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Preparation and activities of macromolecule conjugates of the CCR5 antagonist Maraviroc

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Abstract

CCR5 antagonists are among the most advanced approaches in HIV therapy and may also be relevant to treatment of graft-versus-host disease and *Staphylococcus aureus* infection. To expand the potential of the only approved CCR5 antagonist, Maraviroc, we studied derivatives that would enable functional linkage of Maraviroc to long-lived carriers. Through targeted synthesis, we discovered an effective linkage site on Maraviroc and demonstrate the potential of these derivatives to prepare potent chemically programmed antibodies and PEGylated derivatives. The resulting compounds effectively neutralized a variety of HIV-1 isolates. Both chemically programmed antibody and PEGylation approaches extend the neutralization activity of serum circulating Maraviroc. Derivation of a successful conjugation strategy for Maraviroc should further enable its use in chemically programmed vaccines, novel bispecific antibodies, and topical microbicides.

Keywords

CCR5 antagonist; Maraviroc; Chemically programmed antibody; PEGylation

HIV-1 infection is typically managed by a treatment regimen known as highly active antiretroviral therapy or HAART, which commonly involves the administration of combinations of reverse transcriptase and protease inhibitors. Viral escape, drug side effects and compliance issues, however, continue to drive the development of novel approaches, which most recently have seen the approval of entry, fusion and integrase inhibitors.[1] Of these more recent innovations, entry inhibitors that target conserved host proteins are particularly intriguing. The accepted mechanism of HIV infection involves initial attachment of the virus to the host cell receptor CD4 via interaction with the viral gp120 envelope protein. This binding event then triggers a conformational change in the envelope protein that provides for binding to chemokine co-receptors CCR5 or CXCR4 and finally membrane fusion after viral gp41 insertion into the target cell (Figure 1).[2–7] Blockade of chemokine receptor engagement by the virus therefore blocks infection. To date the only approved chemokine receptor targeted inhibitor is Maraviroc (**1**, Figure 2), a potent CCR5 antagonist

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ASSOCIATED CONTENT

Supporting Information. Synthetic procedure, analytical data, and procedures for neutralization assay and FACS. This material is available free of charge via the Internet at http://pubs.acs.org.

Any additional relevant notes should be placed here.

that received FDA approval in 2007.[8–9] Maraviroc treatment regimens, however, require twice daily dosing with as much as 1.2 grams of drug per day making patient compliance an issue. Significantly, the biology of CCR5 is not limited to HIV-1 and recent studies have demonstrated beneficial activity of Maraviroc therapy in graft-versus-host disease[10] and *Staphylococcus aureus* pathogenesis.[11] Thus the development of Maraviroc derivatives with extended pharmacokinetic profiles could be a valuable contribution to therapy.

Chemically programmed antibodies (cpAbs),[12–19] which link a catalytic antibody to a small molecule drug, peptide, or aptamer dramatically extend the pharmacokinetic profile of the attached molecule. Chemical programming of the monoclonal antibody (mAb) 38C2 is facilitated by a low pKa lysine residue located in the 38C2-binding site. This lysine is key to 38C2 aldolase activity and can be site-selectively labelled with *N*-acyl-β-lactams to produce a chemically programmed antibody.[20–22] The cpAb approach has demonstrated efficacy in a number of disease models including anti-infectives and the relative merits of cpAbs over conventional mAbs have been well documented.[12] For example with a derivative of Zanamivir, a neuraminidase inhibitor, the cpAb approach provided long-term systemic exposure without loss of neuraminidase inhibitory activity.[18] Another approach to extending the pharmacokinetic profiles of drugs involves their conjugation to polyethylene glycol (PEG), a process known as PEGylation. PEGylation often imparts other significant pharmacological advantages, such as improved solubility, minimized proteolytic cleavage, reduced dosage frequency, increased serum half-life, and reduced immunogenicity and antigenicity.[23–25] PEGINTRON®, an α-interferon derivative, is the first FDA-approved, PEG-modified drug. The plasma circulating half-life of PEGINTRON, which is used for treatment of hepatitis C, is about 10 times that of native IFN α-2b and allows weekly subcutaneous dosing.[26] PEGylation also imparts desired properties on small molecule drugs.[27–28] Pepinsky et al. reported that PEGylation dramatically improves pharmacodynamics and pharmacokinetic properties of an integrin α4β1 inhibitor.[28] Key to the development of effective chemically programmed antibodies and PEGylated small molecules is the discovery of a linkage chemistry that minimally impacts activity of the parental drug. Herein, we describe the structure activity relationships of linked Maraviroc derivatives and macromolecular conjugates with mAb 38C2 and polyethylene glycol variants designed to create potent long-lived CCR5 antagonists.

