^-	0.50	70	140
6b	2	2	H	Py^+Br^-	0.95	153	161
6c	1	2	D-CH_3	Py^+Br^-	0.97	200	206

[a]All determinations were performed in duplicate with triplicate determinations for each compound concentration. The variation of individual triplicates was 10% or less.
[b]Zinc finger reactivity was observed at 12 μM using previously described methodologies.[19] EC_{50}: effective concentration resulting in 50% cytoprotection; IC_{50}: cellular growth inhibitory concentration causing 50% cytotoxicity; TI: therapeutic index (IC_{50}/ EC_{50}).

Table 2. Antiviral range of action studies of select TICAs

Compd	PBMCs[a] (EC_{50} μM)	Monocyte[b] (EC_{50} μM)	TNF-α Induced U1 cells[c] (EC_{50} μM)		Chronically infected cells[d] (EC_{50} μM)	Virus inactivation[e] (EC_{50} μM)
			RT	p24		
5g	1.1	13.9	ND	ND	ND	0.9
5h	0.8	28.7	ND	ND	ND	1
6a	2.7	28.4	5.1	2.5	20	ND
6b	1.9	27.1	2.26	0.84	10.8	0.9

Data shown are representative of triplicate assays in at least two separate experiments. ND means not determined.
[a]Expression of supernatant RT activity was determined 6 days post-infection of human PBMCs with the pediatric clinical isolate HIV-1RoJo.
[b]Human peripheral blood monocytes were cultured in vitro for 6 days and infected with HIV-1 Ba-L, and virus replication detected by commercial p24 ELISA.
[c]U1 cells were induced with 10 ng/mL TNF-α and the expression of supernatant RT activity and p24 protein determined at day 3 post-treatment.
[d]CEM-SS cells chronically infected with the SK-1 strain of HIV-1 (CEM/SK-1) were assessed for supernatant RT activity following 6 days of treatment.
[e]Cell-free HIV-1 IIIB was incubated for 4 h incubation with TICAs, removed by centrifugation (18,000*g*, 90 min, 4 °C) and residual infectivity determined on HeLa CD4 LTR β-gal cells by chemiluminescence. EC_{50}: concentration resulting in 50% suppression of virus replication.

Presumably, transacylation of the carbamoyl group to Zn finger cysteine sulfur atoms occurs in the manner shown for thiolesters.[19] This mechanism is currently being investigated. The lead compounds (**5g** and **5h**; TI >300) were evaluated for their ability to eject zinc from purified recombinant NCp7 in vitro in a continuous fluorescence-based assay as previously described.[19] The test compound at 10 or 12 μM concentration resulted in nearly quantitative release of zinc from 1 μM NCp7, while addition of 6 μM released approximately 25% of the zinc (data not shown). The release of zinc occurs at lower concentrations of TICAs than was found for the PATEs.[19] These results demonstrate that TICAs retain NCp7 reactivity and are more active compared to our earlier described PATEs.[16]

The discovery of the susceptibility of the NCp7 protein to electrophilic agents has resulted in the identification of a suite of secondary cell-based assays as NCp7 relevant inhibitor models. The methodologies for and use of cell-based assays to assess potential Zn finger inhibitors has been extensively reported.[1a,11,14–16] Table 2 shows that compounds **5g**, **5h**, **6a**, and **6b** can inactivate cell-free virus and inhibit virus replication in acute (PBMCs and monocytes), chronic and in a post-integrative (latent) model of HIV-1 replication (TNF-α induced U1 cells). The TICAs are shown to be low micromolar inhibitors of virus replication and prevent both new infection and the expression of virus from infected cells. We have preliminary data (not shown) indicating that cross-linking of gag precursors occurred in U1 cells treated with TICAs; visualization was done by native and reducing electrophoresis followed by Western staining for NCp7 and p24.

Thus, as with previously identified NCp7 inhibitors, the TICAs maintain a broad and effective range of antiviral activity with potencies equivalent to or better than previously identified inhibitors (PATEs[16]). The lead compounds were further evaluated for their ability to inhibit a broad range of viruses, ranging from clinical field isolates and drug resistant strains of HIV-1 (laboratory-adapted and clinical isolates) to HIV-2 and simian immunodeficiency virus (SIV), and found to be active against all virus strains tested.[20] In summary, the discovery of this family of potent NCp7 inhibitors holds promise for the development of broad spectrum antiretrovirals. Discovery of TICAs that inhibit virus replication in chronically and latently infected cells should allow for the development of novel antiviral therapies that can address some of the limitations of current therapies.

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18. **5h**: To a well stirred mixture of N^{β}-(2-mercaptobenzoyl)-β-alaninamide (0.45 g, 2.0 mmol) in 8 mL DMF was added *tert*-butyl isocyanate (3.0 mmol). The reaction mixture was stirred for 8–10 h at rt. After completion of the reaction, all of the DMF was removed in vacuo and the residue was treated with ethyl ether. The white precipitate that formed was washed with water, dried in vacuo/$CaCl_2$ and crystallized from acetonitrile. Yield 89%; mp: 170–171 °C; ^{1}H NMR (DMSO-d_6) 1.30 (s, 9H, $3CH_3$), 2.38 (t, 2H, CH_2), 3.40 (q, 2H, CH_2), 6.86 δ (brs, 1H, NH_2), 7.30–7.60 (m, 5H, Ar-H, NH_2), 8.06 (s, 1H, NH), 8.26 (t, 1H, NH); MS (FAB): 324 ($M^+ + 1$).
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