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Discovery of potential dual-target prodrugs of HIV-1 reverse transcriptase and nucleocapsid protein 7

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Abstract

In the present work, we described the design, synthesis and biological evaluation of a novel series of potential dual-target prodrugs targeting the HIV-1 reverse transcriptase (RT) and nucleocapsid protein 7 (NCp7) simultaneously. Among them, the most effective compound **7c** was found to inhibit HIV-1 wild-type (WT) strain at double-digit nanomolar concentration (EC_{50} = 42 nM) in MT-4 cells, and sub-micromole (EC_{50} = 0.308 μM) to inhibit HIV-1 NL4-3 strain in TZM-bl cells. This is a significant improvement over the parent drug MT. In addition, it showed moderate inhibitory potency (EC_{50} = 1.329 μM) against the HIV-1 K103N/Y181C double mutant strain (MT-4 cells). The metabolic stability in human plasma of compound **7c** indicated that it can release the active forms of the parent drugs MT and AZT in a linear time-independent manner and turn out to be a potential prodrug.

Keywords: HIV-1, NCp7, RT, Dual-target prodrug, Anti-HIV activity, Metabolic stability

Acquired immune deficiency syndrome (AIDS), primarily caused by human immunodeficiency virus type 1 (HIV-1), remains an urgent problem in the world. Currently, combination antiretroviral therapy (cART) targeting different proteins/steps in the HIV-1 life

cycle contributes significantly to the success in treating HIV-1 infections [1,2]. Although new antiretroviral therapy for HIV has been successfully applied, infections caused by drug-resistant strains are also observed in treatment-intensive locations at the same time [3]. Therefore, there is an urgent need to exploit novel anti-HIV drugs with new scaffolds and mechanisms of action.

HIV-1 nucleocapsid protein 7 (NCp7), a 55-amino acid portion of the HIV-1 Gag polyprotein, has attracted significant attention as a next-generation target due to its highly conservative feature and pivotal role at many steps of the viral replication cycle [4]. For HIV-1 replication, NCp7 must bind to viral RNA via two zinc knuckle motifs (C-X2-C-X4-H-X4-C pattern), templating the assembly of multiple Gag polyproteins at the membrane surface to direct formation of a new viral particle [5]. NCp7 also plays an important role in HIV-1 reverse transcription. Interactions between RT and NCp7 are required for the formation of the HIV-1 RT initiation complex. In the presence of NCp7, HIV-1 reverse transcriptase (RT) changes conformation after the binding of the protein and primer-template. NCp7-faciliated annealing changes to a high-efficiency conformation after the formation of the double stranded primer-template [6]. At the completion of proviral DNA synthesis, interference in these steps leads to the improper HIV-1 maturation and loss of viral infectivity.

The compound *S*-acyl-2-mercaptobenzamide thioester (SAMT) only binds to the C-terminus of the zinc finger of the NCp7 protein and has a high selectivity. Inhibitors first acetylate Cys39 in zinc knuckle motifs of the protein through nucleophilic interaction, and then transfer to Lys33 and Lys38 through intramolecular acyl groups. Eventually, the zinc finger structure is changed and the zinc ion is expelled to inactivate the protein. Subsequently, SAMT is transferred to intramer acyl group to obtain 2-mercaptobenzamide thioester (MT). MT is acetylated with intracellular acetyl-CoA to obtain SAMT, which plays its role again (**Fig. 1**). Through recycling, the half-life of the drug is increased and the dosage of the drug is reduced. Therefore, SAMT and MT are good anti-HIV drug candidates [7]. However, compared with most drugs used to treat HIV-1 infection, the antiviral activity of NCp7 inhibitors SAMT and MT is significantly weaker. At the same time, the thioester bond in SAMT is highly susceptible to hydrolysis, making them difficult to formulate as a therapeutic product. Therefore, it is generally believed that prodrug modification of MT can improve its antiviral activity and stability [8].

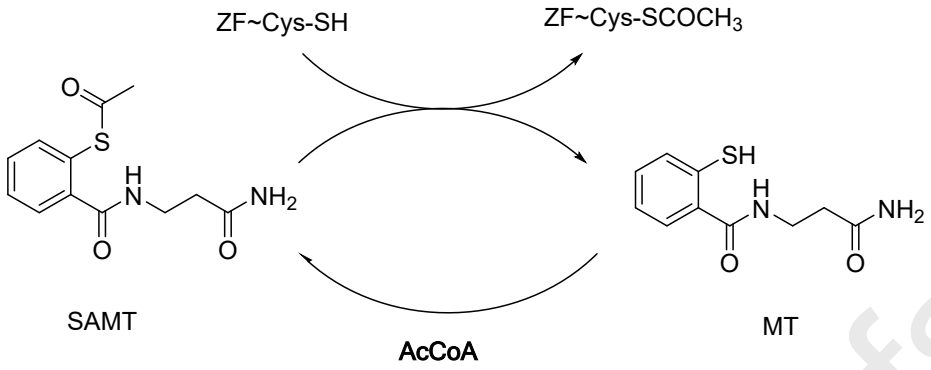


Fig. 1. *In vivo* transformation of inhibitors SAMT and MT [7].