In our previous study of the CCR5 antagonist Aplaviroc, we showed that linkage of the small molecule to mAb 38C2 through a benzoic acid moiety resulted in a cpAb with potent activity.[16] Aplaviroc was dropped from clinical development due to toxicity. To avoid concerns regarding toxicity of Aplaviroc, we were compelled to study the more challenging antagonist Maraviroc. We decided to explore two routes towards linked variants of Maraviroc. The first westerly linkage point was chosen based on ease of synthesis and known structural tolerance at the cyclohexyl position. To explore this linkage directionality we synthesized compounds **2a** and **2b**. A docking study of CCR5 antagonists performed by Kondru et al. suggests that the benzoic acid moiety of Aplaviroc and the triazole moiety of Maraviroc overlap in the putative binding pocket.[29] Since we had successfully introduced a linker on Aplaviroc at the benzoic acid position, the modeling encouraged us to explore introduction of a linker onto the triazole ring of Maraviroc (easterly connection) and we synthesized compound **3** to explore this point of connectivity.

The routes used for the syntheses of the mAb derivatives of Maraviroc are shown in Scheme 1. Azide compounds **2a** and **3**, synthesized as illustrated in Scheme S2 and S3, were converted to *N*-acyl-β-lactam compounds **4** and **5** by click reaction with the corresponding alkyne compounds (**S21**, Scheme S4). *N*-Acyl-β-lactam compounds were conjugated to mAb 38C2 after incubation at room temperature in PBS. The time dependency of the conjugation reaction of **5** with mAb 38C2 was evaluated by measuring the catalytic activity of retro-

aldol reaction of methodol.[30] The reaction was complete in 2 hours (Figure S1). The lack of catalytic activity of the product was confirmed (Figure S2), and cpAbs **6** and **7** were purified by size-exclusion column chromatography to remove excess small molecule.

Linear and branch PEGylated compounds were prepared by simple amidation of *N*-hydroxysuccinimide (NHS) esters (Scheme 2). PEGylation of *n*-propylamine or Maraviroc-attached propylamine (**9**), which was obtained by deprotection of Boc group in compound **8**, with the commercially available PEG-NHS derivatives gave the desired PEGylated compounds (**10**, **11**, **12**, and **14**). All PEGylated compounds were purified by precipitation from Et_2O. None of the small molecule remained in the obtained solid as confirmed by HPLC (Figure S6). MALDI-TOF and/or ESI mass analysis of cpAbs and PEGylated compounds were in agreement with expected values (Figure S3–5).

We initially studied the azide linker derivatives **2a** and **2b** and evaluated their activity for neutralization of HIV-1 JR-FL reporter virus. The activity of **2a** (westerly connection replacing the cylcohexyl group) was two orders of magnitude less than that of parental Maraviroc **1** (Table 1). The activity of compound **2b** with a PEG linker was further reduced relative to **2a**. Previous studies of Maraviroc derivatives showed that the amide site we used for connectivity in these compounds should interact with a predominantly lipophilic binding site on the CCR5 receptor and this result is consistent with the lower activity of **2b** as compared with **2a**.[31] By contrast, compound **3**, modified on the triazole ring had potent activity (easterly connection).

N-Acyl-β-lactam derivatives of these compounds were prepared through a standard click reaction and the lactams used for conjugation to mAb 38C2. Surprisingly, the activity of *N*-acyl-β-lactam-linked Maraviroc derivative **4** was significantly higher than that of **2a**. In contrast, the activity of *N*-acyl-β-lactam **5** was 2.6 nM, less active than **3** and comparable to that of Maraviroc **1**. Unmodified antibody, mAb 38C2, did not neutralize the JR-FL HIV-1 reporter virus. The activity of the antibody conjugate of **5**, cpAb **7**, was higher than that of the conjugate of **4**, cpAb **6**. As shown in Figure 3 and Table S1, we evaluated the binding affinity of both cpAbs by FACS using TZM-bl cells, which derive from a HeLa cell line and are transfected to express CCR5, and CCR5 negative control cells (HeLa). cpAbs **6** and **7** bound to CCR5-expressing TZM-bl cells as did positive control mAb 2D7. None bound CCR5-negative HeLa cells (Figure 3). The IC_{50} of cpAbs **6** was 19 nM and that of **7** was 7.7 nM. The neutralization activity for these cpAbs with the JR-FL HIV-1 strain is summarized in Table 1.

