C8166-CCR5 cells retained sensitivity to small-molecule CD4 mimics and sCD4 comparable to that of HIV-1 MNA.

We then examined whether the synergistic neutralization of HIV-1 MNA by KD-247 antibody in the presence of NBD-556 (Yoshimura *et al.*, 2010) would be reproduced when CD4 mimic was substituted by YYA-021. The synergistic neutralization effect of KD-247 and YYA-021 was reproduced in our experiments (Fig. 1d). At 50 μg ml^{-1}, KD-247 barely achieved 50 % neutralization of HIV-1 MNA but resulted in 50 % neutralization at <0.05 μg ml^{-1} in the presence of 20 μM of YYA-021.

Finally, to examine whether these two agents neutralized SHIV MNA in the same manner as the parental HIV-1, we conducted a neutralization assay with KD-247 in the presence of increasing amounts of YYA-021 (0, 5, 10, 20 and 40 μM) (Fig. 1e). The neutralization curve of KD-247 against SHIV MNA showed an upward shift as the concentration of YYA-021 increased (Fig. 1e), similar to the observations with HIV-1 (Fig. 1d), indicating augmentation of neutralization, and complete neutralization of both viruses was achieved at 20 μM YYA-021 (Fig. 1d, e). Based on these results, we concluded that the neutralization profile of SHIV MNA was comparable to that of HIV-1 MNA.

Reproduction of the neutralization characteristics of HIV-1 MNA in the newly generated SHIV prompted us to assess the ability of SHIV MNA to replicate in monkey cells. SHIV MNA, along with SIV239 and SHIV KS661, were normalized with infectious titres and inoculated into rhesus macaque PBMC preparations from four animals,

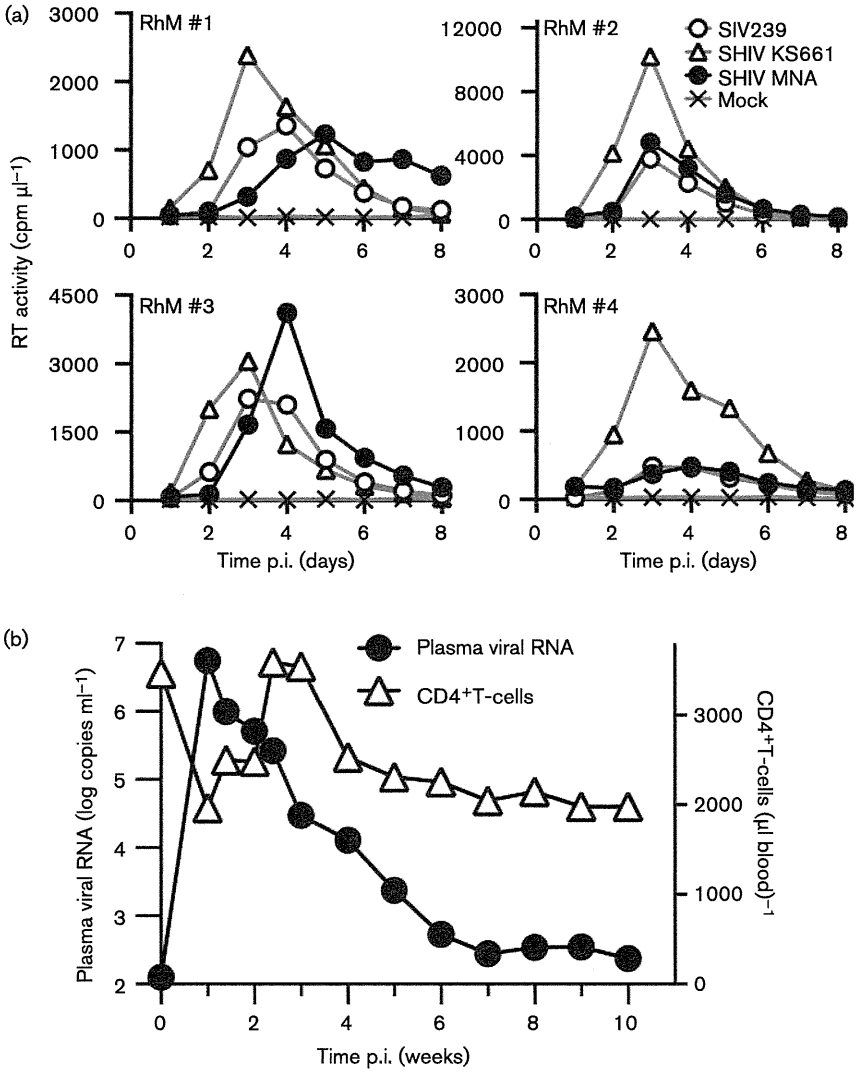


Fig. 2. Replication of SHIV MNA in rhesus macaque PBMCs (a) and *in vivo* (b). (a) M.o.i. was adjusted to 0.01 ($TCID_{50}$ per cell). (b) Experimental infection of a rhesus macaque with SHIV MNA. SHIV MNA (1.75×10^5 $TCID_{50}$) was intravenously inoculated into a rhesus macaque, and the plasma viral RNA burden and circulating $CD4^+$ T-lymphocytes were monitored.

as described previously (Fujita *et al.*, 2013) (Fig. 2a). SHIV KS661, a CXCR4-utilizing virus, replicated to the highest titres of all the viruses in all PBMC preparations. Compared with SHIV KS661, SIV239 replicated to lower titres. Under these experimental conditions, SHIV MNA showed productive replication in the cells with similar replication kinetics and peak titres to SIV239. Based on these results, we concluded that SHIV MNA was replication competent in primary monkey lymphocytes.

Productive replication of SHIV MNA in monkey PBMCs justified experimental infection of the virus *in vivo*. We inoculated 1.75×10^5 $TCID_{50}$ SHIV MNA intravenously into a rhesus macaque and monitored plasma viral RNA burden and circulating $CD4^+$ T-lymphocyte levels (Fig. 2b). Plasma viral RNA burden reached a peak of 5.6×10^6 copies ml^{-1} at 1 week post-infection (p.i.), and declined rapidly thereafter, reaching low levels of detection at 7 weeks p.i. (around 2.8×10^2 copies ml^{-1}). Circulating $CD4^+$ T-cell numbers showed a transient decrease around 1 week p.i., rebounded around 3 weeks p.i. and stabilized around 70 % of the pre-infection level from 4 weeks p.i. During the period of observation, the animal developed no obvious clinical manifestations related to lentivirus infection.

As SHIV MNA replicated *in vivo* without depleting helper T-cells, it was expected that the animal mounted an antiviral immune reaction. The production of antibody directed against Env was assessed by Western blotting, as described previously (Igarashi *et al.*, 1999). Purified Env protein (Advanced Biotechnologies) was used as the antigen (Fig. 3a). Anti-Env antibody was detected at 3 weeks p.i., and the level of antibody judged by the intensity of the band increased gradually with time.

We next examined whether the animal generated neutralizing antibodies against SHIV MNA. Because plasma samples from this specimen exhibited high background activity, IgG was purified from these samples collected on day 0 and in week 24 p.i. using protein G spin columns (GE Healthcare Japan). While the IgG from day 0 exhibited no neutralizing activity (Fig. 3b), as expected, the IgG collected at 24 weeks p.i. neutralized SHIV MNA, although a concentration >100 μg ml^{-1} was required to suppress replication of 100 $TCID_{50}$ of the input virus (Fig. 3c).

We examined whether the observed marginal neutralization by the antibody could be enhanced by the presence of YYA-021. Upon addition of YYA-021 in the assay system, SHIV MNA became sensitive to IgG obtained at 24 weeks p.i. (Fig. 3c), while no enhancement was identified from day 0 (Fig. 3b).

In this study, we generated a replication-competent SHIV MNA strain carrying an Env resistant to the neutralizing mAb KD-247 but conditionally sensitive in the presence of the CD4 mimic YYA-021. As the observed neutralization characteristics were identical to those of HIV-1 MNA, which contributed the majority of the Env sequence to the chimaera, the utility of the CD4 mimic as a means of enhancing antibody-mediated virus neutralization should be assessed in the context of infection *in vivo*. This concept could be tested during the acute phase of SHIV MNA infection, during which the virus undergoes substantial replication. To examine the feasibility of CD4-mimic-mediated enhancement of virus neutralization in the context of chronic infection, the conditions under which this type of intervention should be applied to HIV-1-infected patients in a clinical setting, the virus must be

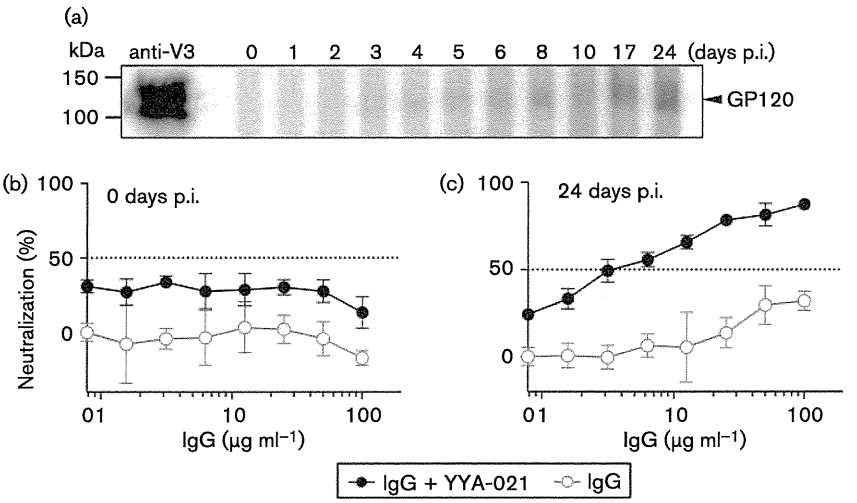


Fig. 3. Antibody induced against SHIV MNA. (a) The anti-HIV-1 gp120 antibody response was assessed by Western blotting with plasma samples collected at the indicated times. An anti-HIV-1 V3 mAb, 4G10 (ascites diluted 1 : 100) (von Brunn *et al.*, 1993), obtained from the NIH AIDS Reagent Program, was used as a positive control (lane anti-V3). (b, c) Neutralization of SHIV MNA with IgG purified from plasma of the infected rhesus macaque (day 0 and week 24 p.i.) with 20 μM YYA-021 or without YYA-021.

modified to sustain productive replication for a longer period. SHIV MNA in the present form does not fulfil this requirement. It is possible that animal-to-animal passage could increase the fitness of the virus in monkeys.

This study demonstrated that a CD4 mimic could modulate viral Env protein to be more susceptible to neutralization by less potent antibodies generated in the context of infection. During the early phase of infection, patients mount high titres of non-neutralizing antibodies directed against the V3 loop (Davis *et al.*, 2009a). Patients with HIV-1 clade C generate anti-Env antibodies, including anti-CD4i antibodies, with poor neutralizing activity against recent infection (Gray *et al.*, 2007). It is possible that the CD4 mimic YYA-021 causes a conformational change in SHIV MNA Env, which renders sequestered epitope(s) accessible to potentially neutralizing IgG, such as ones directed against the V3 loop and CD4i.

The current study extended the previous study by Yoshimura *et al.* (2010) and used HIV-1 MNA belonging to clade B to generate a new SHIV strain carrying Env. The neutralization sensitivity of this strain is characteristically augmented in the presence of a small-molecule CD4 mimic. Similar observations by Decker *et al.* (2005) showed that infections of a wide range of HIV-1 strains of multiple clades or circulating recombinant forms elicit high titres of anti-CD4i antibodies. These anti-CD4i antibodies neutralize viruses as divergent as HIV-2 in the presence of sCD4 (Decker *et al.*, 2005). Taking these observations into account, small-molecule CD4 mimics such as YYA-021 could potentially enhance the neutralization activity of the antibodies directed against autologous viruses belonging not only to clade B but also to multiple HIV-1 strains of various clades and possibly even HIV-2. Our results pave the way for a novel therapeutic intervention based on administration of CD4 mimics to patients with HIV to facilitate control of the virus by their own antibodies.

Acknowledgements

Thanks should be given to: Drs Julie Strizki and Paul Zavodny of the Schering-Plough Research Institute, Kenilworth, NJ, USA, for providing AD101; the NIH AIDS Research & Reference Reagent Program for providing TZM-bl cells, SV-A-MLV-env, 4G10 and soluble CD4; the Chemo-Sero-Therapeutic Research Institute, Kaketsuken, Japan, for providing mAb KD-247; former and current members of the Igarashi Laboratory for discussion and support. This work was supported by a Research on HIV/AIDS grant from the Ministry of Health, Labor and Welfare of Japan (H22-AIDS Research-007 and H24-AIDS Research-008) and by a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (23300156).

References

Davis, K. L., Bibollet-Ruche, F., Li, H., Decker, J. M., Kutsch, O., Morris, L., Salomon, A., Pinter, A., Hoxie, J. A. & other authors (2009a). Human immunodeficiency virus type 2 (HIV-2)/HIV-1 envelope chimeras detect high titers of broadly reactive HIV-1 V3-specific antibodies in human plasma. *J Virol* **83**, 1240–1259.

Davis, K. L., Gray, E. S., Moore, P. L., Decker, J. M., Salomon, A., Montefiori, D. C., Graham, B. S., Keefer, M. C., Pinter, A. & other authors (2009b). High titer HIV-1 V3-specific antibodies with broad reactivity but low neutralizing potency in acute infection and following vaccination. *Virology* **387**, 414–426.

Decker, J. M., Bibollet-Ruche, F., Wei, X., Wang, S., Levy, D. N., Wang, W., Delaporte, E., Peeters, M., Derdeyn, C. A. & other authors (2005). Antigenic conservation and immunogenicity of the HIV coreceptor binding site. *J Exp Med* **201**, 1407–1419.