Nucleoside HIV-1 reverse transcriptase inhibitors (NRTIs), the earliest anti-HIV drug applied in clinical practice, still plays an important role nowadays. Zidovudine (AZT) is the first anti-AIDS drug in the world approved by the US FDA because of its good antiviral activity and is still the most basic component of combination antiretroviral therapy [9]. However, with the appearance of drug-resistant strains, there is an urgent need to modify their structural resistance.

The design idea of dual-target prodrugs is that a single chemical entity molecule with two pharmacophores related to activity can simultaneously act on two targets of the same disease or two domains of a single target [10]. Dual-target prodrugs are composed of four moieties: two parent drugs, a spacer, and a specifier. The specifier, as a substrate for a specific enzyme, is often connected to the parent drug via a selfimmolative spacer. The spacer is incorporated to promote enzymatic cleavage of the specifier, and must spontaneously eliminate to release the desired active agent after specifier removal [11-13]. Single-component multi-target drugs of the same disease have an obvious advantage over single-target drugs in the treatment of dysfunctional diseases [14-16]. There have been many articles reporting dual-target prodrugs of HIV-1 inhibitors [17-19]. Since NCp7 cooperates with reverse transcriptase, we designed to link NCp7 inhibitor (MT) and reverse transcriptase inhibitor (AZT) through an ester bond linker to form a dual-target prodrug. Hydrolyzed by esterases, these prodrugs can release the original drugs to play a role (**Fig. 2**). We expect these compounds to have the advantages of their parent drugs, including activity against HIV-1 wild strain and resistant strain [20]. And we intend to explore how different lengths and types of linkers affect the antiviral activity of compounds.

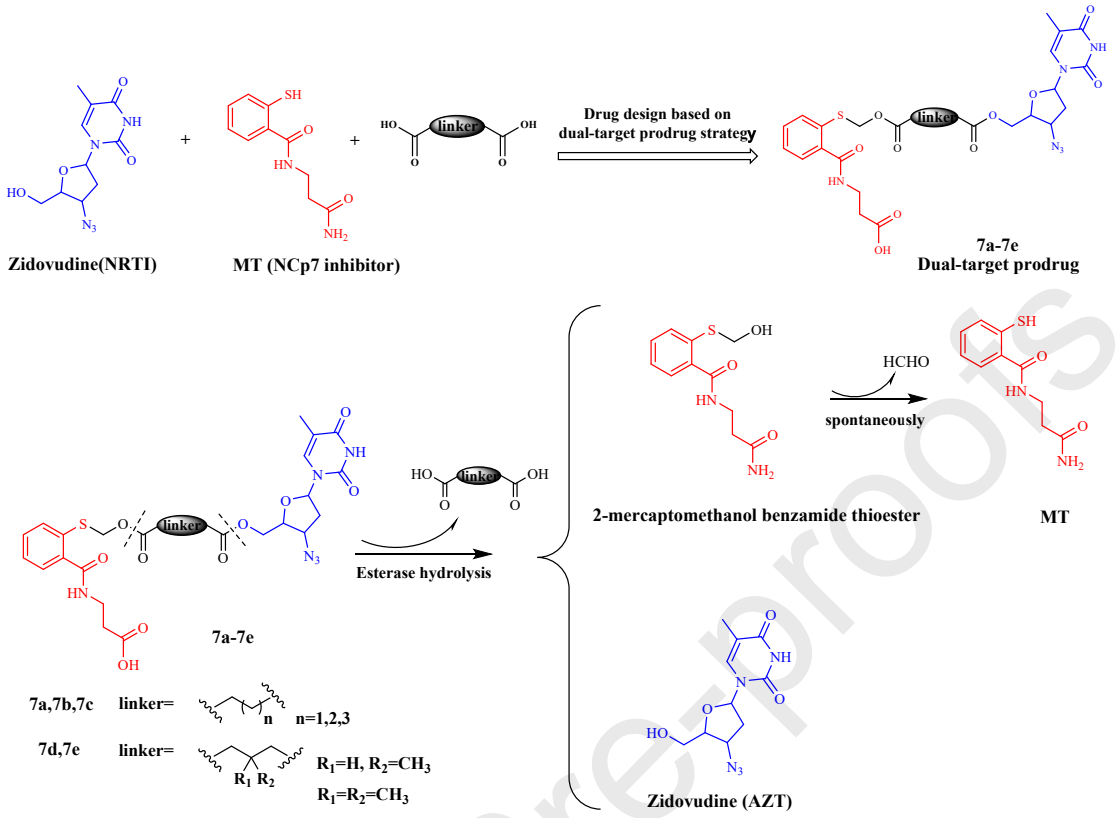
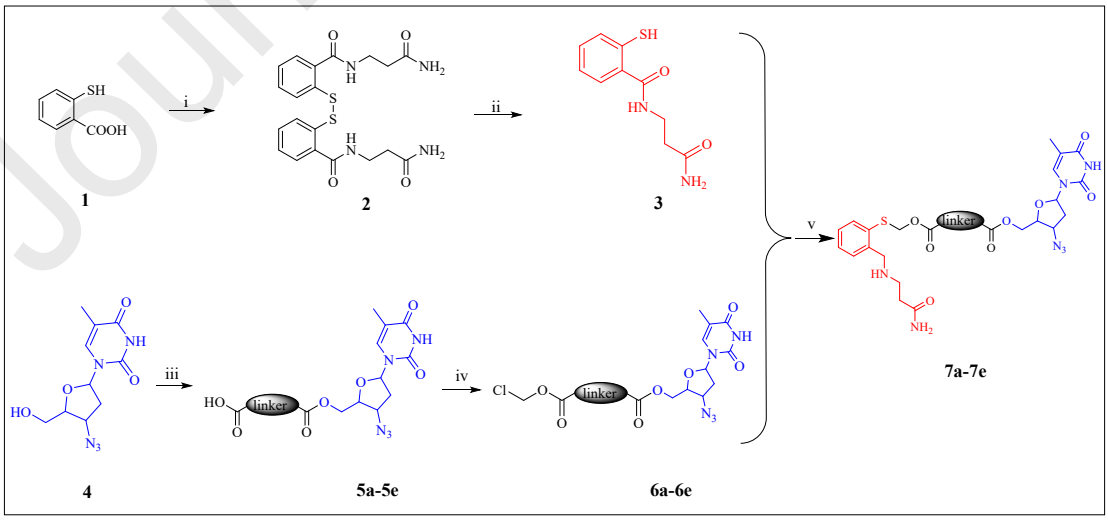


