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Synthesis of the biotinylated anti-HIV compound BMMP and the target identification study

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ABSTRACT

BMMP [2-(benzothiazol-2-ylmethylthio)-4-methylpyrimidine], an inhibitor of HIV-1 replication, was linked to biotin to study the interaction with the presumed target, HIV-1 $Pr55^{Gag}$ or CA, by means of surface plasmon resonance. The synthesized Biotin–BMMP inhibited HIV-1 replication to a similar extent as BMMP alone, but did not interact with $Pr55^{Gag}$ or CA, suggesting that BMMP exerts its activity by a different mechanism.

Acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus type 1 (HIV-1), was previously fatal in all patients. Since the development of anti-retroviral therapy (ART),[1] the use of multiple drugs targeting the viral reverse transcriptase, protease and integrase as well as viral entry has allowed patients to survive the disease. However, the disadvantages of ART therapy include drug resistance and other side effects. Thus, efforts to develop new anti-HIV drugs acting through novel mechanisms are ongoing.

One attractive approach is to treat HIV to block the function of the HIV-1 protein Gag.[2] When it is present in a cell which is producing virus particles, Gag mainly exists as the polyprotein precursor $Pr55^{Gag}$. Concurrently or immediately after budding of the virion, the HIV-1 protease cleaves $Pr55^{Gag}$ into four main proteins (matrix: MA, capsid: CA, nucleocapsid: NC and p6) and two spacer peptides (p2 and p1), to produce the mature virion capable of target cell infection. In both producer and target cells, immature and mature Gag proteins play various central roles.[3]

In 2011, Urano et al. explored small molecule inhibitors of the oligomerization of HIV-1 $Pr55^{Gag}$,[4] a key process essential for virion production,[5] and identified 2-(benzothiazol-2-ylmethylthio)-4-methylpyrimidine (BMMP) (Fig. 1) as an inhibitor of HIV-1 replication by an unknown mechanism. Contrary to the generally accepted prediction of its mechanism of action, BMMP did not inhibit the virion release stage.[4] Instead, BMMP suppressed the reverse transcription of the virus genome in target cells, and the protein responsible for interacting with BMMP was shown to be CA.[4] This suggests the possibility that BMMP directly binds to the CA domain of $Pr55^{Gag}$ or the CA protein. To examine this hypothesis, we synthesized biotinylated BMMP (Biotin–BMMP), confirmed that Biotin–BMMP retains the activity of BMMP alone, and examined its interaction with $Pr55^{Gag}$ or CA using biotin–avidin technology.

Biotin–BMMP (Fig. 1) carries a biotin on the pyrimidyl–methyl group of BMMP through a methylene linker. Synthesis of Biotin–BMMP began with the construction of the mercaptopyrimidine-linker fragment which was then coupled with the benzothiazole moiety, followed by conjugation with biotin as shown in Scheme 1.

Benzothiazole-2-carboxaldehyde **1** was reduced by $NaBH_4$ to afford **2** quantitatively. Treatment of **2** with PBr_3 gave bromide **3** in 57% yield. *N*-(5-Bromopentyl)phthalimide **4** was treated with the sodium enolate of ethyl acetoacetate to furnish **5** in 79% yield. Acid hydrolysis and decarboxylation of **5** afforded **6** in 90% yield. Reaction of **6** and *N*,*N*-dimethylformamide dimethyl acetal in the presence of BF_3 gave **7** in 64% yield. Treatment of **7** with thiourea and sodium ethoxide furnished the mercaptopyrimidine compound **8** that was used without further purification and coupled with the benzothiazole **3** in the presence of Et_3N to afford **9** in 49% overall yield. Treatment of **9** with hydrazine gave the free

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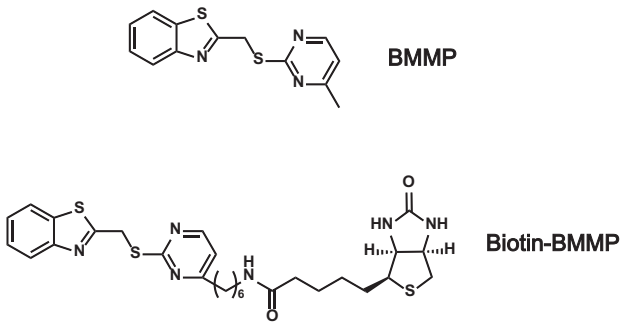


Figure 1. Structures of BMMP and Biotin–BMMP.