Past studies have indicated that short PEG chains may not provide enough protection from metabolism and may not alter clearance rates significantly, however, long PEG chains may interfere with binding to target proteins.[32] The constitution (linear vs. branched) of the polymer also affects half-life.[33] Boc-protected compound **8**, the precursor for the PEGylated compound had activity similar to that of *N*-acyl-β-lactam compound **5**. Derivatives of Maraviroc with 5 kDa and 40 kDa linear PEG chains were synthesized by amidation of **9** with the NHS-PEG derivatives. In this case, the length of a PEG chain did not significantly influence the activity but PEGylated compounds **10** (5 kDa) and **11** (40 kDa) were approximately 15 times less active than the parent small molecule **8**. The antibody conjugate **7**, carries two molar equivalents of Maraviroc, and was approximately 6-fold more active than the monovalent PEGylated Maraviroc derivatives. This difference was likely due to enhanced avidity by the multiple interactions of the cpAb with target.[34] To improve the activity of monovalent PEGylated compound, a branched derivative was synthesized. Attachment of four Maraviroc moieties to a single PEG (compound **12**; >98% purity) dramatically enhanced neutralization activity relative to the linear PEGylated compounds.

The activities of cpAb **7** and PEGylated derivative **12**, which had the highest activity against HIV-1 JR-FL of those derivatives tested were evaluated in neutralization assays against HIV-1 strains from clades A, B, and C. Representative inhibition curves are shown in Figure 4 and Figures S7–S10 and IC_{50} values are listed in Table 2. Both Maraviroc conjugates had broad spectrum of activity similar to that of the parent compound.

In order to investigate the stability of cpAb **7** and the PEGylated Maraviroc derivative **12**, we incubated each compound with human serum at 37 °C and evaluated their HIV neutralization activity at multiple time points. Unlike **1**, both **7** and **12** retained their full activity for up to 10 days (Figure S11), indicating that cpAbs and PEGylation are a potentially effective means for enhancing Maraviroc serum stability and extending neutralization activity. Lastly, while Maraviroc is known to cause only minor side effects, the toxicity of conjugates **7** and **12** is unknown.

In conclusion, we discovered that the triazole ring of the CCR5 antagonist Maraviroc could be derivatized for linkage to macromolecules without significant loss of activity. In contrast, modifications on the westerly side of the molecule, which presents a lipophilic amide in the parental Maraviroc, reduced activity relative to the parent. Antibody conjugate **7** and PEGylated compound **12** effectively neutralized HIV-1 strains from four clades with IC_{50}s similar to those of Maraviroc. As previously reported, cpAbs and PEGylation strategies dramatically extend the circulating serum half-life of conjugated molecules relative to small molecule,[18, 28] peptide, and protein parent drugs. Thus we anticipate that **7** and **12** will have dramatically extended pharmacokinetic properties and warrant further study in anti-HIV models. While the discovery of a viable site of conjugation for this promising drug has allowed us to establish good antiviral activity in the case of a chemically programmed antibody and a PEGylated derivative, their application in chemically programmed vaccine,[35] chemical approaches to bi-specific antibodies[36] and topical microbicides whose construction is also hereby facilitated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CCR5	C-C chemokine receptor type 5
CXCR4	C-X-C chemokine receptor type 4