Fig. 2. Design and metabolism of NCp7 and RT targeted dual prodrugs

The synthetic routes for the newly designed dual-target prodrugs are outlined in **Scheme 1.** We synthesize the compound MT based on previous reports [8]. And then we combined MT, AZT and a linker containing ester bonds to form the target compound. The target compounds **7a-7e** were fully characterized by mass spectra (MS), proton nuclear magnetic resonance (^{1}H NMR) and carbon nuclear magnetic resonance (^{13}C NMR). The purity of compounds **7a-7e** was checked by high performance liquid chromatography (HPLC).

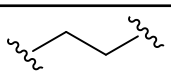
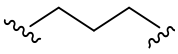
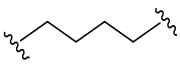
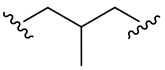


Scheme 1. Reagents and conditions: (i) *β*-alaninamide·HCL, HBTU, DIEA, DMF, r.t., overnight;(ii) TCEP·HCL, DIEA, DCM/water=9/1, r.t.,4h; (iii) Corresponding acid anhydride, DMAP, DMF, r.t.,12h; (iv) $NaHCO_3$, Bu_4N^+ •HSO_4^-, DCM, r.t., 4 h; (v) K_2CO_3, DMF,70℃ reflow, overnight;

The antiviral activity of compounds **7a-7e**, was evaluated in MT-4 cell cultures infected with HIV-1 WT strain (III_B), K103N/Y181C (RES056), one of the reverse transcriptase inhibitor resistant double-mutant strain observed in clinic. Zidovudine (AZT) and 2-mercaptobenzamide thioester (MT) were chosen as reference drugs. The results, including EC_{50}, CC_{50}, SI (selectivity index, CC_{50}/EC_{50} ratio) and FR (fold resistance, $EC_{50}^{mutant}/EC_{50}^{wt}$ ratio), are summarized in **Table 1.**

Table 1.

Antiviral potency against HIV-1 III_B and RES056 strains and cytotoxicity in MT-4 cells.

