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CCR5 Antagonist TD-0680 Uses a Novel Mechanism for Enhanced Potency against HIV-1 Entry, Cell-mediated Infection, and a Resistant Variant*

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Background: Maraviroc-resistant HIV-1 posts challenges to CCR5 antagonist discovery.
Results: CCR5 antagonist TD-0680 employs a novel mechanism for subnanomolar potency against HIV-1 entry, cell-mediated infection, and a TAK-779/Maraviroc-resistant variant.
Conclusion: Distinct binding mode of TD-0680 accounts for its enhanced potency.
Significance: Our findings have implications for drug design and developing TD-0680 as an antiretroviral and/or as a microbicide against HIV-1.

Regardless of the route of transmission, R5-tropic HIV-1 predominates early in infection, rendering C-C chemokine receptor type 5 (CCR5) antagonists as attractive agents not only for antiretroviral therapy but also for prevention. Here, we report the specificity, potency, and underlying mechanism of action of a novel small molecule CCR5 antagonist, TD-0680. TD-0680 displayed the greatest potency against a diverse group of R5-tropic HIV-1 and SIV strains when compared with its prodrug, TD-0232, the Food and Drug Administration-approved CCR5 antagonist Maraviroc, and TAK-779, with EC_{50} values in the subnanomolar range (0.09–2.29 nM). Importantly, TD-0680 was equally potent at blocking envelope-mediated cell-cell fusion and cell-mediated viral transmission as well as the replication of a TAK-779/Maraviroc-resistant HIV-1 variant. Interestingly, TD-0232 and TD-0680 functioned differently despite binding to a similar transmembrane pocket of CCR5. Site-directed mutagenesis, drug combination, and antibody blocking assays identified a novel mechanism of action of TD-0680. In addition to binding to the transmembrane pocket, the unique *exo* configuration of this molecule protrudes and sterically blocks access to the extracellular loop 2 (ECL2) region of CCR5, thereby interrupting the interaction between virus and its co-receptor more effectively. This mechanism of action was supported by the observations of similar TD-0680 potency against CD4-dependent and -independent SIV strains and by molecular docking analysis using a CCR5 model. TD-0680, therefore, merits its development as an anti-HIV-1 agent for therapeutic purposes and/or as a topical microbicide for the prevention of sexual transmission of R5-tropic HIV-1.

After sexual transmission, HIV-1 rapidly establishes persistent infection and latency, posing significant challenges for interventions aimed at achieving sterilizing protection and a therapeutic cure (1). An ideal way to contain the AIDS epidemic, therefore, is to prevent HIV-1 sexual transmission by blocking the virus at the entry stage.

CCR5[2] is one of the major chemokine receptors that serves as a co-receptor for most primary HIV-1 and SIV strains to enter permissive cells (2–4). Blockade of CCR5 is expected to be safe because human individuals and red-capped mangabeys with naturally occurring homozygous *CCR5 Δ32* and *Δ24* deletions, respectively, are apparently healthy (5, 6). Because these hosts are naturally resistant to R5-tropic HIV or SIV infections, targeting CCR5 to block HIV-1 infection has been explored as a preventive strategy. The importance of such strategy is further supported by the observation that sexually transmitted viruses are predominantly R5-tropic (7, 8). For these reasons, considerable efforts have been placed in developing CCR5 antagonists. Several are being tested in clinical trials (9, 10), with Maraviroc already approved by the United States Food and Drug Administration for the treatment of HIV-1-infected individuals who have failed other antiviral reg-

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[2] The abbreviations used are: CCR5, C-C chemokine receptor type 5; EC, effective concentration; TM, transmembrane; ECL, extracellular loop; Env, envelope; CXCR4, C-X-C chemokine receptor type 4; TDF, tenofovir; hu, human; rh, rhesus macaque; MFI, mean fluorescence intensity; Nt, N terminus; ICL, intracellular loop; RLU, relative light units; VSV-G, vesicular stomatitis virus glycoprotein; PBMC, peripheral blood mononuclear cells; SIV, simian immunodeficiency virus.

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imens and harbor only R5-tropic HIV-1 (9, 11). For the prevention of HIV-1 sexual transmission, a CCR5 antagonist-based microbicide is an attractive agent that is being tested in preclinical studies (12). As proof-of-concept, a small molecule CCR5 antagonist CMPD 167, which prevents the interaction of HIV-1 envelope (Env) gp120 with its co-receptor CCR5, was able to completely block $SHIV_{162P4}$ vaginal transmission as a potential microbicide in a macaque model (13).

Because transmission of cell-associated HIV-1 can be several thousand-fold more efficient than cell-free virus (14), we aimed to search for a CCR5 antagonist with significantly improved potency not only in blocking infection with genetically divergent HIV-1 and SIV but also to prevent viral cell-to-cell transmission. In this study, we further examined the specificity, potency, and mechanism of action of two novel CCR5 antagonists, TD-0232 and its derivative TD-0680 (15–17). By investigating TD-0232 and TD-0680 in parallel with the CCR5 antagonists TAK-779 and Maraviroc, we showed that TD-0680 has the highest activity against entry and cell-mediated infection of diverse HIV-1 strains as well as a TAK-779/Maraviroc-resistant variant. Moreover, we uncovered a novel mechanism underlying the enhanced potency of TD-0680.

EXPERIMENTAL PROCEDURES

Materials—TD-0232 and TD-0680 were synthesized by Shanghai Targetdrug Co. Ltd, China. TD-0232 was designated as Compound 30 in Ma *et al.* (15) and nifeviroc in Ben *et al.* (16) and Li *et al.* (17), whereas TD-0680 was designated as Compound 26 in Li *et al.* (17). The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health (Germantown, MD): anti-CCR5 monoclonal antibodies 45502 and 45531; antiretrovirals azidothymidine, emtricitabine, tenofovir (TDF), efavirenz, nevirapine, raltegravir, TAK-779, Maraviroc and JM2987; cell lines TZM-bl, CEM×174 5.25 M7, CEM-NK^r-CCR5, and GHOST(3)-CD4 series. Plasmids encoding Env $HIV\text{-}1_{ADA}$, $HIV\text{-}1_{JR\text{-}FL}$, SIV_{mac239}, and $SIV_{mac1A11}$, Env plus Tat $HIV\text{-}1_{IN08-11}$, vesicular stomatitis virus glycoprotein (VSV-G), Tat, co-receptor huCCR5 and rhCCR5, luciferase reporter backbone $HIV\text{-}1_{NL4-3}R^-E^-luc^+$, and $SIV_{mac239}R^-E^-luc^+$ were obtained from Aaron Diamond AIDS Research Center. Plasmids encoding Env $HIV\text{-}1_{CNE}$ series were kindly provided by Prof. Linqi Zhang (Tsinghua University, Beijing, China). Other antibodies used include anti-CCR5 monoclonal antibodies 2D7 (BD Biosciences), CTC8 (R&D Systems, Minneapolis, MN), isotype controls (eBioscience, San Diego, CA), and Alexa Fluor 488-labeled goat anti-mouse antibody (Invitrogen). Primary HIV-1 strains $HIV\text{-}1_{BaL}$-c2.2wt and $HIV\text{-}1_{BaL}$-c5.6r were kindly provided by Prof. Jose Esté (Universitat Autònoma de Barcelona, Badalona, Spain); 10HK1661, 10HK1447, and 93IN109 were isolated from patient specimens.