Eda, Y., Takizawa, M., Murakami, T., Maeda, H., Kimachi, K., Yonemura, H., Koyanagi, S., Shiosaki, K., Higuchi, H. & other authors (2006). Sequential immunization with V3 peptides from primary human immunodeficiency virus type 1 produces cross-neutralizing antibodies against primary isolates with a matching narrow-neutralization sequence motif. *J Virol* **80**, 5552–5562.

Fujita, Y., Otsuki, H., Watanabe, Y., Yasui, M., Kobayashi, T., Miura, T. & Igarashi, T. (2013). Generation of a replication-competent chimeric simian-human immunodeficiency virus carrying env from subtype C clinical isolate through intracellular homologous recombination. *Virology* **436**, 100–111.

Gray, E. S., Moore, P. L., Choge, I. A., Decker, J. M., Bibollet-Ruche, F., Li, H., Leseka, N., Treurnicht, F., Mlisana, K. & other authors (2007). Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. *J Virol* **81**, 6187–6196.

Igarashi, T., Endo, Y., Englund, G., Sadjadpour, R., Matano, T., Buckler, C., Buckler-White, A., Plishka, R., Theodore, T. & other authors (1999). Emergence of a highly pathogenic simian/human immunodeficiency virus in a rhesus macaque treated with anti-CD8 mAb during a primary infection with a nonpathogenic virus. *Proc Natl Acad Sci U S A* **96**, 14049–14054.

Kong, L. & Sattentau, Q. J. (2012). Antigenicity and immunogenicity in HIV-1 antibody-based vaccine design. *J AIDS Clin Res* **S8**, 3.

Kwong, P. D. & Mascola, J. R. (2012). Human antibodies that neutralize HIV-1: identification, structures, and B cell ontogenies. *Immunity* **37**, 412–425.

Landau, N. R., Page, K. A. & Littman, D. R. (1991). Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. *J Virol* **65**, 162–169.

Li, M., Gao, F., Mascola, J. R., Stamatatos, L., Polonis, V. R., Koutsoukos, M., Voss, G., Goepfert, P., Gilbert, P. & other authors (2005). Human immunodeficiency virus type 1 *env* clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. *J Virol* **79**, 10108–10125.

Lusso, P., Earl, P. L., Sironi, F., Santoro, F., Ripamonti, C., Scarlatti, G., Longhi, R., Berger, E. A. & Burastero, S. E. (2005). Cryptic nature of a conserved, CD4-inducible V3 loop neutralization epitope in the native envelope glycoprotein oligomer of CCR5-restricted, but not CXCR4-using, primary human immunodeficiency virus type 1 strains. *J Virol* **79**, 6957–6968.

Madani, N., Schön, A., Princiotto, A. M., Lalonde, J. M., Courter, J. R., Soeta, T., Ng, D., Wang, L., Brower, E. T. & other authors (2008). Small-molecule CD4 mimics interact with a highly conserved pocket on HIV-1 gp120. *Structure* **16**, 1689–1701.

Mascola, J. R., Stiegler, G., VanCott, T. C., Katinger, H., Carpenter, C. B., Hanson, C. E., Beary, H., Hayes, D., Frankel, S. S. & other authors (2000). Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med* **6**, 207–210.

Moore, J. P., Cao, Y., Qing, L., Sattentau, Q. J., Pyati, J., Koduri, R., Robinson, J., Barbas, C. F., III, Burton, D. R. & Ho, D. D. (1995).

Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. *J Virol* **69**, 101–109.

Narumi, T., Ochiai, C., Yoshimura, K., Harada, S., Tanaka, T., Nomura, W., Arai, H., Ozaki, T., Ohashi, N. & other authors (2010). CD4 mimics targeting the HIV entry mechanism and their hybrid molecules with a CXCR4 antagonist. *Bioorg Med Chem Lett* **20**, 5853–5858.

Narumi, T., Arai, H., Yoshimura, K., Harada, S., Nomura, W., Matsushita, S. & Tamamura, H. (2011). Small molecular CD4 mimics as HIV entry inhibitors. *Bioorg Med Chem* **19**, 6735–6742.

Narumi, T., Arai, H., Yoshimura, K., Harada, S., Hirota, Y., Ohashi, N., Hashimoto, C., Nomura, W., Matsushita, S. & Tamamura, H. (2013). CD4 mimics as HIV entry inhibitors: lead optimization studies of the aromatic substituents. *Bioorg Med Chem* **21**, 2518–2526.

Nishimura, Y., Igarashi, T., Haigwood, N. L., Sadjadpour, R., Donau, O. K., Buckler, C., Plishka, R. J., Buckler-White, A. & Martin, M. A. (2003). Transfer of neutralizing IgG to macaques 6 h but not 24 h after SHIV infection confers sterilizing protection: implications for HIV-1 vaccine development. *Proc Natl Acad Sci U S A* **100**, 15131–15136.

Nishimura, Y., Shingai, M., Willey, R., Sadjadpour, R., Lee, W. R., Brown, C. R., Brenchley, J. M., Buckler-White, A., Petros, R. & other authors (2010). Generation of the pathogenic R5-tropic simian/human immunodeficiency virus SHIVAD8 by serial passaging in rhesus macaques. *J Virol* **84**, 4769–4781.

Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B. & Kabat, D. (1998). Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J Virol* **72**, 2855–2864.

Shibata, R. & Adachi, A. (1992). SIV/HIV recombinants and their use in studying biological properties. *AIDS Res Hum Retroviruses* **8**, 403–409.

Shimizu, Y., Okoba, M., Yamazaki, N., Goto, Y., Miura, T., Hayami, M., Hoshino, H. & Haga, T. (2006). Construction and in vitro characterization of a chimeric simian and human immunodeficiency virus with the RANTES gene. *Microbes Infect* **8**, 105–113.

Shinohara, K., Sakai, K., Ando, S., Ami, Y., Yoshino, N., Takahashi, E., Someya, K., Suzaki, Y., Nakasone, T. & other authors (1999). A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. *J Gen Virol* **80**, 1231–1240.

Thali, M., Moore, J. P., Furman, C., Charles, M., Ho, D. D., Robinson, J. & Sodroski, J. (1993). Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. *J Virol* **67**, 3978–3988.

von Brunn, A., Brand, M., Reichhuber, C., Morys-Wortmann, C., Deinhardt, F. & Schödel, F. (1993). Principal neutralizing domain of HIV-1 is highly immunogenic when expressed on the surface of hepatitis B core particles. *Vaccine* **11**, 817–824.

Wei, X., Decker, J. M., Liu, H., Zhang, Z., Arani, R. B., Kilby, J. M., Saag, M. S., Wu, X., Shaw, G. M. & Kappes, J. C. (2002). Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob Agents Chemother* **46**, 1896–1905.

Wyatt, R., Kwong, P. D., Desjardins, E., Sweet, R. W., Robinson, J., Hendrickson, W. A. & Sodroski, J. G. (1998). The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* **393**, 705–711.

Yamada, Y., Ochiai, C., Yoshimura, K., Tanaka, T., Ohashi, N., Narumi, T., Nomura, W., Harada, S., Matsushita, S. & Tamamura, H. (2010). CD4 mimics targeting the mechanism of HIV entry. *Bioorg Med Chem Lett* **20**, 354–358.

Yoshimura, K., Harada, S., Shibata, J., Hatada, M., Yamada, Y., Ochiai, C., Tamamura, H. & Matsushita, S. (2010). Enhanced exposure of human immunodeficiency virus type 1 primary isolate neutralization epitopes through binding of CD4 mimetic compounds. *J Virol* **84**, 7558–7568.

Yusa, K., Maeda, Y., Fujioka, A., Monde, K. & Harada, S. (2005). Isolation of TAK-779-resistant HIV-1 from an R5 HIV-1 GP120 V3 loop library. *J Biol Chem* **280**, 30083–30090.

Zhao, Q., Ma, L., Jiang, S., Lu, H., Liu, S., He, Y., Strick, N., Neamati, N. & Debnath, A. K. (2005). Identification of *N*-phenyl-*N'*-(2,2,6,6-tetramethyl-piperidin-4-yl)-oxalamides as a new class of HIV-1 entry inhibitors that prevent gp120 binding to CD4. *Virology* **339**, 213–225.

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Abacavir/Lamivudine versus Tenofovir/Emtricitabine with Atazanavir/Ritonavir for Treatment-naive Japanese Patients with HIV-1 Infection: A Randomized Multicenter Trial

Takeshi Nishijima[1,2], Misao Takano[1], Michiyo Ishisaka[1], Hirokazu Komatsu[3], Hiroyuki Gatanaga[1,2], Yoshimi Kikuchi[1], Tomoyuki Endo[4], Masahide Horiba[5], Satoru Kaneda[6], Hideki Uchiumi[7], Tomohiko Koibuchi[8], Toshio Naito[9], Masaki Yoshida[10], Natsuo Tachikawa[11], Mikio Ueda[12], Yoshiyuki Yokomaku[13], Teruhisa Fujii[14], Satoshi Higasa[15], Kiyonori Takada[16], Masahiro Yamamoto[17], Shuzo Matsushita[2], Masao Tateyama[18], Yoshinari Tanabe[19], Hiroaki Mitsuya[20,21], Shinichi Oka[1,2], on behalf of the Epzicom-Truvada study team

Abstract

Objective To compare the efficacy and safety of fixed-dose abacavir/lamivudine (ABC/3TC) and tenofovir/emtricitabine (TDF/FTC) with ritonavir-boosted atazanavir (ATV/r) in treatment-naïve Japanese patients with HIV-1 infection.

Methods A 96-week multicenter, randomized, open-label, parallel group pilot study was conducted. The endpoints were times to virologic failure, safety event and regimen modification.

Results 109 patients were enrolled and randomly allocated (54 patients received ABC/3TC and 55 patients received TDF/FTC). All randomized subjects were analyzed. The time to virologic failure was not significantly different between the two arms by 96 weeks (HR, 2.09; 95% CI, 0.72-6.13; p=0.178). Both regimens showed favorable viral efficacy, as in the intention-to-treat population, 72.2% (ABC/3TC) and 78.2% (TDF/FTC) of the patients had an HIV-1 viral load <50 copies/mL at 96 weeks. The time to the first grade 3 or 4 adverse event and the time to the first regimen modification were not significantly different between the two arms (adverse event: HR 0.66; 95% CI, 0.25-1.75, p=0.407) (regimen modification: HR 1.03; 95% CI, 0.33-3.19, p=0.964). Both regimens were also well-tolerated, as only 11.1% (ABC/3TC) and 10.9% (TDF/FTC) of the patients discontinued the allocated regimen by 96 weeks. Clinically suspected abacavir-associated hypersensitivity reactions occurred in only one (1.9%) patient in the ABC/3TC arm.

Conclusion Although insufficiently powered to show non-inferiority of viral efficacy of ABC/3TC relative to TDF/FTC, this pilot trial suggested that ABC/3TC with ATV/r is a safe and efficacious initial regimen for HLA-B*5701-negative patients, such as the Japanese population.

[1]AIDS Clinical Center, National Center for Global Health and Medicine, Japan, [2]Center for AIDS Research, Kumamoto University Graduate School of Medical Sciences, Japan, [3]Department of Community Care, Saku Central Hospital, Japan, [4]Department of Hematology, Hokkaido University Hospital, Japan, [5]Division of Respiratory Medicine, Higashisaitama National Hospital, Japan, [6]Department of Gastroenterology, National Hospital Organization Chiba Medical Center, Japan, [7]Department of Medicine and Clinical Science, Gunma University Graduate School of Medicine, Japan, [8]Department of Infectious Diseases and Applied Immunology, Research Hospital of the Institute of Medical Science, The University of Tokyo, Japan, [9]Department of General Medicine, Juntendo University School of Medicine, Japan, [10]Department of Infectious Diseases and Infection Control, The Jikei University School of Medicine, Japan, [11]Department of Infectious Diseases, Yokohama Municipal Citizen's Hospital, Japan, [12]Immunology and Infectious Disease, Ishikawa Prefectural Central Hospital, Japan, [13]Clinical Research Center, National Hospital Organization Nagoya Medical Center, Japan, [14]Division of Blood Transfusion, Hiroshima University Hospital, Japan, [15]Division of Hematology, Department of Internal Medicine, Hyogo College of Medicine, Japan, [16]Postgraduate Clinical Training Center, Ehime University Hospital, Japan, [17]Internal Medicine, Clinical Research Institute, National Hospital Organization Kyushu Medical Center, Japan, [18]Department of Infectious, Respiratory, and Digestive Medicine Control and Prevention of Infectious Diseases Faculty of Medicine, University of the Ryukyus, Japan, [19]Division of Infection Control and Prevention, Niigata University Medical and Dental Hospital, Japan, [20]Departments of Infectious Diseases and Hematology, Kumamoto University Graduate School of Medical Sciences, Japan and [21]Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, National Institutes of Health, USA

Received for publication October 18, 2012; Accepted for publication December 17, 2012

Correspondence to Dr. Shinichi Oka, oka@acc.ncgm.go.jp

Key words: HIV-1 infection, tenofovir/emtricitabine, abacavir/lamivudine, ritonavir-boosted atazanavir, treatment-naïve Asian patients, HLA-B*5701-negative

(Intern Med 52: 735-744, 2013)
(DOI: 10.2169/internalmedicine.52.9155)

Introduction

The fixed-dose combinations of tenofovir disoproxil fumarate 300 mg/emtricitabine 200 mg and abacavir sulfate 600 mg/lamivudine 300 mg are components of antiretroviral therapy for treatment-naïve patients with HIV-1 infection in developed countries (1, 2). The efficacy and safety of tenofovir/emtricitabine (TDF/FTC) and abacavir/lamivudine (ABC/3TC) remain the focus of ongoing debate. The ACTG 5202 trial demonstrated that the viral efficacy of ABC/3TC is inferior to that of TDF/FTC among treatment-naïve patients with a baseline HIV viral load of >100,000 copies/mL receiving efavirenz or ritonavir-boosted atazanavir as a key drug (3). On the other hand, the HEAT study showed that the viral efficacy of ABC/3TC is not inferior to that of TDF/FTC, regardless of the baseline viral load when used in combination with lopinavir/ritonavir (4).