Compd.	Linker	EC_{50} (μM)[a]		CC_{50} (μM)[b]	SI[c]		FR[d]
		III_B	RES056		III_B	RES056	
7a		0.129 ± 0.099	2.402 ± 0.36	124.35	959	51.77	18.6
7b		0.068 ± 0.011	1.619 ± 0.06	>202.39	> 2994	> 125.0	23.8
7c		0.042 ± 0.025	1.329 ± 0.39	> 197.89	> 4660	> 148.9	31.6
7d		0.044 ± 0.017	1.963 ± 0.47	> 197.89	> 4500	> 100.8	44.6
7e		0.805 ± 0.511	104.4 ± 21.3	> 193.59	> 240	> 1.854	129.7
MT		5.261 ± 2.319	7.936 ± 0.13	332.44	63	41.89	1.5
AZT		0.011 ± 0.001	1.197 ± 0.07	> 7.48	> 645	> 6.249	108.8

[a] EC_{50}: concentration of compound required to achieve 50% protection of MT-4 cell cultures against HIV-1-induced cytotoxicity, as determined by the MTT method.

[b] CC_{50}: concentration required to reduce the viability of mock-infected cell cultures by 50%, as determined by the MTT method.

[c] SI: selectivity index, the ratio of CC_{50}/EC_{50}.

[d] FR: fold resistance, ratio of EC50 value against K103N/Y181C double mutant type HIV-1 over EC_{50} value against WT HIV-1 ($EC_{50}^{mutant}/EC_{50}^{wt}$).

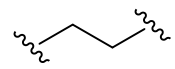
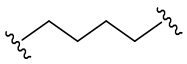
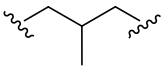
As shown in Table 1, it can be observed that some newly synthesized dual-target prodrugs are more active than the parent drug MT, and are comparable or slightly weaker than AZT. At the same time, the toxicity and selectivity index of these prodrugs on MT4 cells are superior to the two parent drugs. This series of prodrugs showed moderate to excellent activity against the WT HIV-1 strain with EC_{50} values ranging from 0.042 μM to 0.805 μM. Among them, compound **7c** exhibited highest potency against WT HIV-1 strain with an EC_{50} value of 0.042 μM, which was up to 125 times more active than that of parent drug MT (EC_{50} = 5.261 μM) and was comparable to AZT (EC_{50} = 0.011 μM). Moreover, **7c** was found to be a potent inhibitor against the HIV-1 double mutant strain RES056 (EC_{50} = 1.329 μM). It can be seen that **7c** is a potential prodrug based on HIV-1 RT with micromolar inhibitory activity against the most common HIV-1 RT inhibitor-resistant strains. Both anti-HIV activity and cytotoxicity are determined after 5-day incubation in cell-based assays using MTT method [21], the prodrug molecule should possibly transform to its parent drugs entirely and function as HIV-1 inhibitor. In brief, compound **7c** is active against the WT and mutant HIV-1 strains (FR value = 31.6).

Next, in order to verify the rationality of our dual-target prodrug strategy, we sought to test the newly synthesized compounds in a multicycle assay using fully infectious HIV-1 wild-type (NL4-3) virus and TZM-bl target cells for invitro anti-HIV-1 activity. Parent drugs MT and AZT were included as an in-line control to allow for direct comparison with the new compounds. Besides, the toxicity of MT, AZT and the new compounds towards the TZM-bl cells was also assessed. **Table 2** shows the anti- HIV potency (EC_{50}, as measured by a luciferase gene expression assay [22,23]), cytotoxicity (CC_{50}) as well as selectivity index (SI, the ratio of CC_{50}/EC_{50}) for each of the compounds and control. As shown in Table 2, it is obvious that prodrug **7c** exhibited the best activity against HIV-1 NL4-3 virus with the lowest EC_{50} values (EC_{50} = 0.308 μM). Compared with the parent drug MT (EC_{50} = 2.363 μM), the activity has been greatly improved, which proved the rationality of the dual-target prodrug strategy again. However, the activity of this series of compounds has not exceeded AZT, and further modification is needed. In addition, the antiviral activity results suggested that the linker length and the number of branches showed significant impact on the antiviral activity of this series of dual-target prodrugs. To be specific, prodrugs which bear a longer linker exhibited more potent antiviral activity (EC_{50}: 7a>7b>7c), and the activity of the prodrug is similar when there is only one branch in the linker, but the activity decreases significantly when the linker contains two

branches(EC_{50}: 7b≈7d<<7e). We speculate that the steric effect may affects the decomposition of prodrug molecules, thereby affecting the release of the parent drug and reducing the activity.

Table 2.

Anti-HIV-1 activity and cytotoxicity of the novel phenylalanine derivatives in TZM-bl cells infected with the HIV-1 NL4-3 virus.