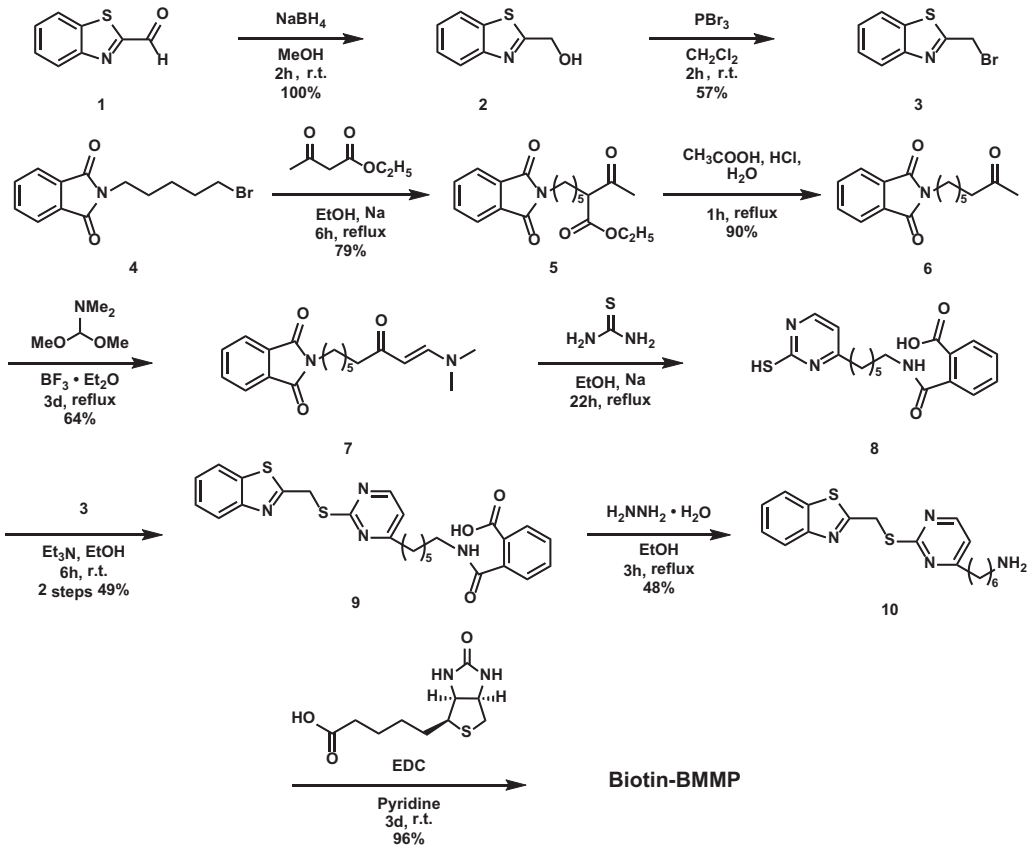
amine **10** in 48% yield. Finally, **10** was conjugated with biotin using EDC to furnish Biotin–BMMP in 96% yield. The total yield of Biotin–BMMP from **4** in 7 steps was 10%.

To confirm that Biotin–BMMP retained the biological activity of BMMP, its inhibitory effect on HIV-1 replication was examined and compared with BMMP, the synthetic intermediate **10**, and the biotin methyl ester. To obtain cell-free HIV-1 for infection, pNL4-3[6] coding the full length HIV-1 was transfected into 293T human embryonic kidney cells and incubated. The supernatant at 2 days post transfection containing virus was inoculated into the human T-lymphoblastoid cell line M8166, and incubated for approximately 5 days in the presence of each chemical (20 μM). The virus amounts in the supernatant were monitored by p24 (Gag CA) ELISA, and the obtained growth curve is shown in Figure 2A.

In agreement with the previous Letter,[4] BMMP suppressed HIV-1 replication. It was shown that Biotin–BMMP and the BMMP-linker compound **10** had inhibitory activity, although the activity of Biotin–BMMP was somewhat weaker than that of BMMP. In contrast, the biotin methyl ester, which represents the biotin moiety of Biotin–BMMP, did not show any activity. This indicated that the BMMP part of Biotin–BMMP was responsible for the inhibition of HIV-1 replication, presumably by binding to the $Pr55^{Gag}$ or CA target protein. It is likely that the 4-methyl group of the pyrimidine ring is located in an appropriate position without affecting the binding and thus, function, of the BMMP.