Cell Culture and Production of Env-pseudotyped Viruses—293T and TZM-bl cells were maintained in culture medium for adherent cells (Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) plus 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen)). CEM-NK^r-CCR5 cells were maintained in culture medium for non-adherent cells (RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin and 2 mM L-glutamine (Invitrogen)). GHOST(3)-CD4 cells were maintained in culture medium for adherent cells supplemented with 500 μg/ml G418, 1 μg/ml puromycin, and 100 μg/ml hygromycin B (Invitrogen). CEM×174 5.25 M7 cells were maintained in culture medium for non-adherent cells supplemented with 500 μg/ml G418, 1 μg/ml puromycin, and 100 μg/ml hygromycin B. Fresh PBMCs were isolated from buffy coats of healthy donors (Hong Kong Red Cross, Hong Kong SAR, China) by gradient centrifugation using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Total PBMCs were maintained in culture medium for non-adherent cells supplemented with 10 units/ml recombinant human interleukin-2 (IL-2) (Roche Applied Science) and stimulated with 5 μg/ml phytohemagglutinin (Sigma) for 3 days before use. Env-pseudotyped viruses were generated by co-transfecting 293T cells with plasmids encoding Env and the $pHIV\text{-}1_{NL4-3}R^-E^-luc^+$ or $pSIV_{mac239}R^-E^-luc^+$ luciferase reporter backbone.

Antiviral Assays—Two assays were performed as previously described (18). Briefly, (i) for single-cycle infectivity assays, GHOST(3)-CD4 cells were seeded into 96-well plates (1×10^4/well) and preincubated in the presence or absence of compounds at 37 °C for 1 h before the addition of 5–30 μl of Env-pseudotyped luciferase reporter virus stock. At 48-h post-infection, cells were lysed, and luciferase activity of lysate was measured by using the Bright-Glo Luciferase Assay System (Promega, Madison, WI). (ii) For the PBMC assay, 2×10^5 phytohemagglutinin and IL-2-stimulated PBMCs were pretreated with or without compound for 1 h and infected by primary HIV-1 virus (0.01 multiplicity of infection) for 7–14 days. Viral replication was measured using a RETRO-TEK HIV-1 p24 Antigen ELISA kit (ZeptoMetrix, Buffalo, NY). Relative light units (RLU) revealing luciferase activity or p24 concentration was measured by Inspire $VICTOR^3$ Multilabel Counter (PerkinElmer Life Sciences). Percentage of viral entry was calculated by (RLU with compound − RLU without virus)/(RLU without compound − RLU without virus) × 100%. Percentage of inhibition of viral infection was calculated as 100% − % viral entry. All experiments were performed in duplicate or triplicate and repeated at least twice. A nonlinear regression sigmoidal dose-response (variable slope) curve was adopted to determine the effective concentration (EC_{50}) value using the GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

Env-mediated Cell-Cell Fusion Assay—(i) 293T cells were transfected with a plasmid encoding both R5-tropic Env and Tat or co-transfected with plasmids encoding Env and Tat individually. After 48 h of incubation, transfected 293T cells (donor) were detached with Enzyme-free PBS-based Cell Dissociation Buffer (Invitrogen). TZM-bl cells that express CD4 and CCR5 and contain a long terminal repeat-driven luciferase reporter gene that could be initiated by Tat protein (19) were preincubated with or without compound for 1 h and used as target cells. Donor and target cells were mixed in a 96-well plate at a 1:1 ratio. During Env-mediated cell-cell fusion, Tat was transferred from 293T cells to TZM-bl cells and induced the expression of luciferase. 18 h later, cells were lysed and assayed for luciferase activity. (ii) 293T cells were transfected with GFP-

tagged HIV-1_{JR-FL}-expressing plasmid. 48 h later, supernatants were discarded, and 293T cells were washed with PBS twice. CEM×174 5.25 M7 cells were preincubated with or without compound for 1 h and used as target cells. Donor 293T cells were mixed with target cells at a 1:2 ratio. 18 h after co-culture, the formation of syncytia was examined under an inverted fluorescent microscope (Nikon, Tokyo, Japan).

Combination Assay—Combination of TD-0680 with other classes of antiviral reagents against R5-tropic HIV-1_{ADA} Env-pseudotyped luciferase reporter virus infection of GHOST(3)-CD4-CCR5 cells was designed following the construction of MacSynergy II software (20). Volumes of synergy and antagonism (nM^2 %) were calculated at 95% confidence intervals using drug combination data from at least two independent assays. Synergy (assigned a positive value) or antagonism (assigned a negative value) was defined as drug combinations yielding mean volumes in excess of 50 nM^2 %, and additive drug interactions were defined by mean volumes of 0–50 nM^2%.

Effect of CCR5 Antagonists on Single-residue Mutants of huCCR5—Single-residue huCCR5 mutants were constructed through site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), and their effects on CCR5 antagonists activity were examined using two assays. (i) GHOST(3)-CD4-CCR1 were transfected with wild-type huCCR5, rhCCR5, or huCCR5 mutants and infected with HIV-1_{CNE3} Env-pseudotyped luciferase reporter viruses with or without CCR5 antagonist at a concentration that causes >95% entry inhibition in this assay (TAK-779, 200 nM; Maraviroc, TD-0232 and TD-0680, 100 nM). At 48 h post-infection, cells were lysed and assayed for luciferase activity. Mutants that support viral entry above 14% were considered to have significantly reduced sensitivity (21, 22). (ii) 293T cells were transfected with wild-type huCCR5, rhCCR5, or huCCR5 mutants and infected with $SIV_{mac1A11}$ Env-pseudotyped luciferase reporter viruses with or without a fixed concentration of 150 nM CCR5 antagonist. At 48 h post-infection, cells were lysed and assayed for luciferase activity. EC_{50} was determined by GraphPad Prism 5 software.

Molecular Modeling and Docking—The model of CCR5 was built by homologic modeling with CXCR4 crystal structure (PDB code 3OE0) using the server SWISS-MODEL (23) with default parameters. The CCR5 model was further analyzed and refined using INSIGHTII (Molecular Simulations, Inc., San Diego, CA), and the Ramachandran plot was examined to prevent any unfavorable regions. Molecular docking of TAK-779, Maraviroc, TD-0232, and TD-0680 to the binding pocket of CCR5 model was performed using the Autodock 4.2 software (24, 25). Only the presumed active conformers were considered. Lamarckian genetic algorithm (LGA) was utilized to search for the conformations using the following docking parameters: population size of 150 individuals, maximum number of generations and energy evaluations of 27,000 and 25 million, respectively, and 50 docking runs and random initial positions and conformations. Binding energy of each conformation was calculated using AMBER force field, and a root mean squared deviation tolerance of 3.0 Å was used to cluster the conformations using the AutoDock Tools software.

Inhibition of Monoclonal Antibody Binding to CCR5—1×10^6 CEM-NK^r-CCR5 cells were preincubated with 100 nM RANTES or CCR5 antagonist at 37 °C for 1 h before incubation with phycoerythrin-labeled or purified anti-CCR5 monoclonal antibody (mAb) at 4 °C for 1 h. Alexa Fluor 488-conjugated goat anti-mouse antibody was used as a secondary antibody against purified anti-CCR5 mAb. Data were acquired using FACSCalibur (BD Biosciences) and analyzed by Flowjo software (Tree Star, Ashland, OR). Percentage of mAb binding was calculated by using the formula $(MFI_{inhibitor} - MFI_{isotype})/(MFI_{diluent} - MFI_{isotype}) \times 100\%$. Inhibition of CCR5 antagonist on antibody binding was reflected by the reduction of percentage of mAb binding (100 % − % mAb binding).