With regard to safety, the occurrence of ABC-associated serious hypersensitivity reactions, the most important adverse effect of ABC affecting 5-8% of patients, has limited its use (5). However, screening for HLA-B*5701 or prescribing ABC in HLA-B*5701-negative populations, such as the Japanese, can reduce the incidence of immunologically-confirmed hypersensitivity to 0% (6, 7). Another negative aspect of ABC use is its association with myocardial infarction, as reported by the D:A:D study (8). However, the possible association of myocardial infarction with ABC was not confirmed by a recent meta-analysis report of the US Food and Drug Administration (9). On the other hand, renal proximal tubular damage leading to renal dysfunction and a loss of phosphate, which can result in decreased bone mineral density, is a well-known adverse effect of TDF (10-14).

Taking this background into account, the American Department of Health and Human Services (DHHS) Guidelines place TDF/FTC as the preferred drug and ABC/3TC as an alternative choice, whereas other international guidelines, including the European AIDS Clinical Society (EACS) Guidelines and the Japanese Guidelines, recommend both TDF/FTC and ABC/3TC as preferred choices (1, 2, 15).

Randomized control trials comparing TDF/FTC and ABC/3TC have been conducted in the US and Europe, but not in other parts of the world (4, 16, 17). The efficacy and safety of these two fixed-dose regimens in patients with different genetic backgrounds and body statures might not be similar to the results of previous trials, especially considering that the prevalence of HLA-B*5701 is zero in the Japanese population (7). Moreover, the degree of decrement in the renal function with TDF is larger in patients with a low body weight, such as the Japanese, which might limit the use of TDF in patients with a high risk for renal dysfunction (18-20).

Based on the above described background, the present randomized trial was originally designed in 2007 to elucidate whether the viral efficacy of ABC/3TC is not inferior to that of TDF/FTC with ritonavir-(100 mg) boosted atazanavir (300 mg) in treatment-naïve Japanese patients, whose body weight is much lower than Whites or Blacks (21). However, the independent data and safety monitoring board (DSMB) recommended that the protocol be modified to examine the efficacy, safety and tolerability among Japanese patients with HIV-1 infection for 96 weeks as a pilot trial because only 109 patients were enrolled and randomized at the end of the enrollment period despite a planned sample size of 240 patients, primarily due to the above mentioned negative reports of ABC use in the D:A:D study and ACTG 5202 (3, 8).

Materials and Methods

This clinical trial was designed and reported according to the recommendations of the Consolidated Standard of Reporting Trials (CONSORT) statement (22). The protocol and supporting CONSORT checklist are available as supplementary files (see Supplementary files 1 and 2).

Ethics statement

The Research Ethics Committee of each participating center approved the study protocol. All patients enrolled in this study provided a written informed consent. This study was conducted according to the principles expressed in the Declaration of Helsinki.

Study design

The Epzicom-Truvada study is a phase 4, multicenter, randomized, open-label, parallel group pilot study conducted in Japan that compared the efficacy and safety of a fixed dose of ABC/3TC and TDF/FTC, both combined with ritonavir-boosted atazanavir (ATV/r) for the initial treatment of HIV-1 infection for 96 weeks. Enrollment of patients began in November 2007 and ended in March 2010, and the follow-up period ended in February 2012. With a one to one ratio, the patients were randomly assigned to receive either a fixed dose of ABC/3TC or TDF/FTC, both administered with ATV/r. The randomization was stratified according to each participating site and conducted at the data center with

independent clinical research coordinators using a computer-generated randomization list prepared by a statistician with no clinical involvement in the trial.

Study patients

This study population included treatment-naïve Japanese patients aged 20 or over with HIV-1 infection who met the eligibility criteria for the commencement of antiretroviral therapy according to the DHHS Guidelines in place in the U.S. at the time of the writing of the study protocol (a CD4 count <350/μL or a history of AIDS-defining illness regardless of the CD4 count) (23). Patients were screened and excluded if they had previously taken lamivudine, tested positive for hepatitis B surface antigens, had comorbidities such as hemophilia or diabetes mellitus that required medical treatment, congestive heart failure or cardiac myopathy or if they were considered not suitable for enrollment by the attending physicians. Candidates were also excluded if their alanine aminotransferase level was 2.5 times greater than the upper limit of normal, they had an estimated glomerular filtration rate (eGFR) calculated using the Cockcroft-Gault equation of <60 mL/min, {creatinine clearance = [(140- age) × weight (kg)]/(serum creatinine ×72)(×0.85 for females)} or a serum phosphate level <2 mg/dL or had active opportunistic diseases that required treatment (24). Each patient's actual body weight was used for the calculation of eGFR. At screening, a genotypic drug resistant test and screening for the HLA-B*5701 allele were permitted but not required because the prevalence of both the drug resistant virus and the HLA-B*5701 allele are low in Japanese patients (7, 25). Medical history, including a history of AIDS-defining illnesses and other comorbidities, was also collected. Enrollment stopped on March 3, 2008 due to the recommendation from the DSMB of the trial based on the interim analysis of the ACTG5202 that ABC/3TC is less effective than TDF/FTC in patients with a baseline viral load >100,000 copies/mL (3). Accordingly, the DSMB recommended that the trial should be restarted with modified inclusion criteria: to enroll patients with an HIV-1 viral load of <100,000 copies/mL at screening, and the enrollment restarted from April 1, 2008.

Study procedures

Required visits for participants for clinical and laboratory assessments were at screening, enrollment and every 4 weeks until the viral load diminished to <50 copies/mL. For patients with a viral load <50 copies/mL, the required visit interval was every 12 weeks for the duration of the study. The evaluation performed at each visit included a physical examination, CD4 cell count, HIV-1 RNA viral load, a complete blood cell count and blood chemistries (total bilirubin, alanine aminotransferase, lactate dehydrogenase, serum creatinine, potassium, phosphate, triglycerides and low-density lipoprotein (LDL) cholesterol) and a urine examination of the levels of phosphate, creatinine and β2 microglobulin. The values of urinary β2 microglobulin were expressed relative to a urinary creatinine level of 1 g/L (/g Cr). The percent tubular resorption of phosphate was calculated using the following formula: {1-[(urine phosphate × serum creatinine)/(urine creatinine × serum phosphate)]} ×100 (26). All data, including the HIV-1 RNA viral load, were collected at each participating site and sent to the data center. Grade 3 or 4 serious adverse events were reported to the DSMB, which made a judgment whether they were caused by the study drugs. Independent research coordinators at the data center visited at least 10 facilities every year to monitor the accuracy of the submitted data and compliance to the study protocol. All authors vouch for the completeness and accuracy of the reported data.

Statistical analysis

The sample size calculation was originally conducted as follows: Assuming a 90% success rate in the TDF/FTC arm at week 48, a sample size of 224 patients (112 patients per arm) provided 80% power (one sided, α=0.05) to establish non-inferiority of ABC/3TC to TDF/FTC each in combination with ATV/r. Non-inferiority was defined as the lower bound of the two-sided 95% confidence interval (CI) with the treatment difference being above -10%. Based on this assumption, the targeted sample size was set to 240 patients (120 in each arm). However, as previously described, due to the shortage of accrued subjects, this study was underpowered and conducted as a pilot trial.

The primary efficacy endpoint was the time from randomization to virologic failure (defined as a confirmed HIV-1 RNA >1,000 copies/mL at or after 16 weeks and before 24 weeks or >200 copies/mL at or after 24 weeks) (3). The secondary efficacy endpoints included the time from randomization to either virologic failure or ART modification and a comparison of the proportions of patients with HIV-1 RNA <50 copies/mL at weeks 48 and 96 regardless of previous virologic failure. The intent-to-treat (ITT) population comprising all randomized subjects was used to assess the efficacy data; however, a comparison of the proportion of virologically-suppressed patients was conducted with both the ITT and a per protocol population while on the initial randomized regimen.

The safety endpoint was the time from randomization to the first occurrence of grade 3 or 4 laboratory data or abnormal symptoms that were at least one grade higher than the baseline. Isolated hyperbilirubinemia was excluded from the safety endpoints. The grade of adverse events was classified according to the Division of AIDS Table for grading the severity of adult and pediatric events, version 2004 (27). The tolerability endpoint was the time from randomization to any regimen modification. The safety and tolerability endpoints were calculated in the ITT population. Changes per protocol in the CD4 cell count, lipid markers and renal tubular markers at weeks 48 and 96 were compared using the Mann-Whitney test. A repeated measures mixed model was used to estimate and compare changes in the renal function between the two arms (17). The renal function was calculated using the Modification of Diet in Renal Disease study

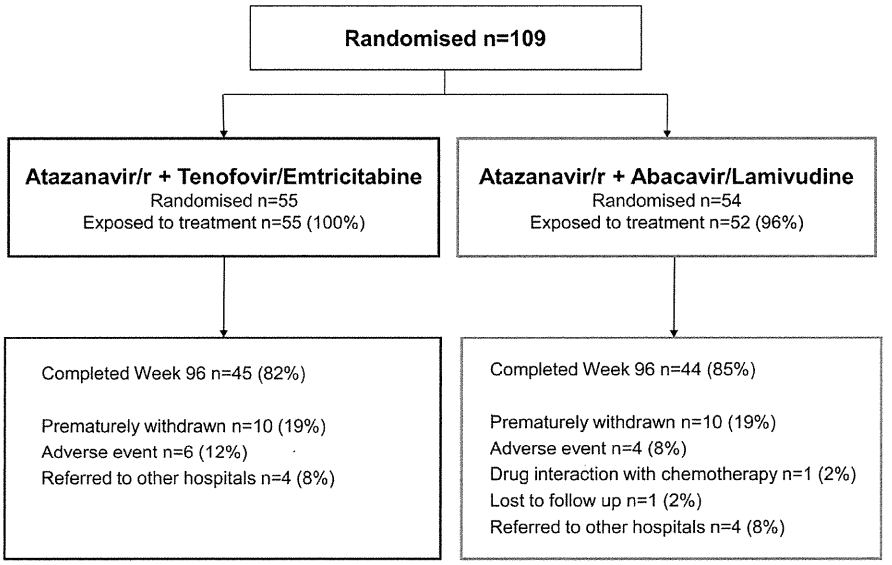


Figure 1. Enrollment, randomization and disposition of patients.

Table 1. Demographic and Baseline Characteristics

	ABC/3TC (n=54)	TDF/FTC (n=55)	Total (n=109)
Sex (male), n (%)	53 (98.1)	54 (98.2)	107 (98.2)
Age (years)†	39 (28.8-44)	35 (29-42)	36 (29-42.5)
CD4 count (/μL)†	236.5 (194-301.3)	269 (177-306)	257 (194-305)
HIV RNA viral load (log_{10}/mL)†	4.29 (3.92-4.67)	4.28 (3.86-4.60)	4.28 (3.89-4.67)
HIV RNA viral load >100,000 log_{10}/mL, n (%)	1 (1.9)	0 (0)	1 (0.9%)
Route of transmission (homosexual contact), n (%)	47 (87)	49 (89.1)	96 (88.1)
History of AIDS n (%)	1 (1.9)	5 (9.1)	6 (5.5)
Body weight (kg)†	64 (59-72.1)	63.1 (58-69)	64 (58.3-70.7)
Body mass index (kg/m^2)†	22.6 (20.4-24.2)	21.9 (20.3-23.6)	22.4 (20.3-23.7)
eGFR (mL/min/1.73 m^2)†	96.9 (82.7-107.3)	94.4 (83.6-105.7)	96.7 (83.0-106.7)
Creatinine clearance (mL/min)†	119.3 (105.4-136.6)	124.6 (103-139.3)	120.3 (104.7-138.3)
Serum creatinine (mg/dL)†	0.76 (0.67-0.83)	0.75 (0.68-0.84)	0.76 (0.68-0.83)
Urinary β2 microglobulin (μg/g Cre)†	195.8 (98.3-505.3)	138.4 (86.8-426.4)	172.9 (88.3-458.7)
Tubular resorption of phosphate (%)†	92.9 (90-95.1)	92.3 (87.7-95.2)	92.7 (89.3-95.1)
LDL-cholesterol (mg/dL)†	91.5 (75-125.5)	94 (72.5-111.5)	94 (74.5-114)
Triglycerides (mg/dL)†	132 (98-170.5)	114 (73-184)	127 (85.5-175)
Hypertension, n (%)	3 (5.6)	1 (1.8)	4 (3.7)
Diabetes mellitus, n (%)	0 (0)	0 (0)	0 (0)
Concurrent use of nephrotoxic drugs, n (%)	10 (18.5)	10 (18.2)	20 (18.3)
Hepatitis C, n (%)	0 (0)	0 (0)	0 (0)

†median (interquartile range)

IQR: interquartile range, AIDS: acquired immunodeficiency syndrome, eGFR: estimated glomerular filtration rate, LDL: low-density lipoprotein

equation adjusted for the Japanese population (28), and a sensitivity analysis was conducted using the above mentioned Cockcroft-Gault equation.