The interaction between BMMP and $Pr55^{Gag}$ or CA protein was examined using Biotin–BMMP. We previously established a highly sensitive surface plasmon resonance method[7] to analyze the hydrophilic and hydrophobic interactions between $Pr55^{Gag}$/MA and inositol phosphates conjugated to biotin. This method was applied to the analysis of the BMMP–$Pr55^{Gag}$ or –CA interaction. Biotin–BMMP in buffer was incubated with streptavidin covalently immobilized on the surface of a BIACORE sensor chip, and conjugation of Biotin–BMMP with the sensor chip was confirmed by its response. Then, $Pr55^{Gag}$ or CA protein, both prepared from 293T cells overexpressing the protein of interest, was passed over the sensor chip. As shown in Figure 2B, no interactions between either of the proteins with the conjugated Biotin–BMMP were observed. Since the interaction of $Pr55^{Gag}$ and phosphoinositide was observed using the same method,[7a] we concluded that BMMP did not interact with $Pr55^{Gag}$ or CA in solution.

There have been reports that the heterocyclic compounds PF-3450074,[8] I-XW-053[9] and BI-1/2[10] interact with CA, and inhibit HIV replication at the post-entry step. These compounds were shown to directly bind to specific portions of CA. In this study, we concluded that BMMP inhibits HIV replication through a mechanism which is different from that employed by the heterocyclic compounds. As BMMP was identified as an inhibitor of HIV replica-



Scheme 1. Synthesis of Biotin–BMMP.

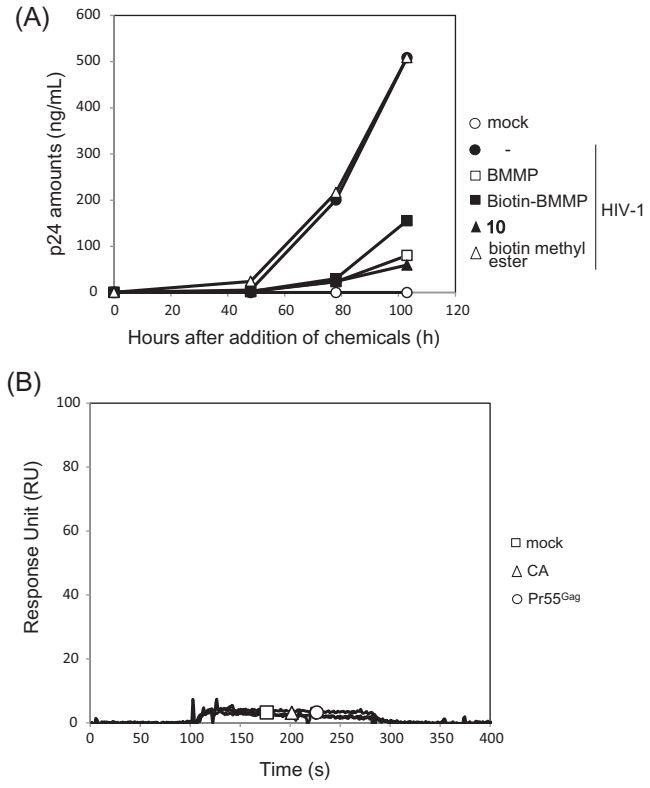


Figure 2. Biological experiments using Biotin–BMMP. (A) Growth kinetics of HIV-1 NL4-3 strain in M8166 cells in the presence of Biotin–BMMP and its related chemicals (20 μM). The supernatant of 293T cells transfected with pNL4-3[6] was inoculated into M8166 cells. After 16 h, chemicals were added and cultured. At the selected time points, the virus amounts in the supernatant were examined by p24 ELISA. As a control, the supernatant of 293T cells transfected with pUC19 was used instead of the virus solution. (B) The binding activity of CA or $Pr55^{Gag}$ protein to Biotin–BMMP. The proteins were prepared as previously described.[7a] Then each protein was incubated on a Biotin–BMMP immobilized BIACORE sensor chip.

tion by cell-based screening and not by a molecular target approach,[4] it is not inconceivable that the direct target of BMMP is something other than $Pr55^{Gag}$ or CA protein.

In summary, we synthesized Biotin–BMMP, and confirmed its usefulness as a tool for HIV target identification. Surface plasmon resonance experiments using Biotin–BMMP suggested the target of BMMP other than $Pr55^{Gag}$ or CA protein. We are now in the process of determining the true target of BMMP by proteomic analysis of viral and host factors using Biotin–BMMP.

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Supplementary data

Supplementary data (experimental procedures and characterization of the compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.11.036.

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