RESULTS

Anti-HIV-1 Specificity of CCR5 Antagonists TD-0232 and TD-0680—We previously reported that TD-0232 is an orally bio-available CCR5 antagonist with potency against R5-tropic HIV-1 infectivity at nanomolar concentrations (15). Structural optimization of TD-0232 by medicinal chemistry resulted in the subsequent synthesis of TD-0680 with four major modifications: (i) substitution of the carbamate linker with a urea linker, (ii) replacement of the nitro-moiety with a $SO_2N(CH_3)_2$ group, (iii) the addition of a tropane bridge (*exo*isomer), and (iv) introduction of a fluoro group at the three-position of the phenyl ring (Fig. 1*A*) (16, 17).

To investigate the specificity of TD-0680, we first adopted a single-cycle infectivity assay using GHOST(3)-CD4-X4R5 as target cells (4, 26). HIV-1_{NL4-3} luciferase reporter viruses were pseudotyped with different Envs, including R5-tropic HIV-1_{ADA}, X4-tropic HIV-1_{HxB2}, and multi-tropic VSV-G. Before exposure to pseudovirions, cells were pretreated with 1 μM maraviroc, TD-0232, TD-0680, or JM2987 (a CXCR4 antagonist) (27). We found that similar to Maraviroc and TD-0232, TD-0680 inhibited R5-tropic HIV-1_{ADA} but not X4-tropic HIV-1_{HxB2}. Conversely, JM2987 blocked X4-tropic HIV-1_{HxB2} but not R5-tropic viruses. All four compounds failed to prevent HIV-1_{VSV-G} infection (Fig. 1*B*, *left*). Next, we sought to determine whether TD-0232 and TD-0680 can block dual-tropic HIV-$1_{89.6}$ using GHOST(3)-CD4-CCR5 (Fig. 1*B*, *middle*) or GHOST(3)-CD4-CXCR4 (Fig. 1*B*, *right*) as target cells. Both TD-0232 and TD-0680 inhibited viral infection of cells expressing CCR5 but not CXCR4. CCR5 antagonists did not completely inhibit HIV-$1_{89.6}$ infection, possibly due to a weak endogenous expression of CXCR4 on GHOST(3)-CD4-CCR5 cells (28). These findings indicated that, like Maraviroc, TD-0232 and TD-0680 are specific HIV-1 entry inhibitors that function only by antagonizing CCR5 and not CXCR4, in line with previous findings that they could block the binding of the chemokine RANTES to CCR5 (15, 17).

TD-0680 Inhibits Diverse R5-tropic HIV-1 and SIV, Env-mediated Cell-Cell Fusion, and Cell-mediated Viral Transmission at Subnanomolar Concentrations—The antiviral potency of TD-0232 and TD-0680 was determined using several assays. First, TD-0232 and TD-0680 were tested in a single-cycle infectivity assay using GHOST(3)-CD4-CCR5 cells against a panel of genetically diverse R5-tropic HIV-1 and SIV Env-pseudotyped viruses, including primary Envs derived from six chronically

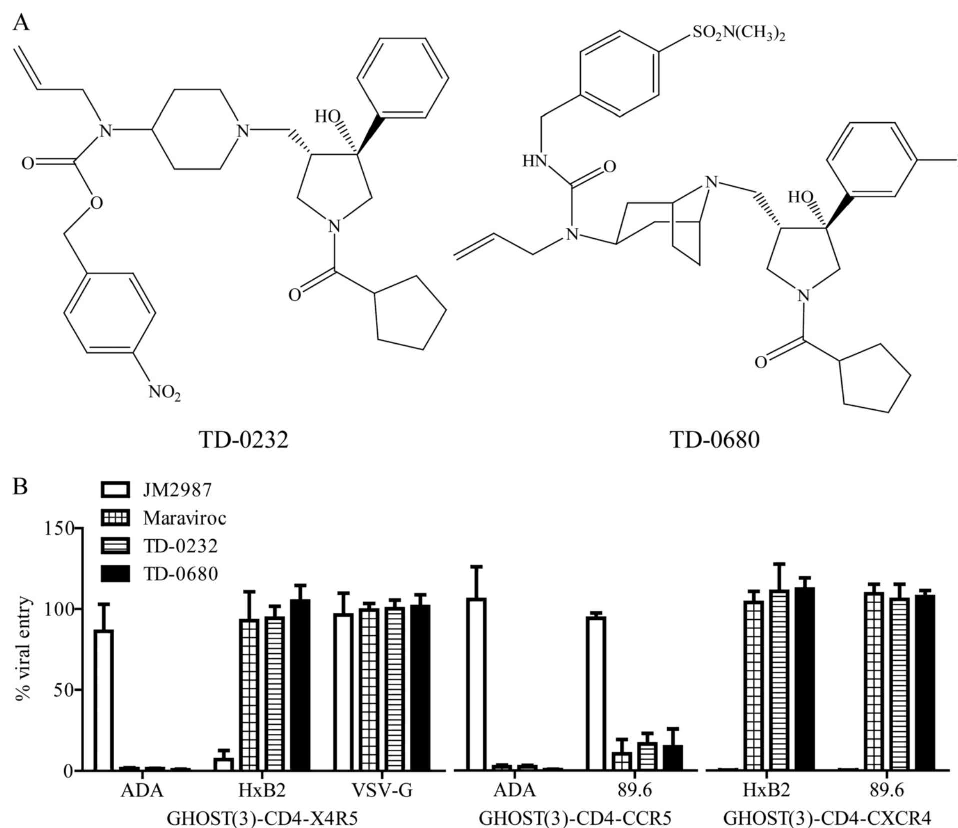

FIGURE 1. **TD-0680 is a novel CCR5 antagonist.** *A*, shown are the chemical structures of TD-0232 (M_r 590.71, *left*) and TD-0680 (M_r 695.89, *right*). *B*, TD-0680 selectively inhibits HIV-1 infection through CCR5 co-receptor. GHOST(3)-CD4-X4R5 (*left*), GHOST(3)-CD4-CCR5 (*middle*), or GHOST(3)-CD4-CXCR4 (*right*) cells were infected with indicated Env-pseudotyped HIV-1 luciferase reporter viruses. All viral entry experiments were conducted in the presence or absence of 1 μM JM2987, Maraviroc, TD-0232, or TD-0680. Each group was tested in triplicate, and data are presented as the mean ± S.D.

TABLE 1
Antiviral activities of small molecule CCR5 antagonists using a single-cycle infectivity assay