Time-to-event distributions were estimated using the Kaplan-Meier method and compared using the two-sided log-rank test. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) were estimated using the Cox proportional hazards model. For grade 3 or 4 serious adverse events caused by the study drugs, the description and severities were recorded. Statistical significance was defined at two-sided p values <0.05. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 17.0 (SPSS, Chicago, IL).

Results

Patient disposition and baseline characteristics

109 patients from 18 centers were enrolled and randomized between November 2007 and March 2010. Of these patients, 54 and 55 were allocated to the ABC/3TC and TDF/FTC arms, respectively (Fig. 1). The baseline demographics and characteristics are shown in Table 1. Most patients were men, with a median body weight of 64 kg. The median CD4 cell count was 257/μL (IQR: 194-305). One patient in the ABC/3TC arm had a baseline HIV-1 RNA level of >100,000

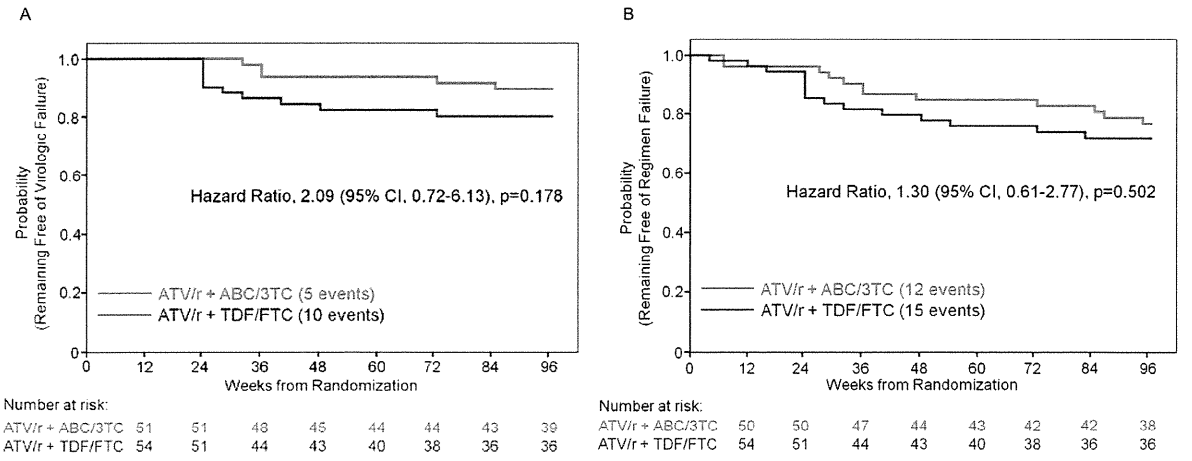


Figure 2. Efficacy results over 96 weeks. (A) Time to protocol-defined virologic failure. (B) Time to the first occurrence of either virologic failure or discontinuation of the initially randomized regimen. ATV/r: ritonavir-boosted atazanavir, ABC/3TC: abacavir/lamivudine, TDF/FTC: tenofovir/emtricitabine

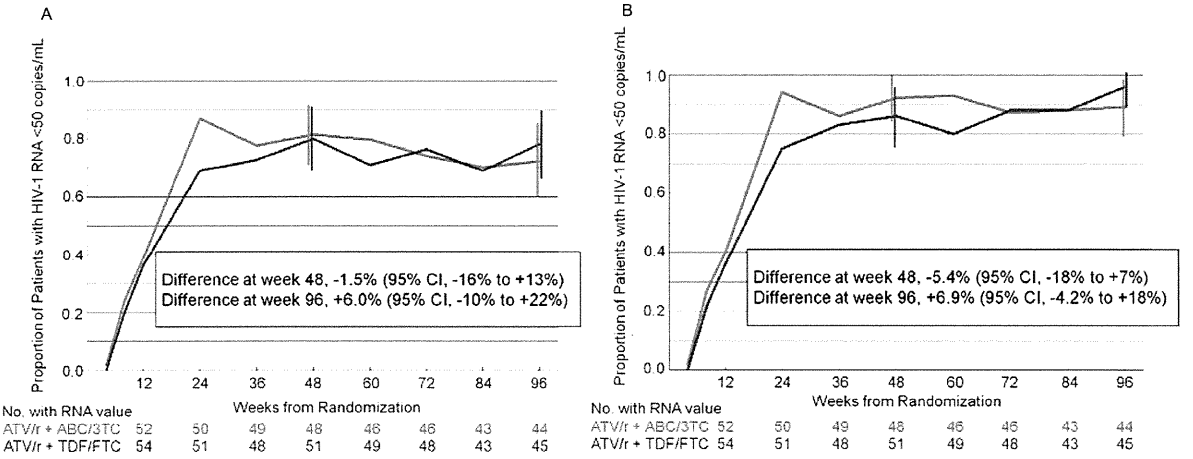


Figure 3. Efficacy results at 48 and 96 weeks. Proportion of patients with an HIV RNA level <50 copies/mL regardless of previous virologic failure with 95% binomial confidence intervals at 48 and 96 weeks. (A) Intention-to-treat analysis. (B) Per protocol analysis. ATV/r: ritonavir-boosted atazanavir, ABC/3TC: abacavir/lamivudine, TDF/FTC: tenofovir/emtricitabine

copies/mL. This patient was enrolled before the announcement of the interim analysis of ACTG5202 in March 2008 and achieved an HIV-1 RNA level of <50 copies/mL by the end of that month. One patient in the TDF/FTC arm had a history of lamivudine use. That patient was included in the analysis because this aspect of the medical history was identified after randomization and initiation of the allocated treatment.

Efficacy results

In the primary efficacy analysis, the time to virologic failure was not significantly different in the ABC/3TC arm from that observed in the TDF/FTC arm by 96 weeks (HR, 2.09; 95% CI, 0.72-6.13; p=0.178). Virologic failure occurred in 5 and 10 patients in the ABC/3TC and TDF/FTC arms, respectively (Fig. 2A). In the secondary efficacy analysis, the times to the first occurrence of confirmed virologic failure or discontinuation of the initially allocated regimen were not different between the two arms (HR, 1.30; 95% CI, 0.61-2.77; p=0.502) (Fig. 2B). Among the ITT population, the proportion of patients with an HIV RNA level <50 copies/mL at week 48 regardless of previous virologic failure was 81.5% in the ABC/3TC group and 80% in the TDF/FTC group, for a difference of -1.5% (95% CI, -16% to 13%), and at week 96, 72.2% and 78.2% for the ABC/3TC and TDF/FTC groups, respectively, for a difference of 6% (95% CI, -10% to 22%) (Fig. 3A). The per protocol analysis showed that the proportions at week 48 were 91.7% and 86.3% for the ABC/3TC and TDF/FTC groups, respectively, for a difference of -5.4% (95% CI, -18% to 7%). At week 96, the proportions were 88.6% and 95.6% for the ABC/3TC and TDF/FTC groups, respectively, for a

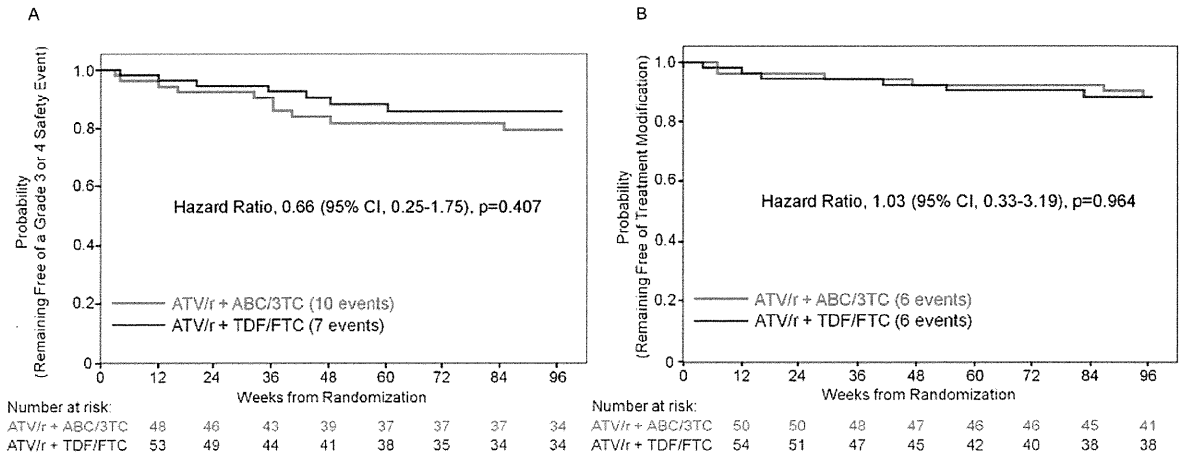


Figure 4. Safety and tolerability results over 96 weeks. (A) Time to first primary safety endpoint, defined as the first grade 3 or 4 event on the initial randomized regimen, which was at least one grade higher than baseline. (B) Time to tolerability endpoint, defined as the first change in regimen. ATV/r: ritonavir-boosted atazanavir, ABC/3TC: abacavir/lamivudine, TDF/FTC: tenofovir/emtricitabine

Table 2. Selected Grade 3 or 4 Events While Receiving Randomized Antiretroviral Drugs

	ABC/3TC (n=54)	TDF/FTC (n=55)	Total (n=109)
Overall, n (%)	13 (24)	10 (18)	23 (21)
Laboratory, n (%)	12 (22)	7 (13)	19 (17)
Alanine aminotransferase, n	0	1	1
LDL-cholesterol, n	6	2	8
Triglycerides, n	0	3	3
Uric acid, n	1	0	1
Serum phosphate, n	2	0	2
Serum calcium, n	1	0	1
Serum creatinine, n	1	0	1
Platelets count, n	1	1	2
Symptoms, n (%)	1 (2)	3 (5)	4 (4)
Depression, n	0	2	2
Fever, n	1	1	2

More than one event occurred in 2 patients.
LDL: low-density lipoprotein

difference of 6.9% (95% CI, -4.2% to 18%) (Fig. 3B). The primary and secondary efficacy analyses did not show a significant difference in viral efficacy between the two arms.

Safety and tolerability results

10 (18.5%) and 7 (12.7%) patients in the ABC/3TC and TDF/FTC groups, respectively, experienced 23 grade 3 or 4 adverse events related to the study drugs while on the initial regimen. The time to the first adverse event was not significantly different between the two arms (HR 0.66; 95% CI, 0.25-1.75, p=0.407) (Fig. 4A). Table 2 shows a list of selected grade 3 or 4 safety events. Among the adverse events, 48% included elevation of lipid markers. The tolerability endpoint, the time to first ART modification, was not significantly different between the two arms (HR 1.03; 95% CI, 0.33-3.19, p=0.964), and only 6 (11.1%) and 6 (10.9%) patients in the ABC/3TC and TDF/FTC arms, respectively, discontinued the initially allocated regimen by 96 weeks (Fig. 4B). The most common reason for regimen modification was drug toxicity (n=10; 4 in ABC/3TC and 6 in TDF/FTC arm; suspected ABC hypersensitivity reactions based on the appearance of rash and fever in HLA-B*5701-negative patient; n=1, depression; n=3, jaundice; n=3, nausea; n=2, and lipodystrophy; n=1). One patient in the ABC/3TC group developed a cerebral infarction during week 39 but was able to continue the study drugs. No deaths were registered during the study period.

Changes in the CD4 cell count and other parameters of interest

The increase in the median CD4 count from baseline to 48 weeks was marginally larger in the ABC/3TC arm than in the TDF/FTC arm (median: ABC/3TC: 216, TDF/FTC: 192, p=0.107). This difference was significantly larger at 96

Table 3. **Median Values of Changes in Parameters of Interest from Baseline to 96 Weeks**

	ABC/3TC (n=54)				TDF/FTC (n=55)				
	Number tested (baseline, week 96)	Baseline	Week 96	Median Δ	Number tested (baseline, week 96)	Baseline	Week 96	Median Δ	p value
CD4 cell count (/μL)	54, 43	236.5	545	328	55, 45	269	493	216	0.031
Lipids									
LDL-cholesterol (mg/dL)	54, 16	91.5	149	31.5	53, 16	94	97	2	0.026
Triglyceride (mg/dL)	54, 29	132	257	111	55, 26	114	202	40.5	0.037
Renal tubular markers									
Urinary β2 microglobulin (μg/g Cre)	49, 32	195.8	99.2	-94.9	52, 38	138.4	303.9	86.6	<0.001
Tubular resorption of phosphate (%)	49, 32	93	92	-1.4	50, 36	92	91	-2.6	0.930

LDL: low-density lipoprotein

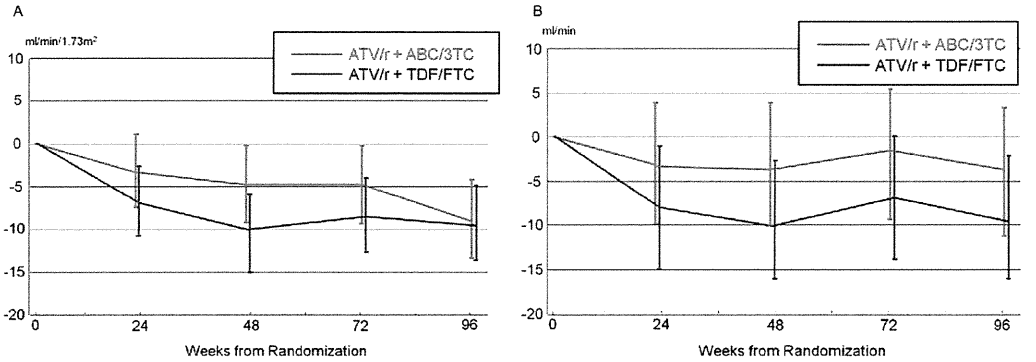


Figure 5. **Changes in the renal function between baseline and 96 weeks. (A) Changes in the estimated glomerular filtration rate calculated with the Modification of Diet in Renal Disease study equation adjusted for the Japanese population. (B) Changes in creatinine clearance calculated with the Cockcroft-Gault equation. The data are presented as the mean±95% confidence interval. ATV/r: ritonavir-boosted atazanavir, ABC/3TC: abacavir/lamivudine, TDF/FTC: tenofovir/emtricitabine**

weeks (ABC/3TC: 328, TDF/FTC: 236, p=0.031, Table 3). The increases in both LDL-cholesterol and triglycerides from baseline to 96 weeks were more significant in the ABC/3TC arm than in the TDF/FTC arm. One patient in the TDF/FTC arm had been treated with lipid-lowering medications prior to study enrollment. Furthermore, 7 patients and 1 patient in the ABC/3TC and TDF/FTC arms, respectively, started lipid-lowering agents during the study period. With regard to renal tubular markers, the levels of urinary β2 microglobulin increased in the TDF/FTC arm (median: 86.6 μg/g Cre), whereas it decreased in the ABC/3TC arm (median: -94.9 μg/g Cre). These changes were significantly different between the two arms (p<0.001). On the other hand, tubular resorption of phosphate did not show changes from baseline to 96 weeks in the two groups, and the levels were not different between the two arms (Table 3).