Compounds	Linker	EC_{50} (μM)[a]	CC_{50} (μM)[b]	SI[c]
7a		0.414 ± 0.084	> 1.656	> 4.00
7b		0.332 ± 0.086	> 1.619	> 5.26
7c		0.308 ± 0.085	> 1.583	> 4.76
7d		0.522 ± 0.131	> 1.583	> 3.03
7e		> 1.55	> 1.548	> 1.00
MT		2.363 ± 0.758	82.486 ± 6.911	> 34.91
AZT		0.016 ± 0.005	> 0.374	> 23.37

[a] EC_{50}: the concentration of the compound required to achieve 50% protection of TZM-bl cells against HIV-1-induced cytopathic effect, determined in at least triplicate against HIV-1 in TZM-bl cells; values are the mean ± SD of at least two parallel tests.

[b] CC_{50}: the concentration of the compound required to reduce the viability of uninfected cells by 50%, determined in at least triplicate against HIV-1 in TZM-bl cells; values were averaged from at least four independent experiments.

[c] SI: selectivity index, the ratio of CC_{50}/EC_{50}.

Most importantly, to verify our hypothesis, **7c** was further assessed for its ability of conversion to the parent drugs MT and AZT *in vitro*. Incubate **7c** in human plasma (pH 7.4 ± 0.1) at 37 °C for 1 hour, and analyze the content of prodrug and parent drugs in plasma by LC-MS/MS. The dual-target prodrug **7c** was completely hydrolyzed by enzyme after 10 minutes of incubation. Accordingly, the content of the parent drugs MT and AZT increased over time (**Fig. 3**). To evaluate whether compound **7c** indeed acts as a potential prodrug, we further checked if the formation amount of MT was linearly with decrease amount of **7c** (within 10min). As revealed in **Fig. 4,** the linear curve fitted well (R^2 = 0.99), indicating that compound **7c** possibly

functions as a prodrug. Definitely, we should admit that the release of the parent MT from **7c** was in a quite fast manner, which is undergoing further investigation in our lab.

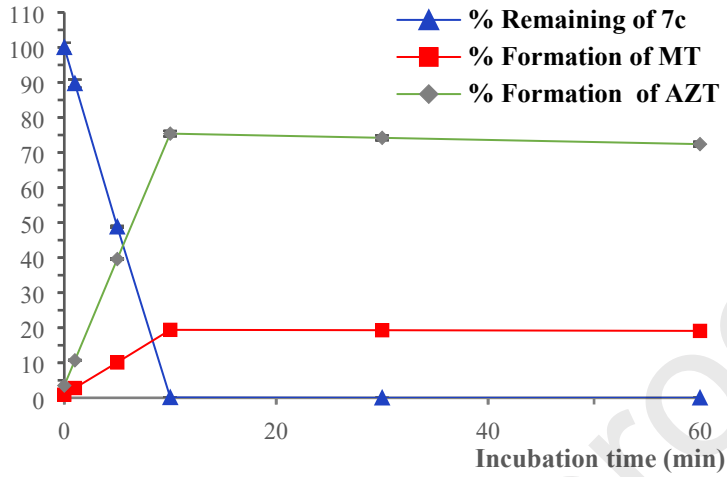


Fig. 3. % Remaining of prodrug **7c** and % formation of its parent drug MT and AZT during the 1 h test. Measured in triplicate for each time point.

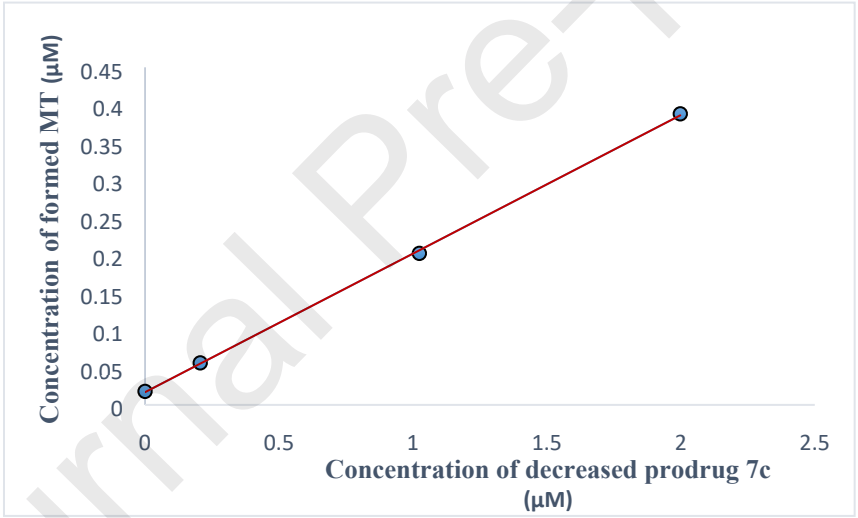


Fig. 4. Linear fit of the reduced micromolar concentration of the prodrug **7c** and the micromolar concentration of the formed MT (within 10 minutes)