Env-pseudotyped virus	Clade	EC_{50} ± S.D.			
		TAK-779	Maraviroc	TD-0232	TD-0680
		nM			
HIV-1_{ADA}	B	29.07 ± 15.08	2.72 ± 2.01	1.00 ± 0.69	0.30 ± 0.17
HIV-1_{JR-FL}	B	88.71 ± 58.89	3.28 ± 0.62	6.85 ± 2.74	1.01 ± 0.28
HIV-1_{CNE6}	B′	53.69 ± 28.55	3.55 ± 0.33	3.36 ± 2.86	0.93 ± 0.31
HIV-1_{CNE11}	B′	203.60 ± 37.34	9.54 ± 3.37	14.09 ± 2.98	1.99 ± 1.28
HIV-1_{CNE15}	B′C	35.74 ± 29.59	3.22 ± 1.71	6.75 ± 7.18	1.35 ± 1.46
HIV-1_{CNE30}	CRF08_BC	14.99 ± 7.88	2.19 ± 0.54	2.08 ± 0.47	0.67 ± 0.33
HIV-1_{CNE3}	CRF01_AE	35.49 ± 4.91	4.22 ± 2.09	2.98 ± 1.15	0.87 ± 0.53
HIV-1_{CNE55}	CRF01_AE	33.53 ± 13.06	2.44 ± 0.88	2.47 ± 2.34	0.61 ± 0.52
Mean		61.85	3.90	4.95	0.97
SIV_{mac239}		95.45 ± 29.49	8.21 ± 0.86	14.45 ± 0.93	2.29 ± 0.66
$SIV_{mac1A11}$		14.09 ± 2.48	1.38 ± 0.10	4.58 ± 1.54	1.43 ± 0.29
Mean		54.77	4.80	9.52	1.86

infected Chinese patients (CNE, Chinese Env) (29). As summarized in Table 1, TD-0680 was found to be the most potent, with broad inhibitory activities against multiple HIV-1 subtypes. In these experiments the EC_{50} of TD-0680 ranged from 0.30 to 1.99 nM with a mean of 0.97 nM, which is significantly more potent than the other CCR5 antagonists ($p < 0.01$, Mann-Whitney test). Interestingly, TD-0680 was also effective against both neutralization-resistant SIV_{mac239} (EC_{50} = 2.29 nM) and CD4-independent neutralization-sensitive $SIV_{mac1A11}$ (EC_{50} = 1.43 nM), likely due to similar CCR5 usage between HIV-1 and SIV as we previously described (4). Second, we determined the EC_{50} values of TD-0232 and TD-0680 against primary HIV-1 isolates in PBMC assays and compared them with TAK-779 or Maraviroc (30). Only TD-0680 displayed subnanomolar EC_{50} values against three genetically divergent R5-tropic HIV-1 clinical isolates: 10HK1661 (clade B), 10HK1447 (clade CRF01_AE), and 93IN109 (clade C) (Fig. 2*A*). Third, taking into consideration the enhanced transmissibility of cell-associated HIV-1 (14), we sought to determine whether TD-0680 could block the virus in an Env-mediated cell-cell fusion and cell-mediated viral transmission model (31). Consistently, TD-0680 exhibited potent inhibitory activity at subnanomolar levels against cell-cell fusion mediated by R5-tropic Envs of three major genotypes, whereas TAK-779, TD-0232, and Maraviroc displayed a reduced efficacy (Fig. 2*B*). As expected, reverse transcriptase inhibitor TDF did not show any inhibitory activity

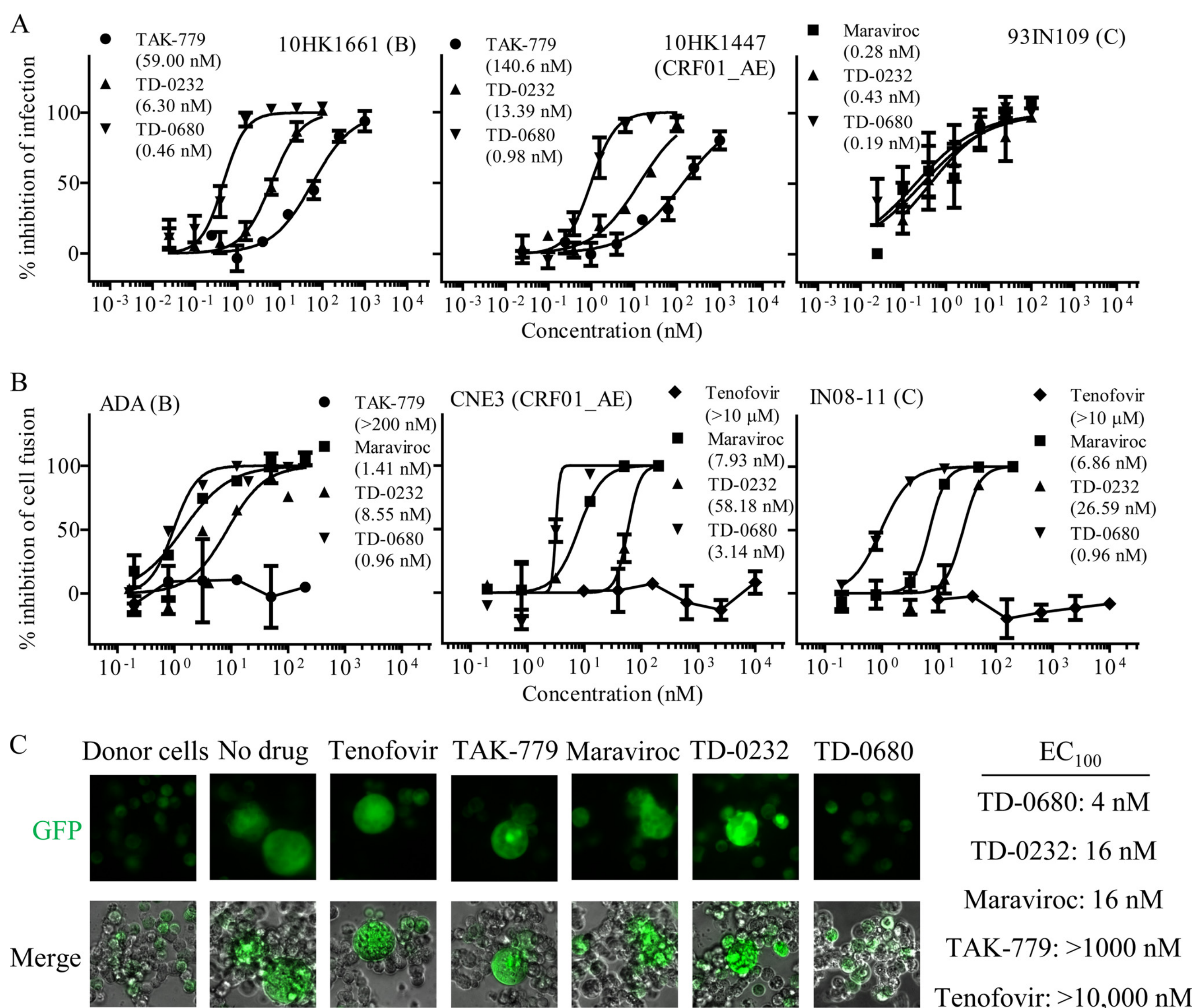

FIGURE 2. **TD-0680 is a potent inhibitor against R5-tropic HIV-1.** *A*, PBMCs were infected with primary R5-tropic HIV-1 infection in increasing concentrations of TAK-779, Maraviroc TD-0232, or TD-0680. p24 concentration of viral culture lysate on day 7 was measured using a p24 ELISA assay. Three independent experiments were conducted using PBMCs from three different healthy donors. *B*, TD-0680 blocks R5-tropic HIV-1 Env-mediated cell-cell fusion and transmission. 293T cells transfected with indicated Env and Tat were co-incubated with TZM-bl cells in the presence or absence of drug. 18 h later, cells were lysed and assayed for luciferase activity. Data represent the mean ± S.E. of two to four independent experiments. *Numbers in parentheses* indicate EC_{50} values. *C*, TD-0680 is effective against the formation of syncytia. 293T cells producing GFP-tagged live HIV-1_{JR-FL} were co-incubated with CEM×174 5.25 M7 cells in the presence or absence of 4 nM indicated drug. 18 h later, Env-mediated cell-cell fusion and formation of syncytia were examined under an inverted fluorescent microscope (Nikon, Tokyo, Japan).

in this model (32). Furthermore, we co-cultured GFP-tagged live HIV-1_{JR-FL}-producing 293T cells with CEM×174 5.25 M7 cells in the presence or absence of various serially diluted inhibitors. As shown in Fig. 2*C*, although micromolar amounts of TDF and TAK-779 were required to achieve 100% inhibition of syncytia formation, the EC_{100} of TD-0680 was 4 nM, 4-fold lower than that required for TD-0232 and Maraviroc. Thus, TD-0680 not only exhibits increased potency against cell-free virus as compared with other CCR5 antagonists but may also offer a major advantage in the prevention of cell-to-cell spread of HIV-1.