Changes in the renal function

A data analysis using repeated measures mixed models showed a significant decrease in the mean eGFR from baseline to 96 weeks in both groups (ABC/3TC: -8.7 mL/min/1.73 m^2, 95%CI -13.3 to -4.2, p<0.001; TDF/FTC: -9.2 mL/min/1.73 m^2, 95%CI -13.7 to -4.7, p<0.001) (Fig. 5A). There was no significant interaction between the trend of the two arms over time (p=0.202), thus indicating that the change in eGFR from baseline to 96 weeks was not significantly different between the two arms. A sensitivity analysis of creatinine clearance calculated using the Cockcroft-Gault equation showed that creatinine clearance decreased significantly from the baseline in the TDF/FTC arm (-9.6 mL/min, 95%CI -16.6 to -2.5, p<0.001) but not in the ABC/3TC arm (-4.1 mL/min, 95%CI -11.2 to 3.0, p=0.466)(Fig. 5B). No significant interaction between the trend of the two arms was observed with respect to creatinine clearance (p=0.403). Two patients in the ABC/3TC arm progressed to more advanced chronic kidney disease (CKD) stage by the last per protocol visit: one patient progressed to stage 4 CKD (eGFR <30 mL/min/1.73 m^2) and the other to stage 3 CKD (eGFR <60 mL/min/1.73 m^2). However, ABC/3TC did not appear to be the causative drug for renal dysfunction in these two cases because the deterioration in the renal function was associated with the development of malignant lymphoma in the former patient and with the commencement of fenofibrate treatment in the latter; renal function recovered rapidly in the latter patient after the discontinuation of fenofibrate.

Discussion

Although insufficiently powered to show the non-inferiority of the viral efficacy of ABC/3TC relative to TDF/

FTC, this pilot study is the first randomized study conducted in Asia to elucidate the efficacy and safety of fixed doses of these two regimens each administered in combination with ATV/r for initial HIV-1 therapy. Viral efficacy, safety, and tolerability were not significantly different in the two arms of Japanese patients with a baseline HIV viral load <100,000 copies/mL over 96 weeks. Both regimens showed favorable viral efficacy, as in the ITT population, 72.2% and 78.2% of the patients in the ABC/3TC and TDF/FTC arms, respectively, had HIV-1 viral loads of <50 copies/mL at 96 weeks. Both regimens were also well-tolerated, as only 11.1% and 10.9% of the patients in the ABC/3TC and TDF/FTC arms, respectively, discontinued the allocated regimen by 96 weeks. Clinically suspected (not immunologically-confirmed) ABC-associated hypersensitivity reaction occurred in only one (1.9%) patient in the ABC/3TC arm, confirming that ABC hypersensitivity is rare in populations in which HLA-B*5701-positive patients are uncommon. Thus, this trial suggests that ABC/3TC may be an efficacious and safe regimen for use in HLA-B*5701-negative populations, such as the Japanese, with a baseline HIV viral load <100,000 copies/mL.

The usefulness of ABC/3TC has recently received higher recognition for two reasons. One, a meta-analysis by the FDA did not confirm the association between ABC use and myocardial infarction (9). Two, it became clear that TDF-induced renal tubulopathy results in decreased bone mineral density due to phosphate wasting and a decreased renal function, both of which might develop into serious complications with long-term TDF use (12-14, 29, 30). On the other hand, greater deteriorations in the levels of lipid markers were noted in ABC/3TC than in TDF/FTC in clinical trials comparing these two agents (16, 17). The present study also demonstrated that the increases in the LDL-cholesterol and triglyceride levels were higher in the ABC/3TC arm than in the TDF/FTC arm.

TDF-induced nephrotoxicity is of particular interest in this study because a low body weight is an important risk factor, and body stature was much smaller in this study population (median baseline body weight 64 kg), than in the ASSERT study (72 kg), which compared the renal function between patients receiving ABC/3TC and TDF/FTC with efavirenz in Europe (17, 18, 20). This study showed that changes in the renal function from baseline were not significantly different between the two arms, similar to the findings of the ASSERT study. None of the patients in the TDF/FTC arm exhibited progression of CKD stage. On the other hand, the levels of urinary β2 microglobulin deteriorated significantly from baseline in the TDF/FTC arm, whereas improvements were observed in the ABC/3TC arm. This is also similar to the findings reported by the ASSERT trial. This suggests that urinary β2 microglobulin is a more sensitive marker for evaluating TDF nephrotoxicity than the renal function calculated by serum creatinine, as also demonstrated in our previous work (31). Tubular resorption of phosphate, another marker examined to evaluate the renal tubular function, did not exhibit any changes from baseline or between the two arms, suggesting that urinary β2 microglobulin may be a better marker for evaluating TDF nephrotoxicity than tubular resorption of phosphate. Of note, in both arms, the renal function did significantly decrease from baseline. To our knowledge, this is the first randomized trial comparing ABC/3TC and TDF/FTC that observed deterioration of the renal function after the initiation of ART. This result highlights the importance of regular monitoring of renal function after initiation of ART, although it is difficult to draw a firm conclusion on the prognosis of the renal function from this study, due to the limited length of the observation period and the small number of enrolled patients.

Only one patient (1.9%) in the ABC/3TC arm developed a clinically suspected ABC-associated hypersensitivity reaction, which was diagnosed based on the appearance of a skin rash and fever six weeks after commencement of the study drug. The patient fully recovered after discontinuation of the drugs. The ASSERT trial of HLA-B*5701-negative patients reported a similar incidence (3%) of clinically suspected ABC hypersensitivity reactions (17). The one case observed in our trial could be a false positive, because ABC hypersensitivity reactions commonly occur 9-11 days after the initiation of therapy (32), and ABC hypersensitivity was not confirmed immunologically. Nonetheless, immediate discontinuation of ABC is highly recommended even in HLA-B*5701-negative patients suspected of ABC hypersensitivity, since ABC hypersensitivity can occur in such patients (33) and errors in genotyping for HLA or reporting a genotype might occur in practice (34).

Several limitations of this trial should be acknowledged. First, due to the shortage of enrolled patients, the trial was insufficiently powered to test non-inferiority of the viral efficacy of ABC/3TC against TDF/FTC, as initially planned. However, the safety and tolerability data of these regimens in Asia are a valuable asset for patients from this region, and efficacy data could be utilized as part of a meta-analysis in the future. Second, the enrolled subjects were mostly men (primarily men who had sex with men and very few injection drug users). Further studies are needed to examine the efficacy and safety of these regimens in women and patients with different routes of transmissions in Asia.

In summary, this randomized trial demonstrated high efficacy and safety of fixed-dose ABC/3TC and TDF/FTC in combination with ATV/r over 96 weeks for treatment-naïve Japanese patients with a baseline HIV-1 viral load <100,000 copies/mL, although it was insufficiently powered to show non-inferiority of the viral efficacy of ABC/3TC compared with TDF/FTC. ABC/3TC with ATV/r is a safe and efficacious initial regimen for treating HLA-B*5701-negative patients with a baseline HIV-1 viral load <100,000 copies/mL.

Author's disclosure of potential Conflicts of Interest (COI).
Uchiumi H: Research funding, ViiV Healthcare. Koibuchi T: Research funding, Nihon Ultmarc Inc. Naito T: Research funding,

MSD K.K. and Janssen Pharmaceutical K.K. Takada K: Research funding, ViiV Healthcare. Oka S: Research funding, MSD K.K., Abbott Japan, Co., Janssen Pharmaceutical K.K., Pfizer, Co., and Roche Diagnostics K.K.

Authors' contributions

SO, MT (Takano), MI, HG, YK and YT designed the study. TE, MH, SK, HU, TK, TN (Naito), MY (Yoshida), NT, MU, YY, TF, SH, KT, MY (Yamamoto), SM, MT (Tateyama) and YT collected the data. HM supervised the study and reviewed and approved study report. TN (Nishijima), HK, HG and SO analyzed and interpreted the data. TN (Nishijima), HK, HG and SO drafted the manuscript and all other authors revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Acknowledgement

We thank the patients for participating in this study. The authors are indebted to Mikiko Ogata and Akiko Nakano for their support in this study as a data manager and research coordinator, respectively. The Epzicom-Truvada study team includes the following members: Takao Koike[1], Mitsufumi Nishio[1], Keisuke Yamaguchi[1], Katsuya Fujimoto[1], Satoshi Yamamoto[1], Ikumi Kasahara[1], Tetsuro Takeda[2], Takafumi Tezuka[2], Hiroshi Moro[2], Takeharu Kotani[3], Mieko Yamada[3], Yoshiyuki Ogawa[4], Kunio Yanagisawa[4], Aikichi Iwamoto[5], Takeshi Fujii[5], Takashi Odawara[5], Nahoko Miyazaki[5], Kazufumi Matsumoto[5], Kumiko Sumino[5], Mizue Saita[6], Mai Suzuki[6], Rino Sakamoto[6], Satoshi Kimura[7], Yukihiro Yoshimura[8], Motohiro Hamaguchi[9], Naoto Mamiya[9], Atsuyoshi Imamura[9], Ayumi Kogure[9], Mayumi Imahashi[9], Takuma Shirasaka[10], Munehiro Yoshino[10], Sawada Akihiro[11], Tazuko Tokugawa[11], Seiji Saito[12], Noboru Takata[12], Fumiko Kagiura[12], Rumi Minami[13], Soichiro Takahama[13], Toshikazu Miyagawa[14], Daisuke Tasato[15], Hideta Nakamura[15], Naoki Ishizuka[16], Katsuji Teruya[16], Miwako Honda[16], Kunihisa Tsukada[16], Hirohisa Yazaki[16], Junko Tanuma[16], Haruhito Honda[16], Ei Kinai[16], Koji Watanabe[16], Takahiro Aoki[16], Tamayo Watanabe[16], Mahoko Kamimura[16], Masako Ito[16], Jiro Mikami[16], Atsushi Kubota[16], Toshikatsu Kawasaki[16]

[1]Hokkaido University Hospital, Japan; [2]Niigata University Medical and Dental Hospital, Japan; [3]Ishikawa Prefectural Central Hospital, Japan; [4]Gunma University Graduate School of Medicine, Japan; [5]Research Hospital of the Institute of Medical Science, The University of Tokyo, Japan; [6]Juntendo University School of Medicine, Japan; [7]Tokyo Teishin Hospital, Japan; [8]Yokohama Municipal Citizen's Hospital, Japan; [9]National Hospital Organization Nagoya Medical Center, Japan; [10]National Hospital Organization Osaka Medical Center, Japan; [11]Hyogo College of Medicine, Japan; [12]Hiroshima University Hospital, Japan; [13]National Hospital Organization Kyushu Medical Center, Japan; [14]Kumamoto University Graduate School of Medical Sciences, Japan; [15]University of the Ryukyus, Okinawa, Japan and [16]National Center for Global Health and Medicine, Japan.