In summary, we have designed compounds **7a-7e** as potential dual-target prodrugs of HIV-1 reverse transcriptase and NCp7 through a self-destructive spacer degradation. We evaluated these compounds, along with the parent drug MT and AZT for their activities against WT and the most challenging K103N/Y181C double-mutant strain of HIV-1 in MT-4 cells, as well as NL4-3 virus in TZM-bl cells. **7c** was found to inhibit the WT HIV-1 strain at nanomolar concentrations and the NL4-3 virus strain at submicromolar concentration. Compared with the parent drug MT, its activity is greatly improved, which is comparable to or slightly weaker than

AZT. Especially, compound **7c** showed potent activity against the HIV-1 K103N/Y181C double mutant, which is the first discovery of a potential dual-target prodrug inhibitor based on HIV-1 RT and NCp7. Metabolic stability test in human plasma demonstrated that **7c** could release its parent drugs MT and AZT in a linearly time-independent manner (during the 10min), and therefore likely to be a potential prodrug. Based on the obtained results, compound **7c** was initially shown to be a promising dual target prodrug of RT and NCp7.

Acknowledgements

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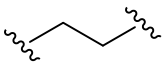
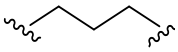
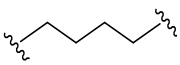
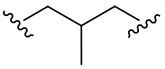
Highlights

1. Dual-target prodrug of HIV-1 reverse transcriptase inhibitor and NCp7 inhibitor.
2. Dual target prodrug modification improves antiviral activity and selectivity index.
3. The compound releases parent drugs linearly in the plasma, with prodrug properties.

Table 1.

Antiviral potency against HIV-1 III_B and RES056 strains and cytotoxicity in MT-4 cells.

Compd.	Linker	EC_{50} (μM)[a]		CC_{50} (μM)[b]	SI[c]		FR[d]
		III_B	RES056		III_B	RES056	
7a		0.129 ± 0.099	2.402 ± 0.36	124.35	959	51.77	18.6
7b		0.068 ± 0.011	1.619 ± 0.06	>202.39	> 2994	> 125.0	23.8
7c		0.042 ± 0.025	1.329 ± 0.39	> 197.89	> 4660	> 148.9	31.6
7d		0.044 ± 0.017	1.963 ± 0.47	> 197.89	> 4500	> 100.8	44.6
7e		0.805 ± 0.511	104.4 ± 21.3	> 193.59	> 240	> 1.854	129.7
MT		5.261 ± 2.319	7.936 ± 0.13	332.44	63	41.89	1.5
AZT		0.011 ± 0.001	1.197 ± 0.07	> 7.48	> 645	> 6.249	108.8

[a] EC_{50}: concentration of compound required to achieve 50% protection of MT-4 cell cultures against HIV-1-induced cytotoxicity, as determined by the MTT method.

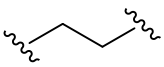
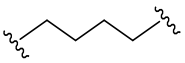
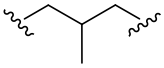
[b] CC_{50}: concentration required to reduce the viability of mock-infected cell cultures by 50%, as determined by the MTT method.

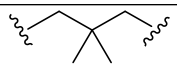
[c] SI: selectivity index, the ratio of CC_{50}/EC_{50}.

[d] FR: fold resistance, ratio of EC50 value against K103N/Y181C double mutant type HIV-1 over EC_{50} value against WT HIV-1 ($EC_{50}^{mutant}/EC_{50}^{wt}$).

Table 2.

Anti-HIV-1 activity and cytotoxicity of the novel phenylalanine derivatives in TZM-bl cells infected with the HIV-1 NL4-3 virus.

Compounds	Linker	EC_{50} (μM)[a]	CC_{50} (μM)[b]	SI[c]
7a		0.414 ± 0.084	> 1.656	> 4.00
7b		0.332 ± 0.086	> 1.619	> 5.26
7c		0.308 ± 0.085	> 1.583	> 4.76
7d		0.522 ± 0.131	> 1.583	> 3.03

7e		> 1.55	> 1.548	> 1.00
MT		2.363 ± 0.758	82.486 ± 6.911	> 34.91
AZT		0.016 ± 0.005	> 0.374	> 23.37

[a] EC_{50}: the concentration of the compound required to achieve 50% protection of TZM-bl cells against HIV-1-induced cytopathic effect, determined in at least triplicate against HIV-1 in TZM-bl cells; values are the mean ± SD of at least two parallel tests.

[b] CC_{50}: the concentration of the compound required to reduce the viability of uninfected cells by 50%, determined in at least triplicate against HIV-1 in TZM-bl cells; values were averaged from at least four independent experiments.