TD-0680 Inhibits TAK-779/Maraviroc-resistant HIV-1 Variant—It has been previously reported that R5-tropic HIV-1_{BaL}-c5.6r is resistant to both TAK-779 and Maraviroc (33). Therefore, we sought to determine whether TD-0680 and TD-0232 are effective against this resistant strain. As shown in Fig. 3*A–D*, all four CCR5 antagonists could inhibit the infection of the parental HIV-1_{BaL}-c2.2wt strain in PBMCs effectively, with TD-0680 showing the most potent activity (EC_{50} = 0.09 nM). Importantly, TD-0680 displayed subnanomolar potency against the TAK-779/Maraviroc-resistant HIV-1_{BaL}-c5.6r strain as well, with only <5-fold reduction in EC_{50} value (0.44 nM) as compared with the parental strain (Fig. 3*D*). In contrast, HIV-1_{BaL}-c5.6r was highly resistant to TAK-779 (>1000-fold), Maraviroc (>40-fold), and TD-0232 (>40-fold) (Fig. 3, *A–C*). These data demonstrate that TD-0680 is effective against a TAK-779/Maraviroc-resistant mutant virus and suggests an antiviral mechanism that is distinct from TAK-779, Maraviroc, and its prodrug TD-0232.

TD-0680 Uses Distinct Binding Motif to Human and Rhesus CCR5—To evaluate the potential of TD-0680 for clinical development, we performed viral inhibitory assays using a combina-

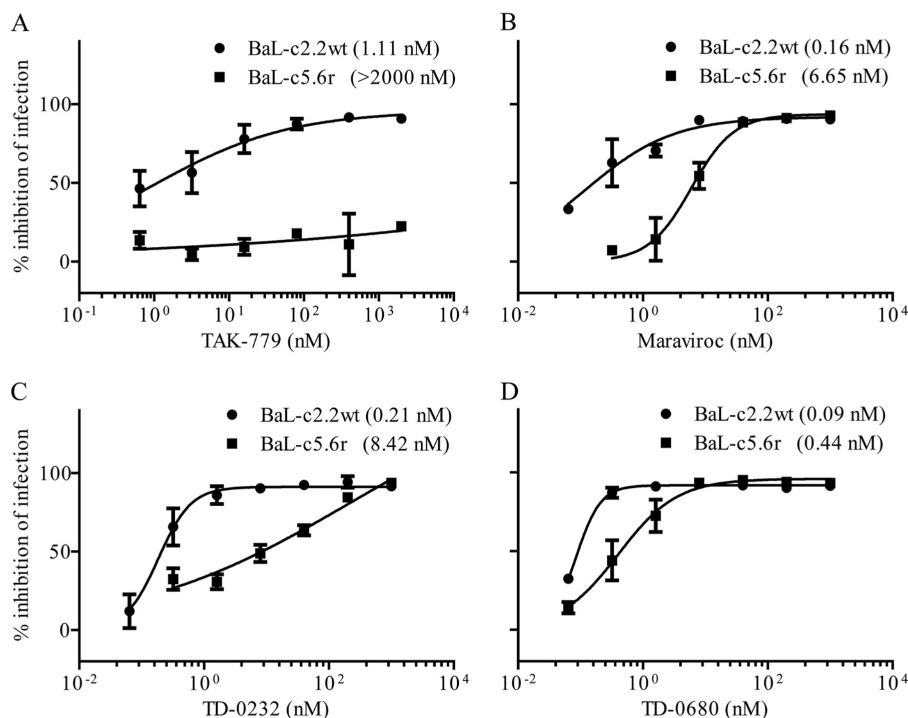

FIGURE 3. **TAK-779/Maraviroc-resistant HIV-1 strain remains sensitive to TD-0680.** HIV-1_{BaL}-c2.2wt and HIV-1_{BaL}-c5.6r replication on day 14 was measured using a p24 ELISA assay after treatment with an increasing concentration of indicated drug. Data represent the mean ± S.E. from at least two independent experiments with duplicates. *Numbers in parentheses* indicate EC_{50} values.

tion with several classes of antiretroviral compounds including nucleoside reverse transcriptase inhibitors azidothymidine, emtricitabine and TDF, nonnucleoside reverse transcriptase inhibitors efavirenz and nevirapine, and integrase inhibitor raltegravir (20). As expected, TD-0680 displayed no antagonistic effects when combined with any of these antiretrovirals, suggesting a possible application in mixture therapy (Table 2). TD-0680, however, had strong antagonism with TD-0232 and moderate antagonism with Maraviroc, likely due to targeting of a common region in CCR5 by these drugs.

To further define the binding motif on CCR5 of TD-0680, a panel of single-residue mutants of human CCR5 (huCCR5) was generated spanning the N terminus (Nt), intracellular loop 2, ECL2, and TM domains (Fig. 4). GHOST(3)-CD4-CCR1 cells, which do not support HIV-1 infection, were transfected with each of the huCCR5 mutants followed by treatment with a fixed concentration of CCR5 antagonist and subsequent infection with an R5-tropic HIV-1_{CNE3}. In accordance with previous studies, a 14% viral entry was used as the cut-off level, above which a CCR5 mutant is considered insensitive to the respective antagonist (21, 22). Individual amino acid substitutions tested in the ECL2 of CCR5 seemed to have little effects on the activity of all three CCR5 antagonists tested. Amino acid residues previously reported to be critical for the inhibitory activity of Maraviroc (*e.g.* Trp-86 in TM2, Tyr-108 in TM3, Ile-198 in TM5, Tyr-251 in TM6, and Glu-283 in TM7) were confirmed in this assay, validating its utility (Fig. 4*A*) (34, 35). Interestingly, these residues also significantly affected TD-0232 activity, indicating that Maraviroc and TD-0232 use an overlapping binding domain within the CCR5 TM pocket. Surprisingly, TD-0680 was affected by W86A in TM2, I198A in TM5, and E283A in TM7 but not by Y108A in TM3, I198M in TM5, or Y251A in TM6, postulating that TD-0680 uses a binding domain that differs from TD-0232 and Maraviroc.

TABLE 2

Anti-HIV-1 effect of TD-0680 in combination with different classes of antiretroviral agent

Antiretroviral agent	TD-0680: Synergy vol/antagonism vol[a]	TD-0680: Combined effect[b]
	nM^2%	
Nucleoside reverse transcriptase inhibitor		
Azidothymidine	0.23/−14.05	Additive
Emtricitabine	0.67/−10.79	Additive
TDF	12.80/−11.13	Additive
Nonnucleoside reverse transcriptase inhibitor		
Efavirenz	0.51/−3.2	Additive
Nevirapine	0/−2.14	Additive
Integrase inhibitor		
Raltegravir	0.48/−0.28	Additive
CCR5 neutralization antibody		
2D7	0.78/−39.43	Additive
CCR5 antagonist		
Maraviroc	0/−83.03	Antagonistic
TD-0232	0/−117.19	Antagonistic

[a] Volumes of synergy and antagonism (nM^2%) were calculated at 95% confidence intervals using data from at least two independent assays.