References

1. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services [http : / / www. aidsinfo. nih. gov / contentfiles / lvguidelines / adultandadolescentgl.pdf].
2. European AIDS Clinical Society Guidelines version 6 [http://www.europeanaidsclinicalsociety. org / images / stories / EACS-Pdf / EACSGuidelines-v6.0-English.pdf].
3. Sax PE, Tierney C, Collier AC, et al. Abacavir-lamivudine versus tenofovir-emtricitabine for initial HIV-1 therapy. N Engl J Med **361**: 2230-2240, 2009.
4. Smith KY, Patel P, Fine D, et al. Randomized, double-blind, placebo-matched, multicenter trial of abacavir/lamivudine or tenofovir/emtricitabine with lopinavir/ritonavir for initial HIV treatment. AIDS **23**: 1547-1556, 2009.
5. Hetherington S, McGuirk S, Powell G, et al. Hypersensitivity reactions during therapy with the nucleoside reverse transcriptase inhibitor abacavir. Clin Ther **23**: 1603-1614, 2001.
6. Mallal S, Phillips E, Carosi G, et al. HLA-B*5701 screening for hypersensitivity to abacavir. N Engl J Med **358**: 568-579, 2008.
7. Gatanaga H, Honda H, Oka S. Pharmacogenetic information derived from analysis of HLA alleles. Pharmacogenomics **9**: 207-214, 2008.
8. Sabin CA, Worm SW, Weber R, et al. Use of nucleoside reverse transcriptase inhibitors and risk of myocardial infarction in HIV-infected patients enrolled in the D:A:D study: a multi-cohort collaboration. Lancet **371**: 1417-1426, 2008.
9. Ding X, Andraca-Carrera E, Cooper C, et al. No association of abacavir use with myocardial infarction: findings of an FDA meta-analysis. J Acquir Immune Defic Syndr **61**: 441-447, 2012.
10. Peyrière H, Reynes J, Rouanet I, et al. Renal tubular dysfunction associated with tenofovir therapy: report of 7 cases. J Acquir Immune Defic Syndr **35**: 269-273, 2004.
11. Verhelst D, Monge M, Meynard JL, et al. Fanconi syndrome and renal failure induced by tenofovir: a first case report. Am J Kidney Dis **40**: 1331-1333, 2002.
12. Gallant JE, Winston JA, DeJesus E, et al. The 3-year renal safety of a tenofovir disoproxil fumarate vs. a thymidine analogue-containing regimen in antiretroviral-naive patients. AIDS **22**: 2155-2163, 2008.
13. Cooper RD, Wiebe N, Smith N, Keiser P, Naicker S, Tonelli M. Systematic review and meta-analysis: renal safety of tenofovir disoproxil fumarate in HIV-infected patients. Clin Infect Dis **51**: 496-505, 2010.
14. McComsey GA, Kitch D, Daar ES, et al. Bone mineral density and fractures in antiretroviral-naive persons randomized to receive abacavir-lamivudine or tenofovir disoproxil fumarate-emtricitabine along with efavirenz or atazanavir-ritonavir: Aids Clinical Trials Group A5224s, a substudy of ACTG A5202. J Infect Dis **203**: 1791-1801, 2011.
15. Guidelines for antiretroviral therapy. Japanese Ministry of Health and Welfare, in Japanese. [http://www.haart-support.jp/pdf/guideline2012.pdf].
16. Sax PE, Tierney C, Collier AC, et al. Abacavir/lamivudine versus tenofovir DF/emtricitabine as part of combination regimens for initial treatment of HIV: final results. J Infect Dis **204**: 1191-1201, 2011.
17. Post FA, Moyle GJ, Stellbrink HJ, et al. Randomized comparison of renal effects, efficacy, and safety with once-daily abacavir/lamivudine versus tenofovir/emtricitabine, administered with efavirenz, in antiretroviral-naive, HIV-1-infected adults: 48-week results from the ASSERT study. J Acquir Immune Defic Syndr **55**: 49-57, 2010.
18. Nishijima T, Gatanaga H, Komatsu H, et al. Renal function de-

clines more in tenofovir- than abacavir-based antiretroviral therapy in low-body weight treatment-naïve patients with HIV infection. PLoS One **7**: e29977, 2012.

19. Chaisiri K, Bowonwatanuwong C, Kasettratat N, Kiertiburanakul S. Incidence and risk factors for tenofovir-associated renal function decline among Thai HIV-infected patients with low-body weight. Curr HIV Res **8**: 504-509, 2010.

20. Nelson MR, Katlama C, Montaner JS, et al. The safety of tenofovir disoproxil fumarate for the treatment of HIV infection in adults: the first 4 years. AIDS **21**: 1273-1281, 2007.

21. Nishijima T, Komatsu H, Gatanaga H, et al. Impact of small body weight on tenofovir-associated renal dysfunction in HIV-infected patients: a retrospective cohort study of Japanese patients. PLoS One **6**: e22661, 2011.

22. Moher D, Hopewell S, Schulz KF, et al. CONSORT 2010 explanation and elaboration: updated guidelines for reporting parallel group randomised trials. BMJ **340**: c869, 2010.

23. Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents October 10, 2006. [http://aidsinfo.nih.gov/contentfiles/AdultandAdolescentGL000629.pdf].

24. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. Nephron **16**: 31-41, 1976.

25. Hattori J, Shiino T, Gatanaga H, et al. Trends in transmitted drug-resistant HIV-1 and demographic characteristics of newly diagnosed patients: nationwide surveillance from 2003 to 2008 in Japan. Antiviral Res **88**: 72-79, 2010.

26. Rodriguez-Novoa S, Labarga P, Soriano V, et al. Predictors of kidney tubular dysfunction in HIV-infected patients treated with tenofovir: a pharmacogenetic study. Clin Infect Dis **48**: e108-e116, 2009.

27. DIVISION OF AIDS TABLE FOR GRADING THE SEVERITY OF ADULT AND PEDIATRIC ADVERSE EVENTS VERSION 1.0, DECEMBER, 2004; CLARIFICATION AUGUST 2009. [http://www.mtnstopshiv.org/sites/default/files/attachments/Table_for_Grading_Severity_of_Adult_Pediatric_Adverse_Events.pdf].

28. Matsuo S, Imai E, Horio M, et al. Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis **53**: 982-992, 2009.

29. Kudo K, Konta T, Mashima Y, et al. The association between renal tubular damage and rapid renal deterioration in the Japanese population: the Takahata study. Clin Exp Nephrol **15**: 235-241, 2011.

30. Ando M, Yanagisawa N, Ajisawa A, Tsuchiya K, Nitta K. Kidney tubular damage in the absence of glomerular defects in HIV-infected patients on highly active antiretroviral therapy. Nephrol Dial Transplant **26**: 3224-3229, 2011.

31. Gatanaga H, Tachikawa N, Kikuchi Y, et al. Urinary β_2-microglobulin as a possible sensitive marker for renal injury caused by tenofovir disoproxil fumarate. AIDS Res Hum Retroviruses **22**: 744-748, 2006.

32. Phillips EJ. Genetic screening to prevent abacavir hypersensitivity reaction: are we there yet? Clin Infect Dis **43**: 103-105, 2006.

33. Sun HY, Hung CC, Lin PH, et al. Incidence of abacavir hypersensitivity and its relationship with HLA-B*5701 in HIV-infected patients in Taiwan. J Antimicrob Chemother **60**: 599-604, 2007.

34. Martin MA, Klein TE, Dong BJ, Pirmohamed M, Haas DW, Kroetz DL. Clinical pharmacogenetics implementation consortium guidelines for HLA-B genotype and abacavir dosing. Clin Pharmacol Ther **91**: 734-738, 2012.

Bioorganic & Medicinal Chemistry 21 (2013) 2518–2526

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

CD4 mimics as HIV entry inhibitors: Lead optimization studies of the aromatic substituents

Tetsuo Narumi [a], Hiroshi Arai [a], Kazuhisa Yoshimura [b,c], Shigeyoshi Harada [b,c], Yuki Hirota [a], Nami Ohashi [a], Chie Hashimoto [a], Wataru Nomura [a], Shuzo Matsushita [b], Hirokazu Tamamura [a,*]

[a] Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan
[b] Center for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan
[c] AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan

ARTICLE INFO

Article history:
Received 22 January 2013
Revised 25 February 2013
Accepted 26 February 2013
Available online 7 March 2013

Keywords:
CD4 mimicry
Conformational change in gp120
HIV entry inhibitor
Envelope protein opener

ABSTRACT

Several CD4 mimics have been reported as HIV-1 entry inhibitors that can intervene in the interaction between a viral envelope glycoprotein gp120 and a cell surface protein CD4. Our previous SAR studies led to a finding of a highly potent analogue **3** with bulky hydrophobic groups on a piperidine moiety. In the present study, the aromatic ring of **3** was modified systematically in an attempt to improve its antiviral activity and CD4 mimicry which induces the conformational changes in gp120 that can render the envelope more sensitive to neutralizing antibodies. Biological assays of the synthetic compounds revealed that the introduction of a fluorine group as a *meta*-substituent of the aromatic ring caused an increase of anti-HIV activity and an enhancement of a CD4 mimicry, and led to a novel compound **13a** that showed twice as potent anti-HIV activity compared to **3** and a substantial increase in a CD4 mimicry even at lower concentrations.

1. Introduction

The first step of HIV entry into host cells is the interaction of a viral envelope glycoprotein gp120 with the cell surface protein CD4.[1] Such a viral attachment process is an attractive target for the development of the drugs to prevent the HIV-1 infection of its target cells.[2] Several small molecules including BMS-806,[3] IC-9564[4] and NBDs[5] have been identified that inhibit the viral attachment process by binding to gp120. Recently, we and others have been exploring the potentials of NBDs-derived CD4 mimics as a novel class of HIV entry inhibitors (Fig. 1).[6–8]

Small molecular CD4 mimics identified by an HIV syncytium formation assay showed potent cell fusion and virus cell fusion inhibitory activity against several HIV-1 laboratory and primary isolates.[5] Furthermore, the interaction of CD4 mimics with a highly conserved and functionally important pocket on gp120, known as the 'Phe43 cavity', induces conformational changes in gp120,[9] a process which occurs with unfavorable binding entropy, leading to a favorable enthalpy change similar to those caused by binding of the soluble CD4 binding to gp120. These unique properties render CD4 mimics valuable not only for the development of entry inhibitors, but which also, when combined with neutralizing antibodies function as envelope protein openers-putatively, stimulants.[10]

The structure of the complex formed by NBD-556 (**1**) bound to the gp120 core from an HIV-1 clade C strain (C1086) was recently determined by X-ray analysis (PDB: 3TGS).[11] As expected with molecular modeling by us[8a] and others,[6a] NBD-556 binds with Phe43 cavity with its *p*-chlorophenyl ring inserted into the cavity, and in addition multiple contacts were observed, with Trp112, Val255, Phe382, Ile424, Asn425, Trp427, Gly473, and Val430 of gp120 were observed (Fig. 2). However, no obvious interaction with Arg59 of CD4 was observed, although the salt bridge formation between Arg59 of CD4 and Asp368 of gp120 is a critical interaction of the viral attachment.[12] Based on this binding model, several potent compounds were recently identified.[6c,7]

Prior to those studies, we performed structure–activity relationship (SAR) studies based on the modification of the piperidine moiety of CD4 mimics to interact with Val430 and/or Asp368. These resulted in the discovery of a potent compound **3** which has bulky hydrophobic groups on its piperidine ring, and shows significant anti-HIV activity and lower cytotoxicity than other known CD4 mimics.[8c] Our study of the docking of **3** into the Phe43 cavity of gp120 suggests that the cyclohexyl group of **3** can interact hydrophobically with the isopropyl group of Val430.

We hypothesized that the optimization of the aromatic ring of **3** would lead to an increase of antiviral activity and CD4 mimicry, the latter inducing the conformational changes in gp120. Here, we de-

* Corresponding author. Tel.: +81 3 5280 8036; fax: +81 3 5280 8039.
E-mail address: tamamura.mr@tmd.ac.jp (H. Tamamura).

0968-0896/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.bmc.2013.02.041

scribe the systematic modification of the aromatic ring of **3** for further optimization to evaluate substituent effects on anti-HIV activity, cytotoxicity and CD4 mimicry.

2. Results and discussion

The co-crystal structure of **1** with the gp120 core revealed that the aromatic group of **1** binds to gp120 by several aromatic–aromatic and hydrophobic interactions (Fig. 2). In particular, hydrophobic space surrounded by the hydrophobic amino acid residues Trp112, Val255, Phe382, and Ile424 is likely to be affected by substituents at the *meta*- and *para*-positions of the aromatic ring, and consequently we decided to investigate substituents at these positions (Fig. 3).

Initially, we selected a chlorine or a methyl group to serve as the *para*-substituent of the aromatic group because CD4 mimic compounds such as **1** (NBD-556) with a *p*-chloro substituent, and because **3** showed significant anti-HIV activity compared to other substituents. Further, CD4 mimic structures such as **2** with a *p*-methyl substituent also showed potent anti-HIV activity and exhibits lower cytotoxicity than those with the *p*-chlorophenyl derivatives.[8a] Next, we chose several halogens including F, Cl and Br, to be the *meta*-substituent on the aromatic group since previous SAR studies revealed that the introduction of an appropriate group with an electron-withdrawing ability at the *meta*-position leads to an increase of binding affinity and antiviral activity.[6a] Furthermore, to investigate whether electron withdrawal and hydrophobicity of the *meta*-position are appropriate, the CD4 mimics with a *meta*-methyl substituent, which has electron-donating properties and is similar in size to bromine, were also synthesized. Finally, two piperidine scaffolds (the 2,2,6,6-tetramethylpiperidine **A** and the dicyclohexylpiperidine **B**) were combined with these aromatics via the oxalamide linker.

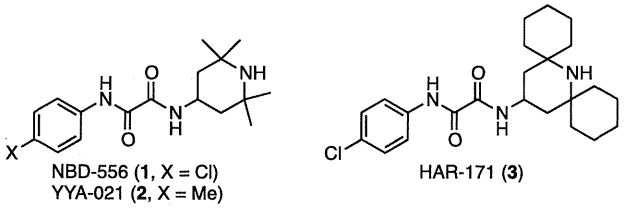


Figure 1. Structures of NBD-556 (**1**), YYA-021 (**2**) and HAR-171 (**3**).

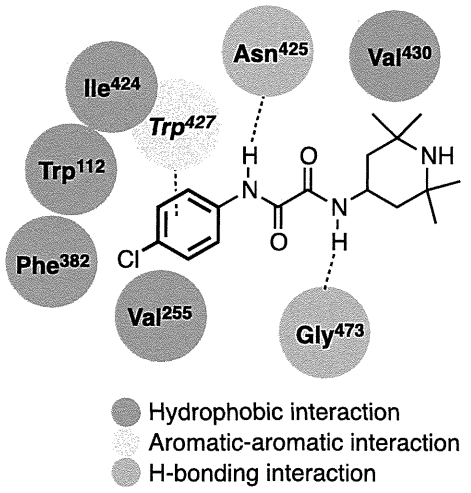


Figure 2. Major interactions between NBD-556 and Phe43 cavity of gp120.