[c] SI: selectivity index, the ratio of CC_{50}/EC_{50}.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

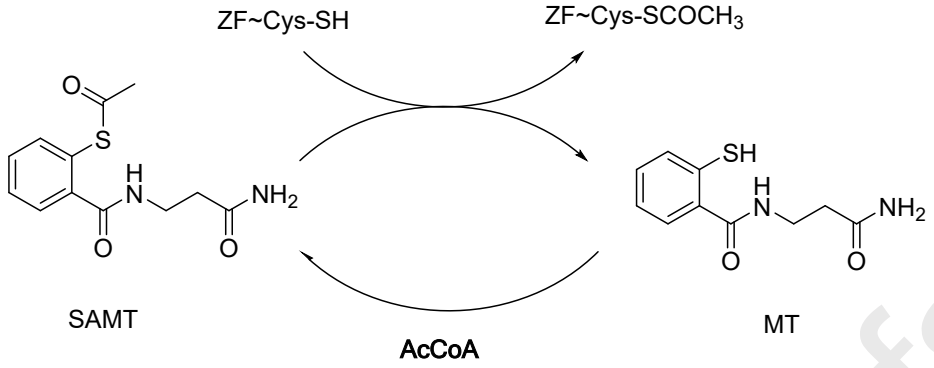


Fig. 1. *In vivo* transformation of inhibitors SAMT and MT.