[b] Synergy (assigned a positive value) or antagonism (assigned a negative value) was defined as drug combinations yielding mean volumes in excess of 50 nM^2%, and additive drug interactions were defined by mean volumes of 0 to 50 nM^2%.

HuCCR5 and its rhesus macaque homolog (rhCCR5) had been reported to exhibit differential sensitivity to CCR5 antagonists such as SCH-C and CMPD 167 (13, 22). Thus, we focused on the impact of eight human-to-rhesus CCR5 mutants on the efficacy of TD-0232 and TD-0680 against HIV-1_{CNE3} infection. We found that the I198M mutation in TM5 reduced the anti-

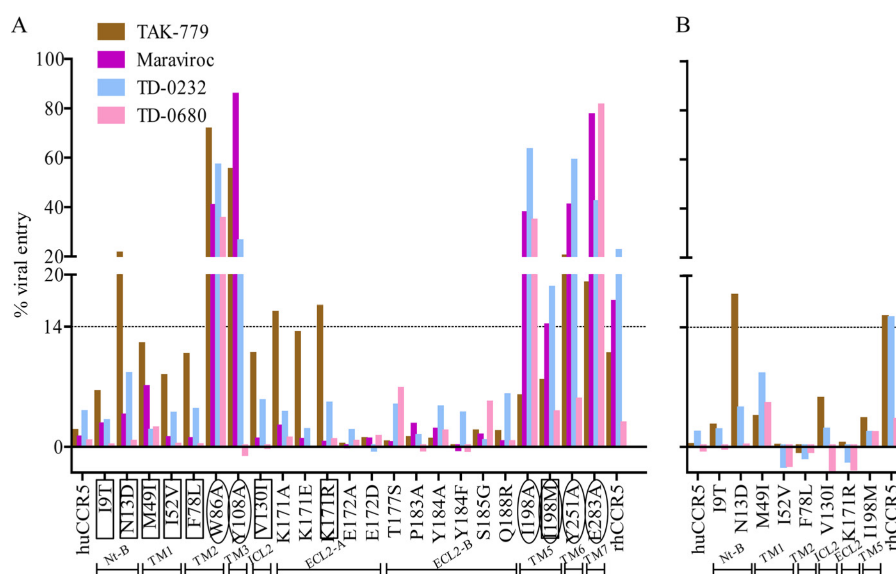

FIGURE 4. **Antiviral activity of CCR5 antagonists on huCCR5 single-residue mutants.** GHOST(3)-CD4-CCR1 (*A*) or 293T (*B*) cells transfected with each indicated CCR5 construct were evaluated for their ability to support the entry of R5-tropic HIV-1_{CNE3} or $SIV_{mac1A11}$ Env-pseudotyped luciferase reporter viruses, respectively, in the presence of a fixed concentration of CCR5 antagonist (for HIV-1: TAK-779, 200 nM; others: 100 nM; for SIV: all drugs, 150 nM). Mutants indicated in *rectangles* are natural human-to-rhesus CCR5 mutants, whereas mutants in *ellipses* are at sites involved in the binding pocket of Maraviroc (34, 35). The mutant that supports viral entry *above the dot line* (14%) is indicative to have significantly reduced sensitivity to the respective CCR5 antagonist (21, 22). Data represent means from one to two independent experiments in triplicate or quintuplicate. *ICL2*, intracellular loop 2.

viral activity of Maraviroc and TD-0232 but had little effects on TD-0680 (Fig. 4*A*). Critically, TD-0232 and Maraviroc were significantly less effective than TD-0680 in blocking HIV-1_{CNE3} entry into GHOST(3)-CD4-CCR1 cells expressing rhCCR5 (Fig. 4*A*). Considering that CD4 can modulate CCR5 expression (36, 37), we further examined the sensitivity of CCR5 antagonists against CD4-independent R5-tropic $SIV_{mac1A11}$ on 293T cells expressing CCR5 alone. In this case, TAK-779 and TD-0232 were also less active on rhCCR5, whereas TD-0680 remained potent (Fig. 4*B*). Collectively, TD-0680 functions effectively on both human and rhesus CCR5 as compared with other CCR5 antagonists.

Structural Modeling Uncovered Unique Binding Mechanism Employed by TD-0680—A CCR5 model was built based on sequence homology of a recently determined CXCR4 crystal structure (35, 38). TAK-779, Maraviroc, TD-0232, and TD-0680 were docked using this model, and the lowest energy/highest cluster conformations of each compound were subsequently analyzed. As shown in Fig. 5, *A–D*, each of the four CCR5 antagonists penetrated into a pocket formed by TM domains. Consistent with mutagenesis analysis, TD-0232 and Maraviroc interacted with key residues such as Trp-86, Tyr-108, Ile-198, Tyr-251, and Glu-283 (Fig. 5, *B*, *C*, and *E*) (21, 34, 35). As for TD-0680, Tyr-108, Phe-112, Ile-198, Trp-248, and Tyr-251, which locate in the deep TM pocket, provided a hydrophobic environment to the binding of TD-0680. Moreover, π-π interactions were observed from the rings stacking between Phe-112 and the phenyl group of TD-0680. Intriguingly, besides the TM binding of TD-0680, its pyrrolidine core and the substituted groups at the 1 and 3 positions uniquely outreached to the ECL2 region, close to Glu-172, Thr-177, Cys-178, and Ser-179 (Fig. 5*D*). This unique protruding structure relied on its rigid *exo* configuration, which was conferred by the introduction of a tropane bridge in TD-0680 (Fig. 5, *D* and *F*). The tropane bridge of TD-0680 was stabilized by the hydrophobicity of Trp-86, Leu-104, and Thr-105, and the fluorophenyl ring of TD-0680 was sandwiched between indole group of Trp-86 and phenyl group of Tyr-89. Thr-177, Cys-178, and Ser-179, which locate at ECL2, interacted and stabilized the pyrrolidine core of TD-0680 possibly through hydrophobic interactions. In contrast, the *endo* isomer of TD-0680, which also binds the same TM pocket, exhibited no ECL2 blockade similar to TD-0232 (Fig. 5*D*).

To further determine the mechanism of ECL2 blockade, we investigated the influence of TD-0680 on the binding of mAb to CCR5 extracellular domains Nt and ECL2, which are essential for HIV-1 entry (39–41). 45502, CTC8, 2D7, and 45531 are mouse anti-human CCR5 mAbs targeting the first half of Nt (Nt-A), the second half of Nt (Nt-B), the first half of ECL2 (ECL2-A), and the second half of ECL2 (ECL2-B), respectively (40). Their binding affinities to CCR5-expressing leukemic lymphoid CEM-NK^r cells (CEM-NK^r-CCR5) were determined by measuring the MFI using flow cytometry in the presence of 100 nM CCR5 antagonists as previously described (41). β-Chemokine RANTES, a natural ligand of CCR5, was included as a positive control. Indeed, RANTES significantly affected the binding of all anti-CCR5 mAbs by inducing CCR5 internalization, whereas CCR5 antagonists did not show similar effects (Fig. 6) (42). None of the CCR5 antagonists affected the Nt-A and Nt-B binding of 45502 and CTC8, respectively, suggesting that their inhibitory activities were unlikely Nt-dependent. The ECL2-B binding of 45531, however, was reduced by all four CCR5 antagonists, with TD-0680 showing the most significant loss (80.2% reduction). Moreover, the ECL2-A binding of 2D7 was also most significantly blocked by TD-0680 (51.7% reduction). Overall, these results demonstrated that due to its unique structure, TD-0680 exerts its function by interfering with both the ECL2-A and ECL2-B regions of CCR5.