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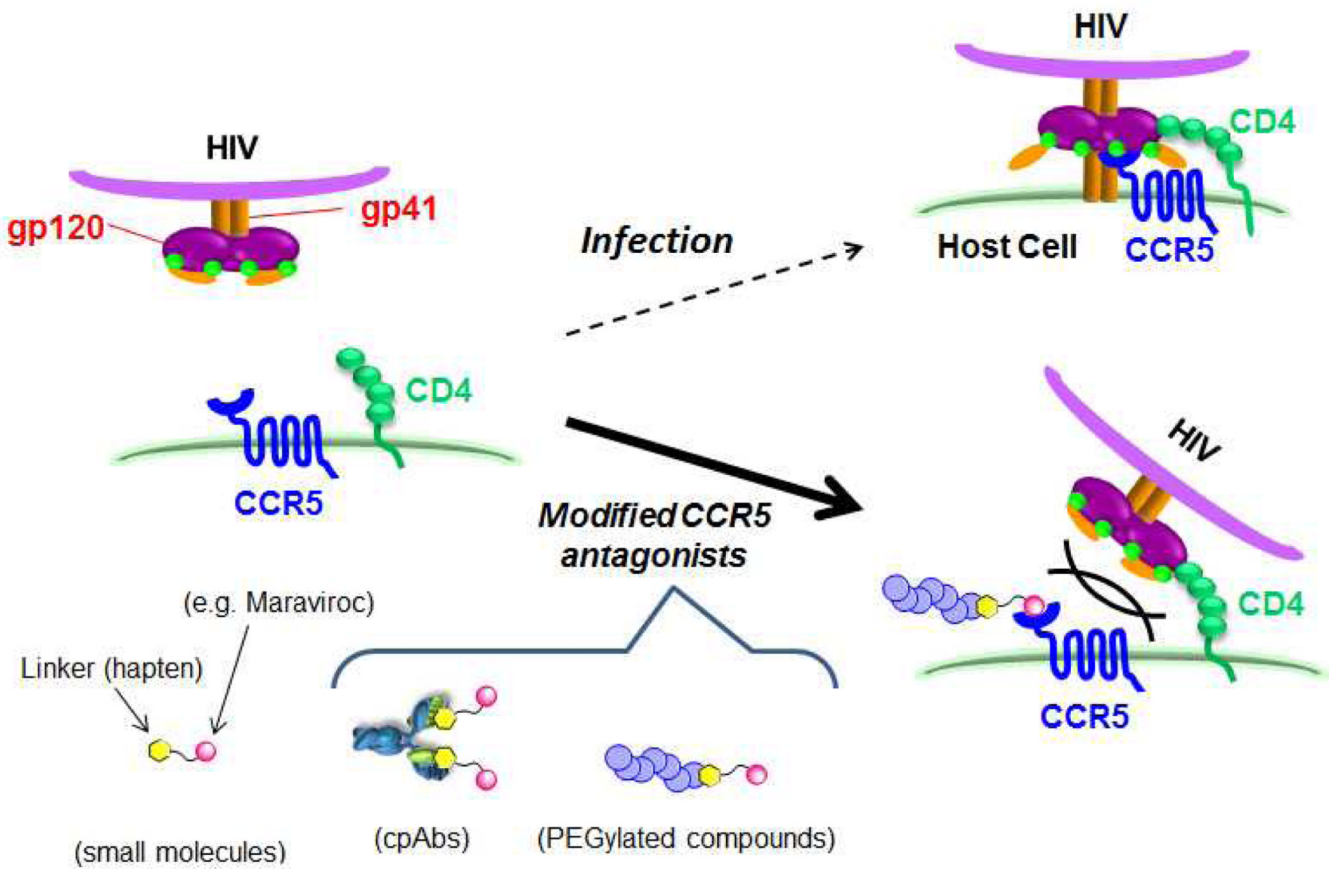


Figure 1.
Schematic representations of mechanism of HIV-1 infection of a host cell and inhibition of the viral entry by CCR5 antagonists.