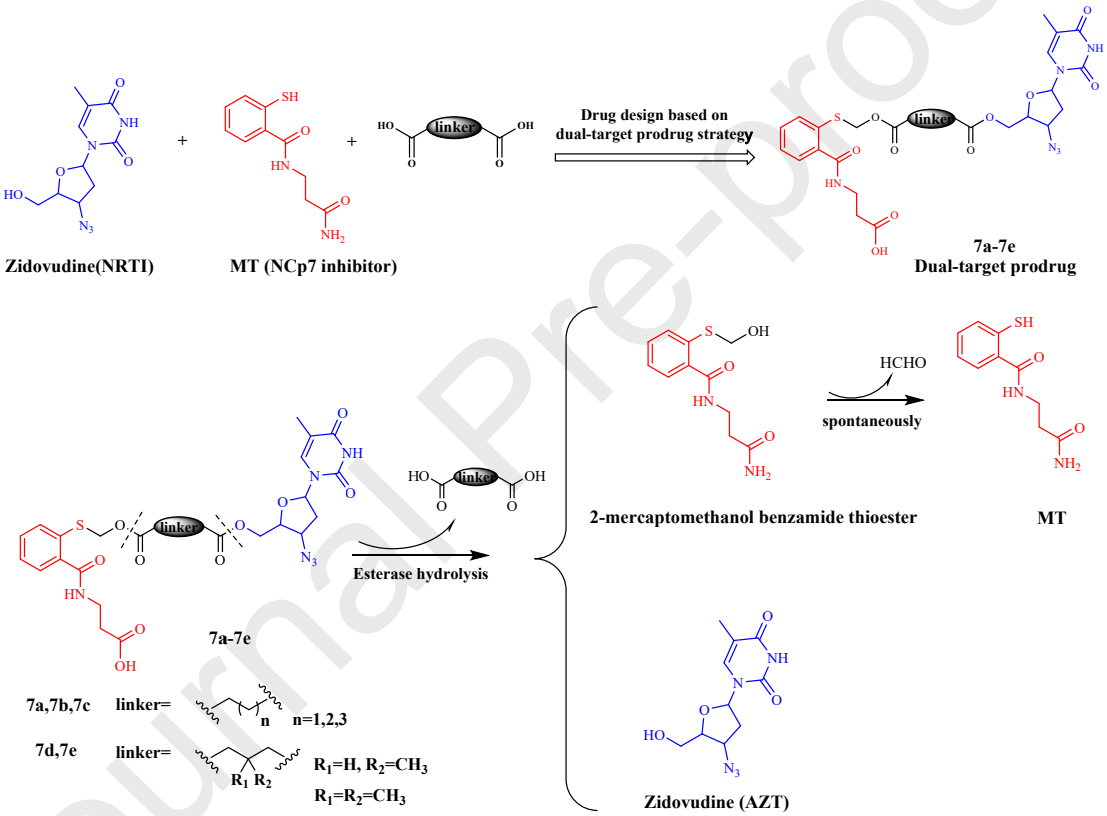


Fig. 2. Design and metabolism of NCp7 and RT targeted dual prodrugs

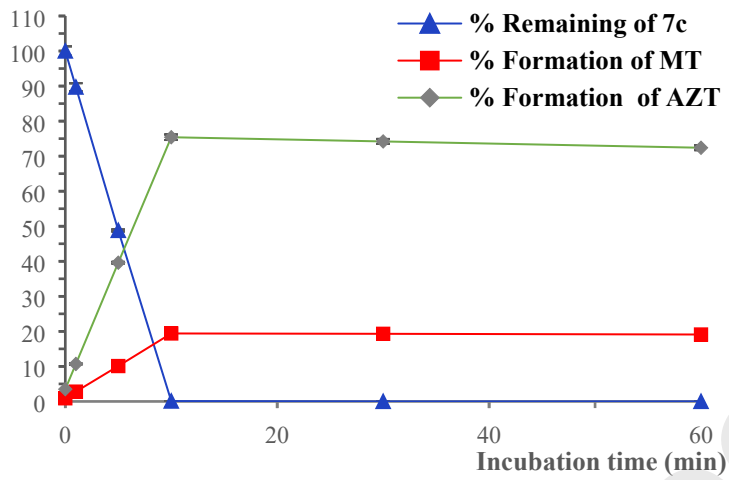


Fig. 3. % Remaining of prodrug **7c** and % formation of its parent drug MT and AZT during the 1 h test. Measured in triplicate for each time point.

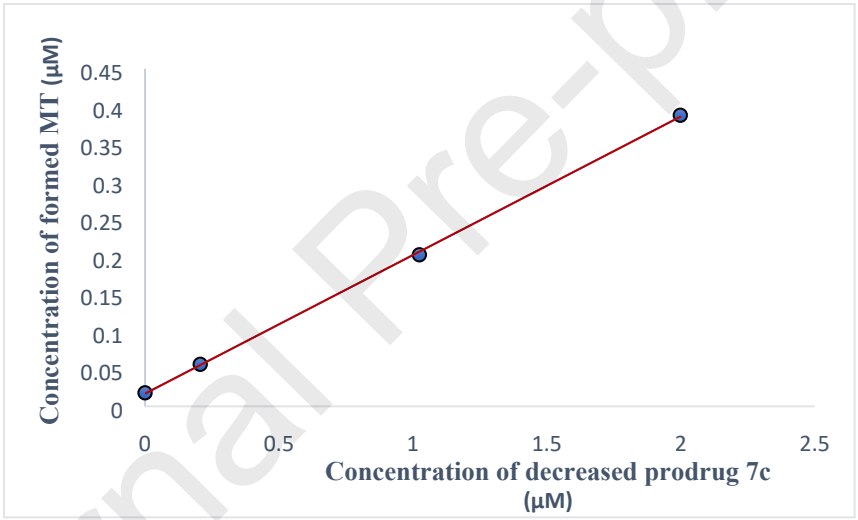
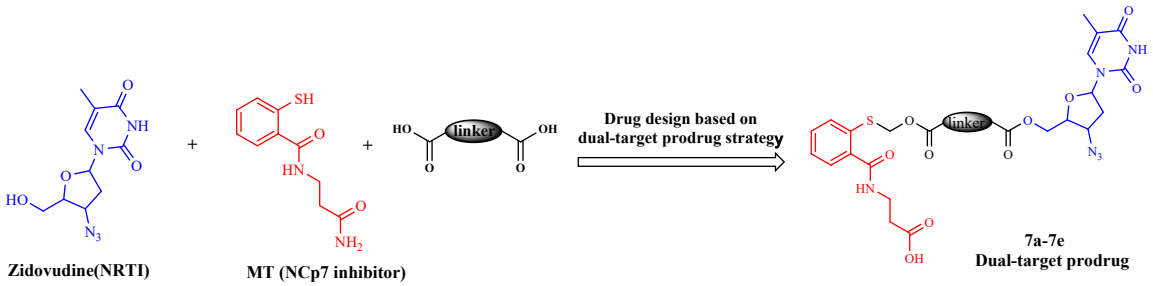


Fig. 4. Linear fit of the reduced micromolar concentration of the prodrug **7c** and the micromolar concentration of the formed MT (within 10 minutes)

Graphical abstract



Zidovudine(NRTI)

EC_{50} (μM) =

0.011 (MT4, III_B)
1.197 (MT4,RES056)
0.016 (TZM-bl, NL4-3)

CC_{50} (μM) =

> 7.48 (MT4)
> 0.347 (TZM-bl)

MT (NCp7 inhibitor)

EC_{50} (μM) =

5.261 (MT4, III_B)
7.936 (MT4,RES056)
2.363 (TZM-bl, NL4-3)

CC_{50} (μM) =

332.44 (MT4)
82.49 (TZM-bl)

7a-7e
Dual-target prodrug

EC_{50} (μM) =

0.042 ~ 0.129 (MT4, III_B)
1.329 ~ 104.4 (MT4,RES056)
0.308 ~ >1.55 (TZM-bl, NL4-3)

CC_{50} (μM) =

124.53 ~ >202.39 (MT4)
> 1.548 (TZM-bl)