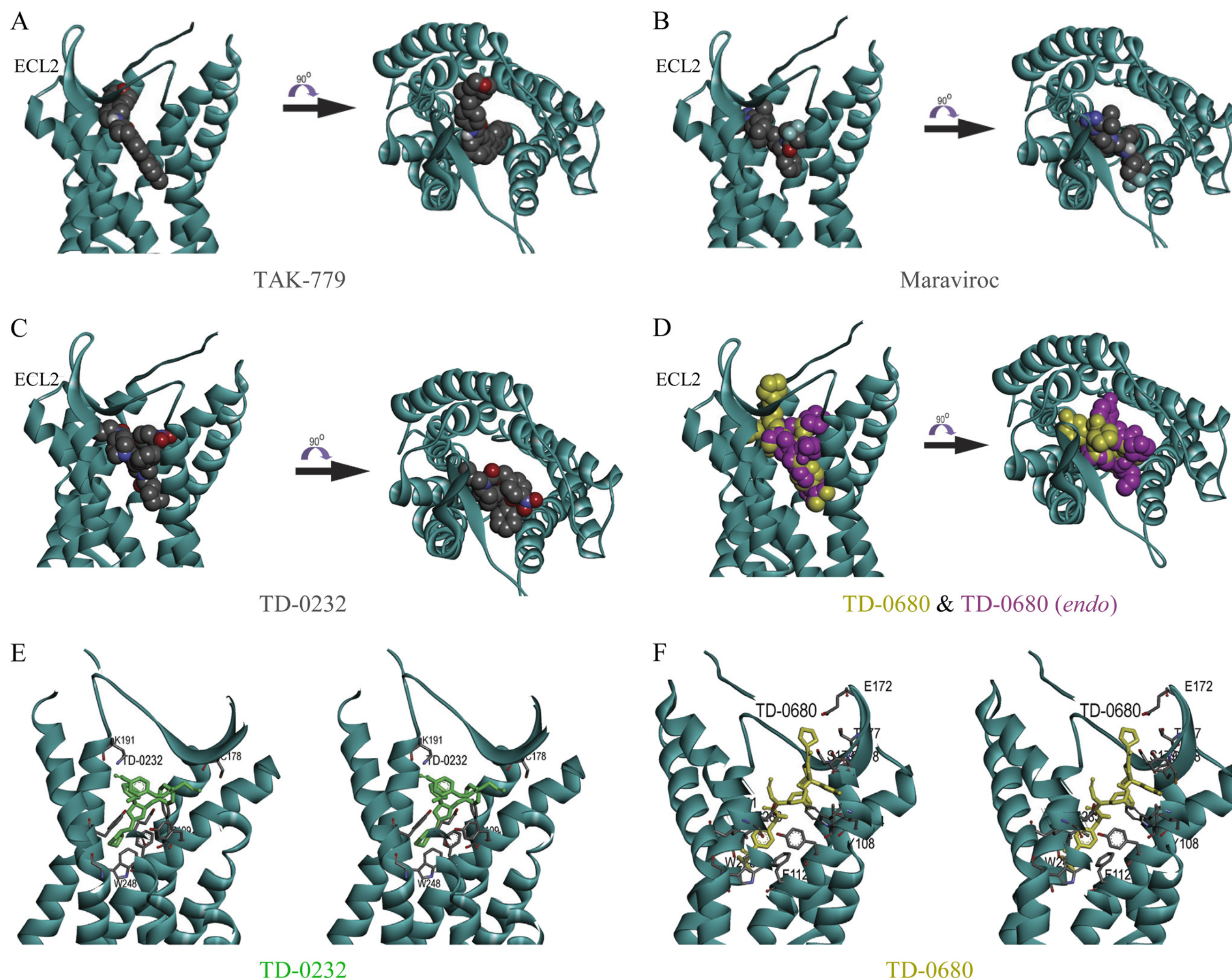

FIGURE 5. **Binding mode of TD-0232 and TD-0680 to CCR5.** Molecular docking of TAK-779 (*A*), Maraviroc (*B*), TD-0232 (*C*), and color-coded TD-0680 and its *endo* isomer (*D*) are shown with the CCR5 model. The structure of CCR5 is shown in ribbon format. Molecules are shown in Corey-Pauling-Koltun format with indicated coloring: carbon (*gray*), oxygen (*red*), and fluoro (*light blue*). The ECL2 region is indicated. The figure is generated using Discovery studio visualizer (Accelrys, San Diego, CA). Detailed interaction of TD-0232 (*E*) and TD-0680 (*F*) with CCR5 model in a stereo view is presented. The interacting residues are highlighted in the figure with the same coloring scheme as described.

DISCUSSION

In this study we report the anti-HIV-1 specificity, potency, and mechanism of action of two novel small molecule CCR5 antagonists, TD-0232 and TD-0680. We demonstrated that both TD-0232 and TD-0680 inhibited HIV-1 entry by specifically blocking virus interaction with the CCR5 co-receptor (Fig. 1). Compared with TAK-779, Maraviroc, and TD-0232, TD-0680 showed the highest anti-HIV-1 activity against a panel of genetically divergent R5-tropic HIV-1 (clades B, B′, B′C, CRF08_BC, and CRF01_AE) and SIV_{mac} strains in both pseudoviral (Table 1) and PBMC assays (Figs. 2*A* and 3), with EC_{50} values in the subnanomolar range (0.09–2.29 nM). The EC_{50} values of TD-0680 were consistently 2–28-fold better than TD-0232 or Maraviroc, whereas the 50% cytotoxic concentrations (CC_{50}) of TD-0680 and TD-0232 were comparable (113 *versus* 98 μM) in Vero cells in our experiments. Moreover, TD-0680 was able to block Env-mediated cell-cell fusion and cell-mediated viral transmission as well as infection of PBMCs with a TAK-779/Maraviroc-resistant HIV-1 variant at the same subnanomolar concentrations (Figs. 2, *B* and *C*, and 3). These features together with the lack of antagonistic effects with commonly used antiretrovirals (Table 2) and a significantly improved selectivity index and reduction of 50% hERG (human ether-*a-go-go* related gene) affinity as compared with TD-0232 (16, 17), render TD-0680 an attractive drug candidate for AIDS treatment and prevention.