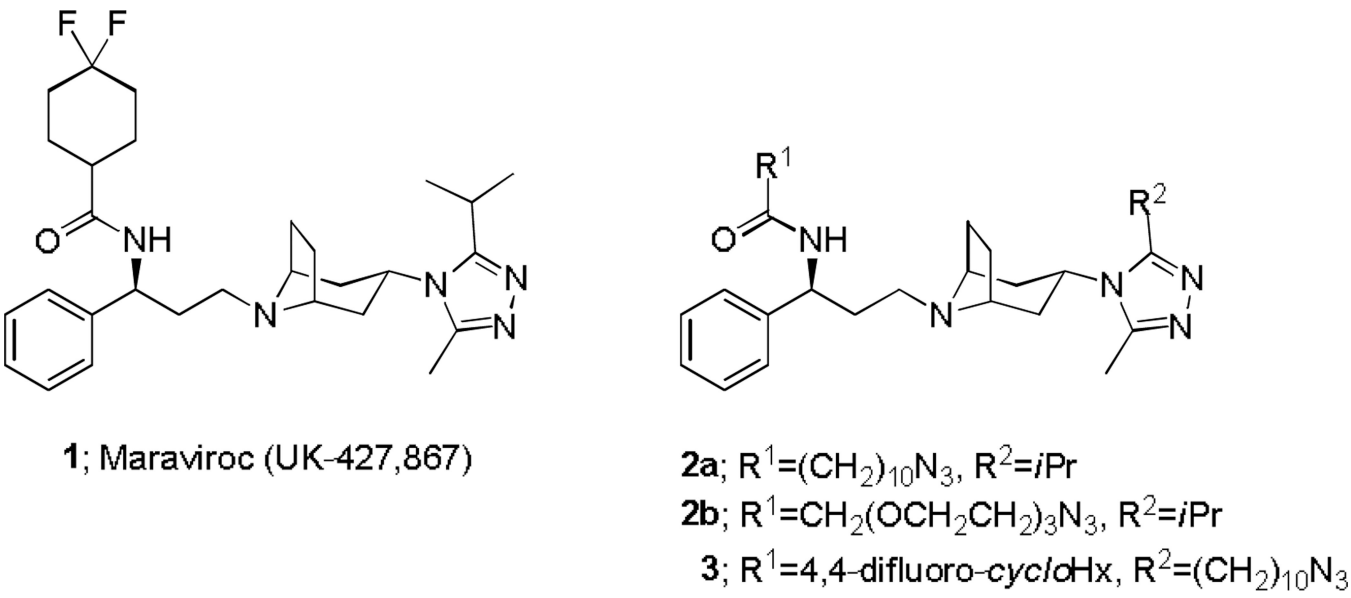


Figure 2.
Structure of Maraviroc (1) and linker-attached Maraviroc (2a, 2b, and 3).