2.1. Chemistry

The syntheses of novel compounds are depicted in Schemes 1 and 2. Starting from the appropriate aniline with *m*- and *p*-substituents, coupling with ethyl chloroglyoxylate in the presence of Et_3N gave the corresponding amidoesters **6a–c** and **7a–c**. Subsequently, microwave-assisted aminolysis[13] of **6a–c** and **7a–c** with commercially available 4-amino-2,2,6,6-tetramethylpiperidines afforded the desired compounds **8a–c** and **9a–c** (Scheme 1). A series of CD4 mimics with two cyclohexyl groups **13a–c** and **14a–c** were prepared from 2,2,6,6-tetramethylpiperidin-4-one **10** by the method previously reported,[8c] with slight modification (Scheme 2). Briefly, treatment of **10** with cyclohexanone in the presence of ammonium chloride gave a 2,6-substituted piperidin-4-one **11** via Grob fragmentation followed by intramolecular cyclization.[14] Reductive amination with *p*-methoxybenzyl amine, acidic treatment with TMSBr/TFA, and oxidative cleavage of *p*-methoxybenzyl group with cerium(IV) ammonium nitrates (CAN) furnished the corresponding 4-aminopiperidines (**12**) with higher yields and less burdensome purifications than the previous method. Finally, coupling of **12** with the corresponding esters **6a–c** and **7a–c** under microwave irradiation provided the desired compounds **13a–c** and **14a–c**.

2.2. Biological evaluation

The anti-HIV activity of the synthetic compounds was evaluated against an R5 primary isolate YTA strain. IC_{50} values were determined by the WST-8 method as the concentrations of the compounds that conferred 50% protection against HIV-1-induced cytopathogenicity in PM1/CCR5 cells. Cytotoxicity of the compounds based on the viability of mock-infected PM1/CCR5 cells was also evaluated using the WST-8 method. The assay results for compounds **8a–c** and **13a–c** with a *p*-chlorophenyl group are shown in Table 1. The parent compound **1** and compound **8a**,[6a] known as JRC-II-191, showed significant anti-HIV activities (IC_{50}

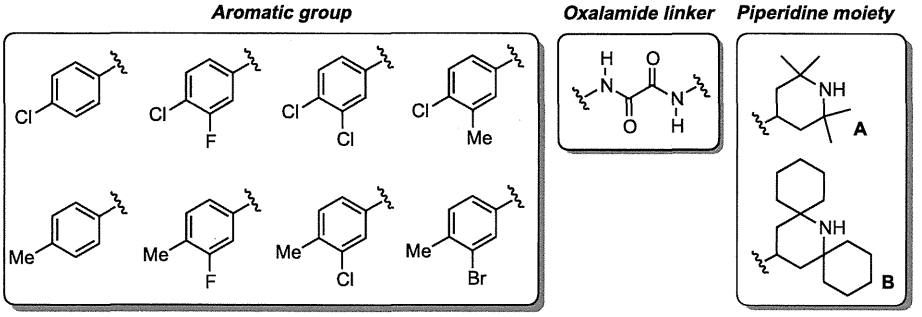
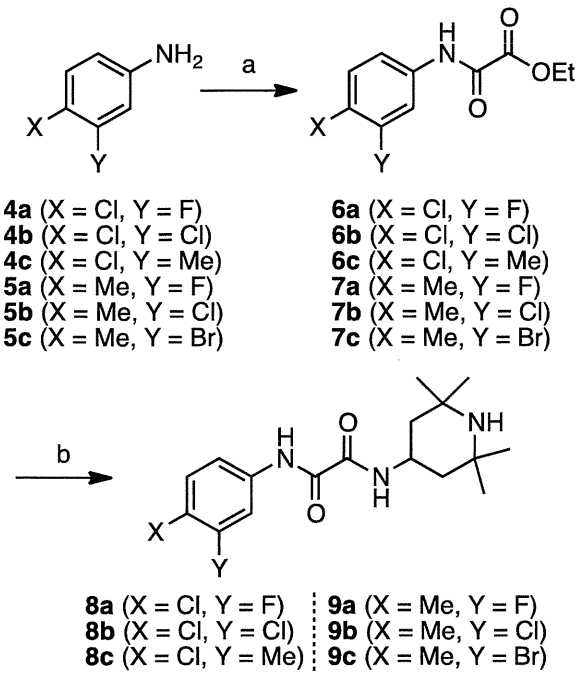
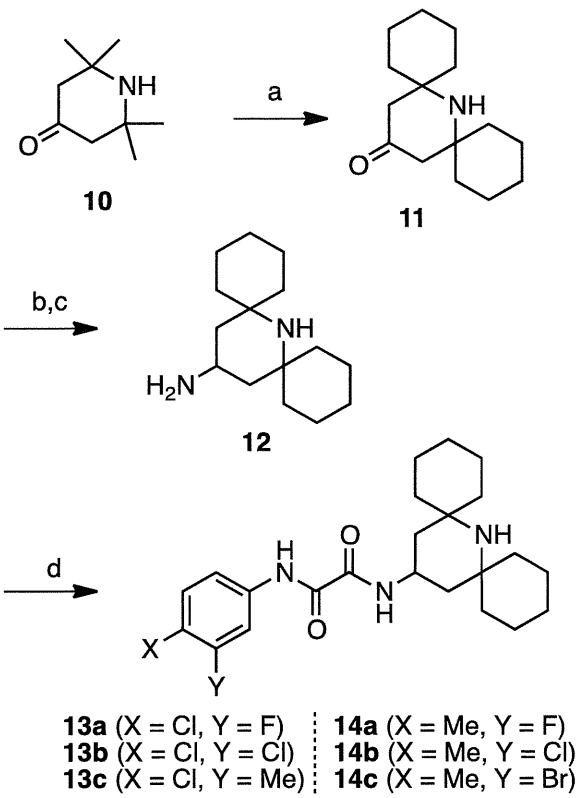


Figure 3. The structures of scaffolds in the design of novel CD4 mimics.



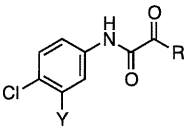
Scheme 1. Reagents and conditions: (a) ethyl chloroglyoxylate, Et_3N, THF; (b) 4-amino-2,2,6,6-tetramethylpiperidine, Et_3N, EtOH, 150 °C, microwave.

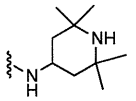
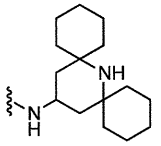


Scheme 2. Reagents and conditions: (a) cyclohexanone, NH_4Cl, DMSO, 60 °C; (b) *p*-methoxybenzylamine, $NaBH_3CN$, MeOH, then 1 M TMSBr in TFA; (c) CAN, CH_3CN/H_2O (v:v = 2:1); (d) **6** or **7**, Et_3N, EtOH, 150 °C, microwave.

of **1** = 0.61 μM and IC_{50} of **8a** = 0.32 μM). Compound **8b**[6a] having a *m,p*-dichlorophenyl group and compound **8c**[6a] (JRC-II-193) having a *p*-chloro-*m*-tolyl group showed moderate anti-HIV activity (IC_{50} of **8b** = 4.1 μM and IC_{50} of **8c** = 3.3 μM) but their potency was

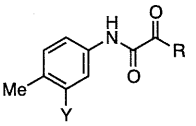
Table 1
Anti-HIV activity and cytotoxicity of compounds **8a–c** and **13a–c** containing a *p*-chlorophenyl group[a]

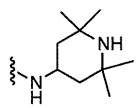
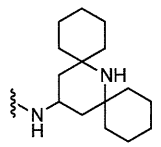


Compd	R	Y	IC_{50}[b] (μM) YTA48P	CC_{50}[c] (μM)
1		H	0.61	110
8a	**A**	F	0.32	94
8b	**A**	Cl	4.1	36
8c	**A**	Me	3.3	38
3		H	0.43	120
13a	**B**	F	0.23	11
13b	**B**	Cl	0.62	11
13c	**B**	Me	2.6	15

[a] All data are the mean values from three of more independent experiments.
[b] IC_{50} values of the multi-round assay are based on the inhibition of HIV-1-induced cytopathogenicity in PM1/CCR5 cells.
[c] CC_{50} values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.

Table 2
Anti-HIV activity and cytotoxicity of compounds **9a–c** and **14a–c** containing a *p*-tolyl group[a]



Compd	R	Y	IC_{50}[b] (μM) YTA48P	CC_{50}[c] (μM)
2		H	9.0	260
9a	**A**	F	2.8	110
9b	**A**	Cl	3.2	62
9c	**A**	Br	>10	32
14a		F	0.54	91
14b	**B**	Cl	6.2	11
14c	**B**	Br	3.2	11

[a] All data are the mean values from three of more independent experiments.
[b] IC_{50} values of the multi-round assay are based on the inhibition of HIV-1-induced cytopathogenicity in PM1/CCR5 cells.
[c] CC_{50} values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.

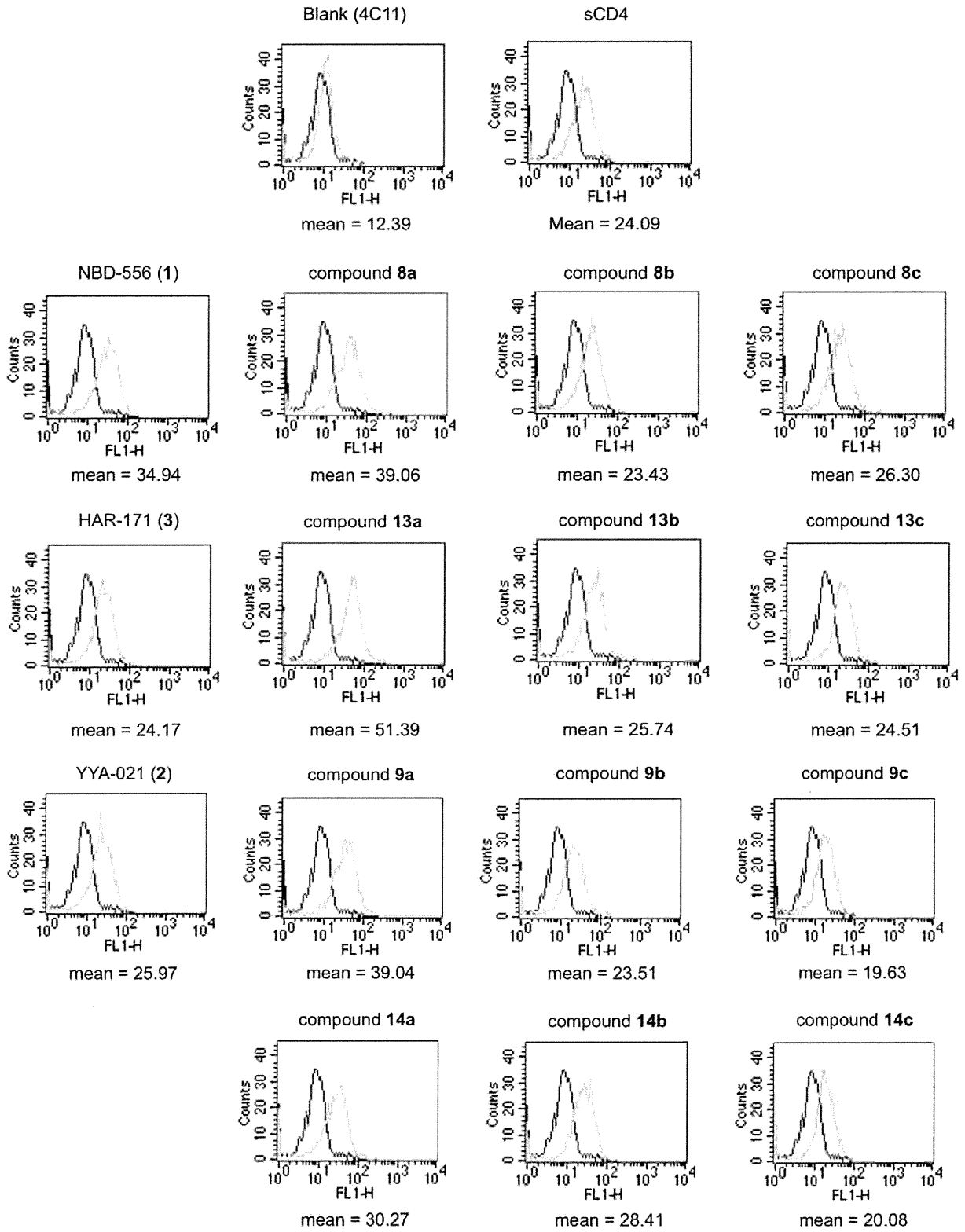


Figure 4. FACS analysis of synthetic compounds **8**, **9**, **13** and **14**.

approximately 10-fold lower than that of compound **8a**. The cytotoxicity of **8b** and **8c** is relatively stronger than that of **8a** (CC_{50} of **8b** = 36 μM and CC_{50} of **8c** = 38 μM). Compounds **13a–c** with hydrophobic cyclohexyl groups in the piperidine moiety showed more potent anti-HIV activity than the corresponding compounds **8a–c**, confirming the contribution of the bulky hydrophobic group(s) to an increase of antiviral activity. Our lead compound **3** showed significant anti-HIV activity comparable to that of compound **8a** (IC_{50} = 0.43 μM) but, consistent with previous results, exhibited lower cytotoxicity. In particular, compound **13a** with a *m*-fluoro-*p*-chlorophenyl group exhibited the highest anti-HIV activity. The IC_{50} value of **13a** was 0.23 μM, whose potency was