TD-0680 showed antagonistic effects with Maraviroc and TD-0232 in drug combination assays (Table 2), suggesting that it functions by binding into the TM pocket of CCR5. Indeed, the antiviral activity of TD-0680 remained sensitive to some amino acid changes (*e.g.* W86A, I198A, and E283A) located within the pocket (Fig. 4*A*). However, several lines of evidence indicated that TD-0680 employed a binding motif that is distinct from TD-0232 and other CCR5 antagonists. First, besides improved antiviral potency as discussed above, TD-0680 remained effective against a human-to-rhesus CCR5 mutant I198M that was previously found to be the cause for the insensitivity of rhCCR5 to SCH-C (22), explaining its potency in blocking HIV/SIV infections into rhCCR5-expressing cells (Fig. 4). Second, mutagenesis study showed that TD-0680 was either insensitive

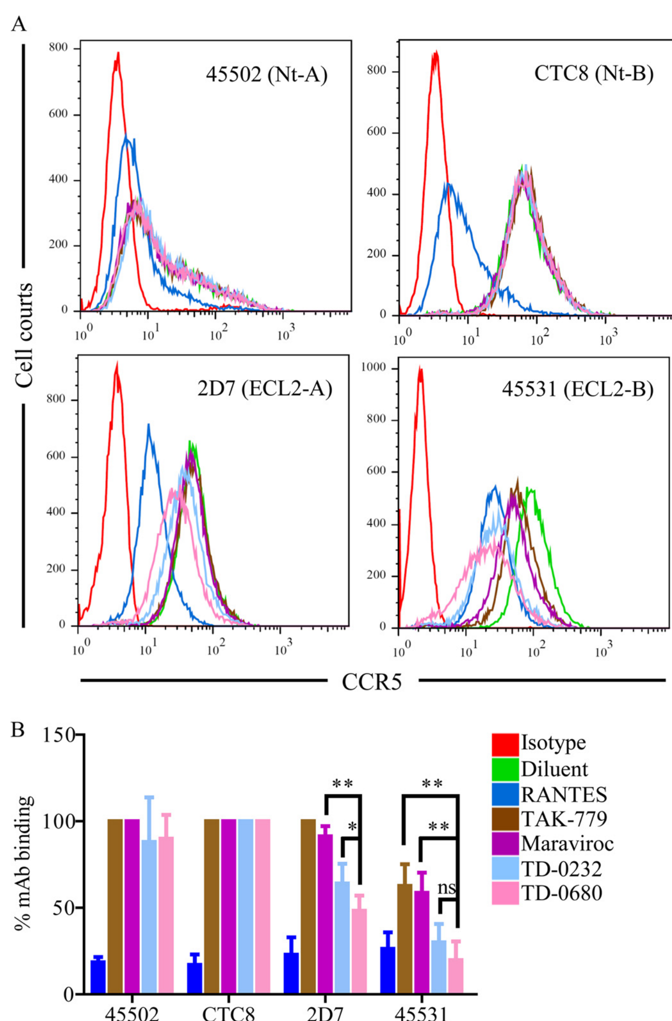

FIGURE 6. **Inhibition of mAb binding by CCR5 antagonists.** *A*, representative results of mAb binding are shown. mAb binding affinity was revealed by MFI using flow cytometry. *B*, statistics of antibody binding are shown. Data represent the mean ± S.D. from two to six independent experiments. For clarity, mean values >100% (not significant) are plotted as 100%. Two-tailed Student's *t* test was used for group comparison. *, $p < 0.05$; **, $p < 0.01$; *ns*, not significant.

or was less dependent on amino acids such as Tyr-108 in TM3 and Tyr-251 in TM6 within the pocket (Fig. 4*A*). Docking analysis suggested that the loss of hydrophobicity due to Y108A or Y251A mutations was likely compensated by the extensively interactions of TD-0680 to CCR5, which was extended from the deep hydrophobic TM pocket to the ECL2 region. Last, TD-0680 was effective against both wild-type HIV-1_{BaL}-c2.2wt and the TAK-779/Maraviroc-resistant HIV-1_{BaL}-c5.6r strains at subnanomolar concentrations, with a less than 5-fold change in EC_{50} values as compared with the >40-fold (*e.g.* TD-0232 and Maraviroc) and 100-fold changes (*e.g.* TAK-779) for the other CCR5 antagonists (Fig. 3). Taken together, these findings suggested that TD-0680 acts upon CCR5 through an overlapping yet distinct mechanism from TD-0232 or Maraviroc.

The unique mechanism of action of TD-0680 is closely linked to its *exo* configuration. Molecular docking analysis using CXCR4 as a template revealed that this rigid *exo* configuration formed a unique physical protruding obstacle from the ECL2 of CCR5 (Fig. 5, *D* and *F*), a region that is required to interact with the V3 loop of gp120 in viral entry (43). This mechanism of action is further supported by the observation that analogs of TD-0680 with an *endo* configuration displayed significantly reduced anti-HIV-1 activity despite comparable CCR5 binding affinity (17). Moreover, in line with this inhibitory mechanism, antibody-blocking data showed that TD-0680 affected mAb 45531 binding to ECL2-B as well as the binding of mAb 2D7 to ECL2-A (Fig. 6), the latter being a potent neutralizing antibody against R5-tropic HIV-1 infection (40). As the affinity of TD-0680 to CCR5 was actually lower than that of Maraviroc, with an IC_{50} of 11.4 nM *versus* 5.2 nM in inhibition of RANTES-induced GTP binding assay (11, 17), the enhanced potency of TD-0680 may simply be a function of its extensive physical protrusion after binding to CCR5 to block both the ECL2-B and ECL2-A regions more effectively. This mechanism may explain the superior activity of TD-0680 against the TAK-779/Maraviroc-resistant HIV-1_{BaL}-c5.6r strain, which is a competitive resistant virus and may use a different yet ECL2-dependent conformation for entry (44). Besides the physical barrier, it is possible that the inhibitory mechanism of TD-0680 also involves a bigger conformational change in the ECL2 of CCR5 that is no longer recognizable by HIV-1 through an allosteric binding to the transmembrane domains (22, 41, 45). These novel findings have important implications for drug design. However, because the identity of TMs between CCR5 and CXCR4 is about 35.4% (35), our CCR5 model is approximate, and molecular docking of inhibitors is mainly tentative. Our hypothetical mechanism remains to be confirmed by future structural and functional analysis.

TD-0680 can be a salvage choice for patients who have developed a competitive resistant virus like HIV-1_{BaL}-c5.6r (46, 47). Although rare, treatment failure has been documented due to Maraviroc-resistant R5-tropic-remaining viruses detected in Maraviroc-treated patients (45). It should be mentioned that despite rigorous efforts, we have not been able to obtain a TD-0680-resistant virus even though non-nucleoside reverse transcriptase inhibitor F18-resistant viruses were readily generated in our laboratory (18). Given the steep slopes of TD-0680 inhibition kinetics (Figs. 2, *A* and *B*, and 3*D*), this failure might be related to the difficulty of capturing a suboptimal drug condition for resistant viruses to emerge, resulting in a high resistance barrier. Continuing efforts will be given to address this issue in future studies. Schanzer *et al.* (48) recently demonstrated that a bi-specific CCR5 antibody displays potent neutralizing activity against resistant viruses by targeting both the Nt and ECL2. Our future studies should investigate the potency of TD-0680 on other R5-tropic non-competitive CCR5 antagonist-resistant viruses, which might be more dependent on the Nt of CCR5 for entry (41, 45).

TD-0680 is >1000-fold better than TDF in blocking cell-mediated HIV-1 transmission. A recent landmark work by Abdool Karim *et al.* (49) demonstrated that a TDF microbicide gel used by women before sex reduced their risk of HIV infection by 39%. Although TDF blocks cell-free virus effectively, it failed to completely inhibit HIV-1 cell-to-cell spread (32), a much more efficient mode of virus transmission (14). In this regard we also demonstrated that TDF was unable to block HIV-1 Env-mediated cell-cell fusion or cell-mediated viral

transmission up to 10 μM concentration, which contrasted with the single-digit 4 nM EC_{100} achieved by TD-0680 (Fig. 2*C*), a >1000-fold difference. Conceivably, TD-0680 will be an excellent microbicide candidate for preventing HIV-1 sexual transmission. To this end, unlike Maraviroc, TD-0680 blocked both huCCR5 and rhCCR5 almost equally well (Fig. 4). This property will greatly facilitate a TD-0680-based drug or microbicide gel to be tested for safety and efficacy evaluation in SHIV/macaque models.

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