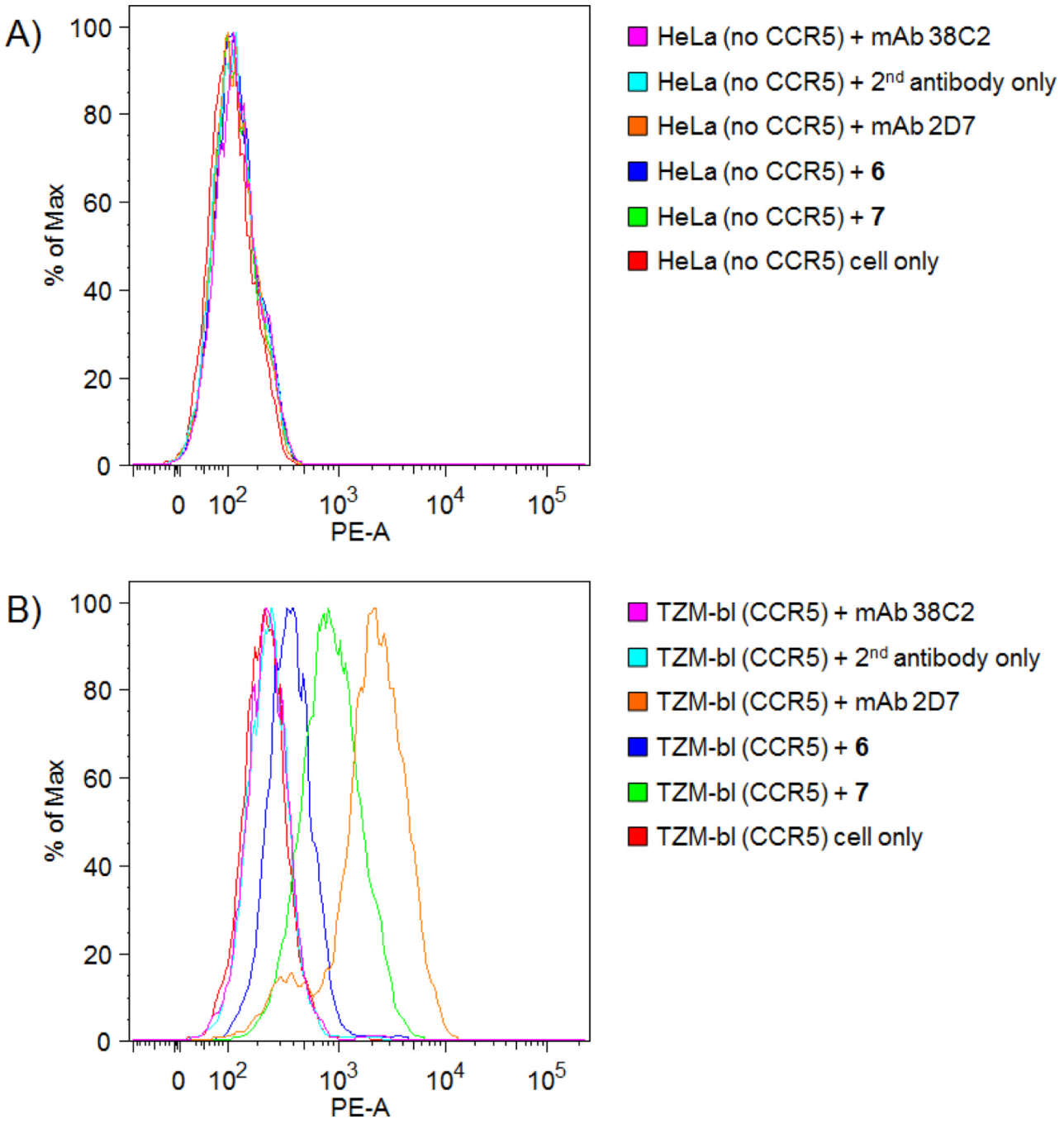


Figure 3.

Binding of mAb 38C2, mAb conjugates **6** and **7** and positive control mAb 2D7 to A) HeLa cells, which do not express CCR5 and to B) TZM-bl cells, which are CCR5-positive, by FACS. mAb 38C2 (pink), secondary antibody (light blue), mAb 2D7 (orange), compound **6** (blue), compound **7** (light green), cell only (red).