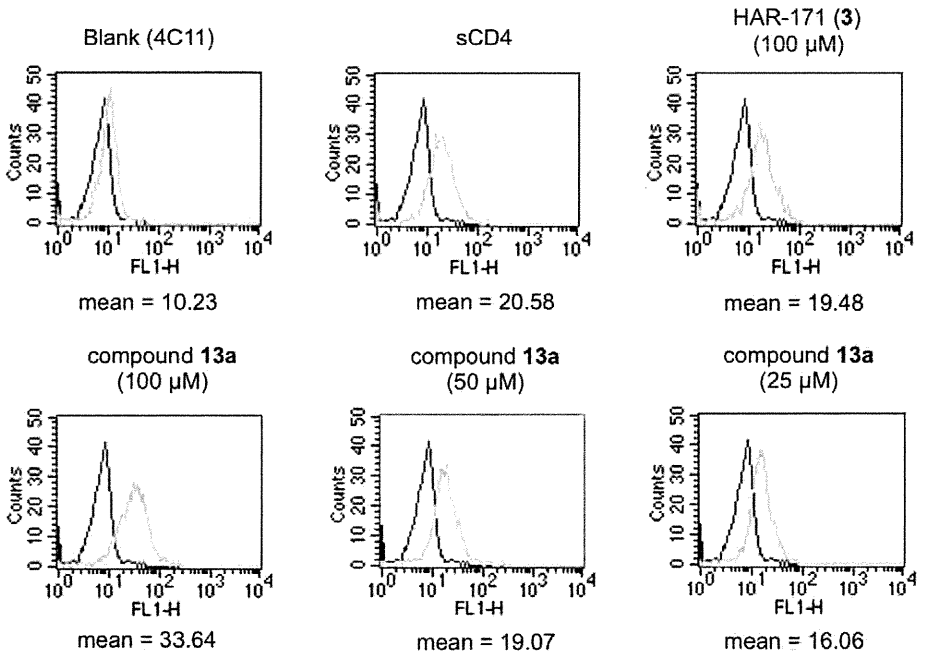


Figure 5. FACS analysis of **3** and **13a** in different concentrations.

approximately twice as high as that of compound **3**. Notably, compound **13b** with a *m,p*-dichlorophenyl group showed 7-fold more potent anti-HIV activity than the corresponding compound **8b**. Compound **13c**, which has a *p*-chloro-*m*-tolyl group, showed potent anti-HIV activity comparable to that of the corresponding compound **8c** and an increase of cytotoxicity (CC_{50} = 15 μM). We observed a tendency for compounds **13a–c** with both hydrophobic cyclohexyl groups and a *m,p*-disubstituted phenyl group to exhibit higher cytotoxicity than the corresponding tetramethyl-type compounds **8a–c**. No clear reason for an increase of cytotoxicity in the *m,p*-disubstituted phenyl group-containing compounds is apparent.

Assay results for the compounds **9a–c** and **14a–c** with a *p*-tolyl group are shown in Table 2. As expected, replacement of the *p*-chloro substituent with a *p*-methyl group resulted in somewhat reduction of anti-HIV activity. Compound **2**, YYA-021 has significant anti-HIV activity (IC_{50} = 9.0 μM) and exhibits the lowest cytotoxicity among all of the compounds tested (CC_{50} = 260 μM). These results are consistent with our previous SAR studies involving the aromatic ring. Introduction of a fluorine at the *meta*-position of the *p*-tolyl group, e.g. in compound **9a** and **14a**, improved the antiviral activity, as observed with **8a** and **13a** and a similar tendency was observed for compound **9b** with a *m*-chloro-*p*-tolyl group. In particular, compound **14a** with cyclohexyl groups and a *m*-fluoro-*p*-tolyl group showed slightly higher anti-HIV activity than the parent compound **1**. Among the compounds with *m*-bromo-*p*-tolyl groups, it was found that compound **9c**, with a 2,2,6,6-tetramethylpiperidine group, showed no anti-HIV activity at a concentration below 10 μM, whereas compound **14c** with hydrophobic cyclohexyl groups attached to the piperidine moiety, showed moderate activity (IC_{50} = 3.2 μM), indicating that the hydrophobic modification of piperidine ring can contribute to an increase in anti-HIV activity.

All the synthetic compounds were evaluated for their CD4 mimicry on the conformational changes in gp120 by fluorescence activated cell sorting (FACS) analysis, and the results are shown in Figure 4. The profile of binding of a CD4-induced (CD4i) monoclonal antibody (4C11) to the Env-expressing cell surface pretreated with the synthetic compounds was assessed in terms of the mean fluorescence intensity (MFI). The increase in binding affinity for 4C11 (by the pretreatment with synthetic compounds) suggests that those compounds can reflect the CD4 mimicry as a consequence of the conformational changes in gp120. Our previous studies disclosed that the profiles of the binding to the cell surface pretreated with **1**, **2**, or **3** were similar to those observed in pretreatment with soluble CD4, indicating that these compounds offer a significant enhancement of binding affinity for 4C11.[8] As shown in Figure 4, similar results were obtained with those compounds in this FACS analysis (MFI of **1**, **2**, and **3** = 34.94, 25.97, and 24.17, respectively). A notable increase in binding affinity for 4C11 was observed in essentially all the synthetic compounds. The compounds **8a**, **9a**, **13a** and **14a** with a *meta*-fluorine in the aromatic ring, showed significant anti-HIV activity, and produced a substantial increase in binding affinity for 4C11. These results suggested that the introduction of a fluorine group at the meta position of the aromatic ring is significant not only for the increase of anti-HIV activity, but also for the enhancement of a CD4 mimicry. In particular, a remarkable improvement in binding affinity for 4C11 was observed with **13a** (MFI = 51.39) which has twofold more potent anti-HIV activity than the lead compound **3** (HAR-171), and is the most active compound in terms of both anti-HIV activity and the CD4 mimicry resulting from the conformational change in gp120. The profiles of pretreatment of the cell surface with compounds **8b** and **13b** having a *m,p*-dichlorophenyl group, compounds **8c** and **13c** having a *p*-chloro-*m*-tolyl group, and compounds **9b** and **14b** with a *m*-chloro-*p*-tolyl group were similar to results obtained for **3**, suggesting that these compounds produced slightly lower enhancement compared to those of compounds **8a**, **9a**, **13a** and **14a** but significant levels of binding affinity for 4C11. On the other hand, pretreatment with compounds **9c**, which failed to show significant anti-HIV activity and **14c**, which had moderate anti-HIV activity resulted in a slight decrease of binding affinity for 4C11, suggesting that the introduction of a Br group at the *meta*-position of *p*-tolyl group is not advantageous to a CD4 mimicry, possibly due to the steric hindrance caused by the two bulky substituents. These results are consistent with previous observations that a limited size and electron-withdrawing ability of the aromatic substituents are required for potent anti-HIV activity and CD4 mimicry.[8a]

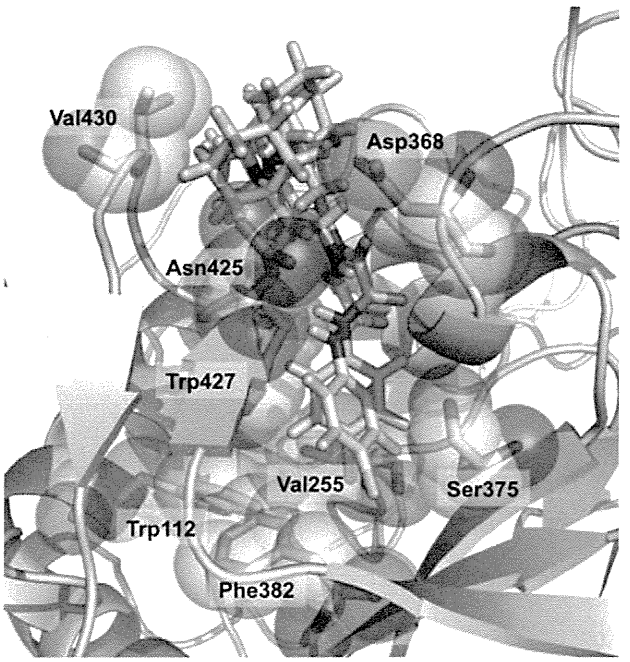


Figure 6. The modeled structure of **13a** (yellow carbon atoms) in the complex with the Phe43 cavity in gp120 (3TGS) overlaid with the modeled structure of **3** (green carbon atoms).

Since **13a** showed higher CD4 mimicry than the other compounds tested, the effect of the solution concentration of **13a** on the binding affinity for 4C11 was investigated. As shown in Figure 5, pretreatment of the cell surface with a 100 μM solution of **13a** produced a higher increase in the binding affinity for 4C11 than pretreatment with the same concentration of compound **3**. Interestingly, the profile pretreated with a 50 μM solution of **13a** was similar to that with a 100 μM of compound **3**, and even with a 25 μM solution of **13a** a potent enhancement of the binding affinity for 4C11 was observed: MFI of **13a** at concentrations of 50 μM and 25 μM = 19.07 and 16.06, respectively. This observation suggests that **13a** could serve as a novel lead compound for the development of envelope protein openers for the use combined with neutralizing antibodies because of its effectiveness at low concentrations.

The substantial increase in the CD4 mimicry of **13a** even at a low concentration is not easily explained because HAR-171 (**3**) and **13a** would be expected to form the similar binding modes with gp120. A probable contribution of **13a** is suggested by modeling studies docked into the Phe43 cavity in gp120 (3TGS) in which the depth and direction of the aromatic ring of **13a** is slightly different from those in compound **3** (Fig. 6), leading to the possible formation of appropriate interactions with the hydrophobic amino acid residues such as Val255 and Phe382, and therefore explaining the increased potency observed in the anti-HIV activity and CD4 mimicry of **13a**.

3. Conclusion

CD4 mimics are attractive agents not only for the development of a novel class of HIV entry inhibitors but also as possible cooperating agents for the neutralizing antibodies—that is, envelope protein openers. In the present study, a structure–activity relationship study of a series of CD4 mimic compounds was performed with a view to improving the biological activity of HAR-171 (**3**), which was identified in our previous studies as a promising lead compound with anti-HIV activity, cytotoxicity and CD4 mimicry resulting from the conformational change in gp120. Systematic modification of the *meta*- and *para*-substituents of the aromatic ring of **3** led to some potent compounds. In particular, **13a**, which has a bulky hydrophobic group on its piperidine ring and a *m*-fluoro-*p*-chlorophenyl group, demonstrated twofold more potent anti-HIV activity and much higher CD4 mimicry than **2** following the conformational changes in gp120, although the cytotoxicity of **13a** is relatively high. Further structural modification studies of the aromatic ring and the oxalamide linker to improve pharmaceutical profiles will be the subject of future reports.

4. Experimentals

^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker Avance III spectrometer. Chemical shifts are reported in δ (ppm) relative to Me_4Si (in $CDCl_3$) as internal standard. Low- and high-resolution mass spectra were recorded on a Bruker Daltonics microTOF focus in the positive and negative detection mode. For flash chromatography, silica gel 60 N (Kanto Chemical Co., Inc.) was employed. Microwave reactions were performed in Biotage Microwave Reaction Kit (sealed vials) in an Initiator™ (Biotage). The wattage was automatically adjusted to maintain the desired temperature for the desired period of time.

4.1. Chemistry

4.1.1. Ethyl 2-((4-chloro-3-fluorophenyl)amino)-2-oxoacetate (6a)

To a stirred solution of 3-fluoroaniline (1.11 g, 10.0 mmol) in $CHCl_3$ (30.0 mL) was added dropwise *N*-chlorosuccinimide (NCS) in $CHCl_3$ (20.0 mL) at 0 °C. The mixture was stirred at 0 °C for 42 h. After the reaction mixture was concentrated under reduced pressure, the residue was dissolved in Et_2O. The mixture was washed with water, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc/*n*-hexane gave 4-chloro-3-fluoroaniline (259.4 g, 18% yield) as crystalline solids. To a stirred solution of the above aniline (259.4 mg, 1.78 mmol) in THF (8.9 mL) were added at 0 °C ethyl chloroglyoxylate (237.3 μL, 2.14 mmol) and Et_3N (296.6 μL, 2.14 mmol). The mixture was stirred at room temperature for 12 h. After the precipitate was filtrated off, the filtrate solution was concentrated under reduced pressure. The residue was dissolved in EtOAc, and washed with 1.0 M HCl, saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Concentration under reduced pressure to provide the title compound **6a** (435.2 mg, 99% yield) as brown crystals, which was used without further purification.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (t, J = 7.50 Hz, 3H), 4.43 (q, J = 7.50 Hz, 2H), 7.24–7.25 (m, 1H), 7.35–7.40 (m, 1H), 7.70–7.75 (m, 1H), 8.93 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.0, 64.1, 108.5 (d, J = 26.3 Hz), 115.9 (d, J = 3.75 Hz), 117.3 (d, J = 18.8 Hz), 130.9 (d, J = 10.0 Hz), 135.9, 153.9, 158.1 (d, J = 246.3 Hz), 160.5; HRMS (ESI), *m*/*z* calcd for $C_{10}H_{10}ClFNO_3$ (MH^-) 244.0182, found 244.0183.

4.1.2. Ethyl 2-((3,4-dichlorophenyl)amino)-2-oxoacetate (6b)

To a stirred solution of 3,4-dichloroaniline **4b** (1.94 g, 12.0 mmol) in THF (20.0 mL) were added ethyl chloroglyoxylate (1.11 mL, 10.0 mmol) and Et_3N (15.2 mL, 11.0 mmol) at 0 °C. The mixture was stirred at room temperature for 6 h. After the precipitate was filtrated off, the filtrate solution was concentrated under reduced pressure. The residue was dissolved in EtOAc, and washed with 1.0 M HCl, saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Concentration under reduced pressure to provide the title compound **6b** (1.58 g, 95% yield) as white powder, which was used without further purification.