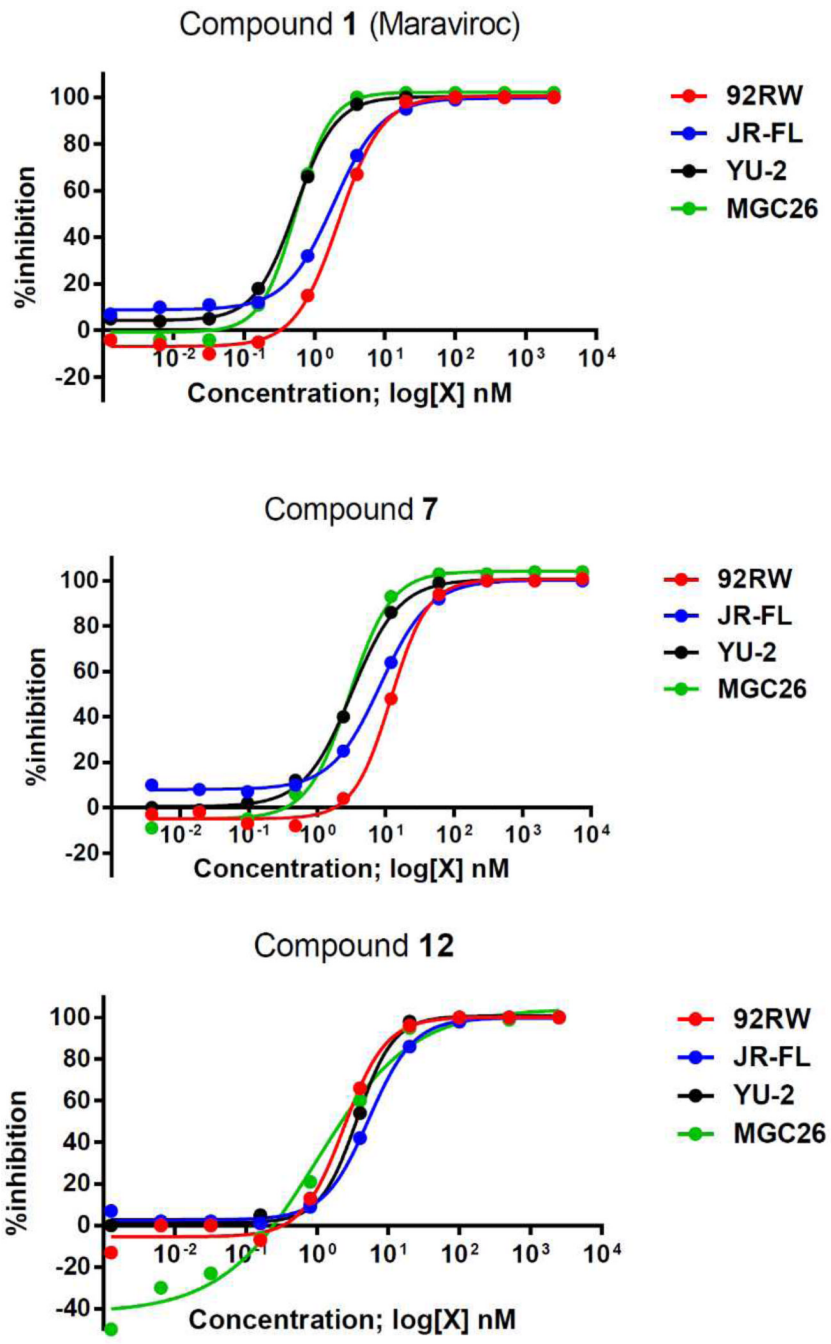
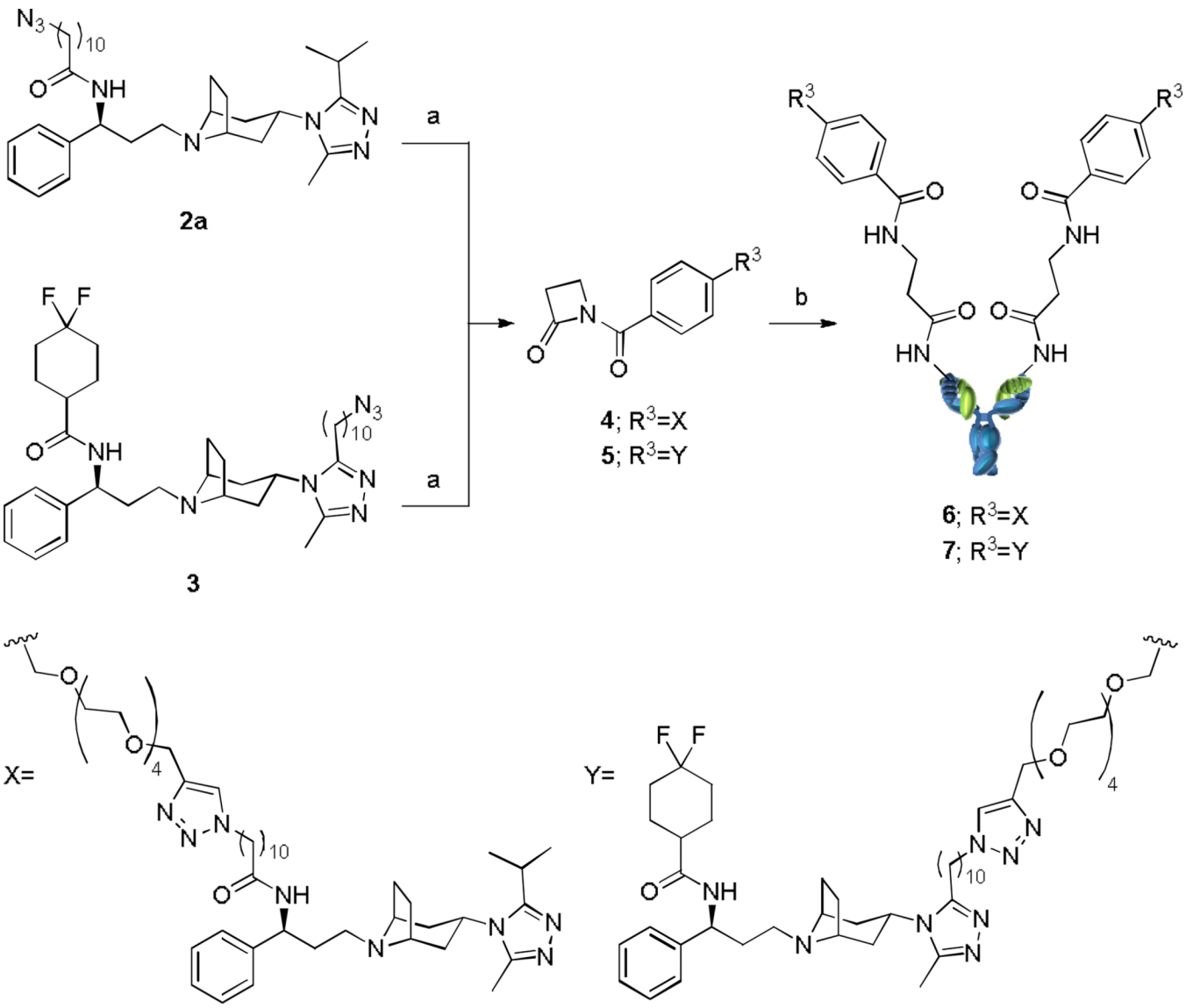


Figure 4.

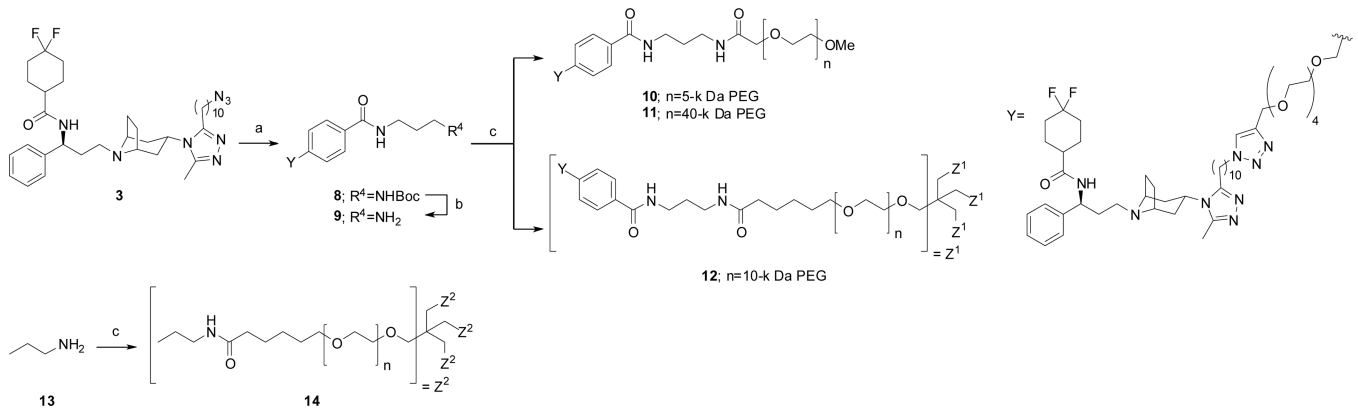
Evaluation of inhibition of HIV-1 replication in CCR5-positive cells by Maraviroc 1, mAb conjugate 7, and PEGylated compound 12; clade A 92RW020 (red line), clade B JR-FL (blue line), clade B YU-2 (black line), and clade C MGC26 (green line). All tests were performed in duplicate.



Scheme 1.

Syntheses of the cpAbs a

[a]Reagents and Conditions: (a) **S21**, THPTA, $CuSO_4$, sodium ascorbate, H_2O, *t*-BuOH, rt, 2 h, **4** 100%, **5** 67%; (b) mouse mAb 38C2, 100 mM PBS solution (pH 7.4), rt, 2 h.



Scheme 2.

Synthesis of the PEGylated compounds a

[a]Reagents and Conditions: (a) **S22**, THPTA, $CuSO_4$, sodium ascorbate, H_2O, *t*-BuOH, rt, 2 h, 82%; (b) TFA, CH_2Cl_2, rt, 1 h, quant; (c) SUNBRIGHT® ME-050AS, ME-400AS, or PTE-400HS, DMF, rt, 3 h, **10**, 46%, **11** 82%, **12** 92%, **14**, 92%.

Table 1

IC_{50} values of Maraviroc derivatives in neutralization assay using HIV-1 pseudotype JR-FL

Compound	Compound-type	IC_{50} (nM) [a]
1	Maraviroc	1.6 ± 0.25 [b]
2a	small molecule	360 ± 34 [a]
2b	small molecule	>1000 [a]
3	small molecule	0.96 ± 0.26 [a]
4	small molecule	27 ± 2.3 [a]
5	small molecule	2.6 ± 0.62 [a]
6	cpAbs	19 ± 2.6 [a]
7	cpAbs	7.7 ± 0.50 [a]
38C2	mAb carrier control	>1000 [a]
8	small molecule	3.0 ± 0.58 [a]
10	PEGylated compound	43 ± 9.6 [a]
11	PEGylated compound	49 ± 13 [a]
12	PEGylated compound	5.6 ± 2.0 [c]
14	PEGylated control	>1000 [a]

[a] Mean ± SE (N=3),

[b] Mean ± SE (N=12),

[c] Mean ± SE (N=5)

Table 2

IC_{50} values of Maraviroc derivatives in neutralization assays against indicated strains.

HIV-1 strain	IC_{50} (nM) of Maraviroc derivatives [a]		
	1	7	12
92RW (clade A)	1.3 ± 0.5 [b]	8.1 [a]	3.0 [a]
JR-FL (clade B)	1.6 ± 0.3 [c]	7.7 ± 0.5 [b]	5.6 ± 2.0 [d]
YU-2 (clade B)	0.43 ± 0.1 [b]	2.8 [a]	2.5 [a]
MGC26 (clade C)	0.37 ± 0.01 [b]	2.7 [a]	0.8 [a]

[a] Average (N=2),

[b] Mean ± SE (N=3),

[c] Mean ± SE (N=12),

[d] Mean ± SE (N=5)