ORIGINAL RESEARCH ARTICLE

High-risk status of HIV-1 infection in the very low epidemic country, Mongolia, 2007

J Davaalkham MD*†, **P Unenchimeg** MD‡, **Ch Baigalmaa** MD‡, **B Oyunbileg** MD‡, **K Tsuchiya** PhD*, **A Hachiya** MS*, **H Gatanaga** MD PhD*†, **D Nyamkhuu** MD PhD‡ **and S Oka** MD PhD*†

*AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan; †Center for AIDS Research, Kumamoto University, Kumamoto, Japan; ‡National Center for Communicable Diseases, Ministry of Health, Ulaanbaatar, Mongolia

Summary: Thirty-six HIV-1 cases had been reported by December 2007 in Mongolia. Therefore, Mongolia has been regarded as a very low HIV-1 epidemic country, although the surveillance system is not fully developed. The aim of this study was to evaluate the risk status of HIV-1 infection in Mongolia. A total of 1415 blood samples from high-risk populations including female sex workers, men who have sex with men, mobile men, tuberculosis patients and male sexually transmitted infection (STI) clinic clients and 1050 samples from healthy controls were collected. The seroprevalences of anti-HIV-1/2, anti-*Treponema pallidum*, hepatitis B surface antigen (HBs Ag), anti-hepatitis C virus and hepatitis B surface antibody in the high-risk populations were 0%, 23.1%, 15.5%, 8.0% and 48.2%, and those in the controls were 0%, 3.1%, 14.7%, 4.4% and 44.4%, respectively. HIV-1 prevalence is currently low. However, according to the high prevalence of STIs in the high-risk populations, the risk status for HIV-1 infection is estimated to be high.

Keywords: seroprevalence of HIV, syphilis, HCV and HBV, high-risk population, Mongolia

INTRODUCTION

Mongolia is located in Central Asia bordered by Russia and China. The population of Mongolia is 2635 million, of which 61.0% live in cities and the remaining are nomadic.[1] Geographical conditions and a very low population density make communication, transport and health service provision difficult. Mongolia has witnessed radical changes in its economic and social policies since the democratic revolution of 1990. Along with independence from the former Soviet Union and loss of Soviet support, there has been an increase in unemployment, alcoholism and prostitution and a steady increase in the prevalence of sexually transmitted infections (STIs) and other communicable diseases.[2-8] A recent study demonstrated that syphilis, gonorrhoea and trichomonas were detected in 57 (43%), 18 (14%) and 37 (28%) subjects, respectively, among 132 low-income female commercial sex workers (FSWs) in Mongolia.[9] Mongolia also has a high prevalence of hepatitis B and C viral infection. In a previous study, hepatitis B surface antigen (HBs Ag) and antibodies to hepatitis C virus (anti-HCV) were detected in 24 (10%) and 41 (16%) subjects, respectively, among 249 apparently healthy individuals in Mongolia.[10] However, most of these data were obtained from convenient or non-generalized samples. There is a lack of information regarding exposures and the burden of diseases in the high-risk populations for HIV and STIs. High-risk populations such as FSWs and their sexual contacts with high rates of STIs are important populations contributing to the transmission of HIV and other STIs in developing countries.[11-13]

Since 1992, when data on HIV/AIDS began to be compiled in Mongolia, there had been only five cases reported as of December 2004. Mongolia is considered as an HIV/AIDS low-prevalence country. However, annual new cases of HIV/AIDS have been increasing in recent years. For example, 11, 9 and 11 new cases were detected in 2005, 2006 and 2007, respectively. Among them, 22 (61.1%) cases were men who have sex with men (MSM), seven (19.4%) were heterosexually transmitted and six (16.7%) were FSWs (Mongolian National Center for Communicable Diseases [NCCD], unpublished data). Owing to the lack of a sound surveillance system, the actual situation is uncertain. The primary objective of this study was to evaluate the current risk status of HIV-1 among high-risk populations in Mongolia, examining the seroprevalence of other STIs concomitantly. These data are crucial for taking future preventive measures against HIV-1 infection.

Correspondence to: Dr S Oka MD PhD, AIDS Clinical Center, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan
Email: oka@imcj.hosp.go.jp

METHODS

Study design and study population

This study was conducted from September through December 2007. The study protocol was approved by the ethics committees of the International Medical Center of Japan (H19-448) and of the Ministry of Health, Mongolia. After explaining this study and obtaining informed consent, blood samples were

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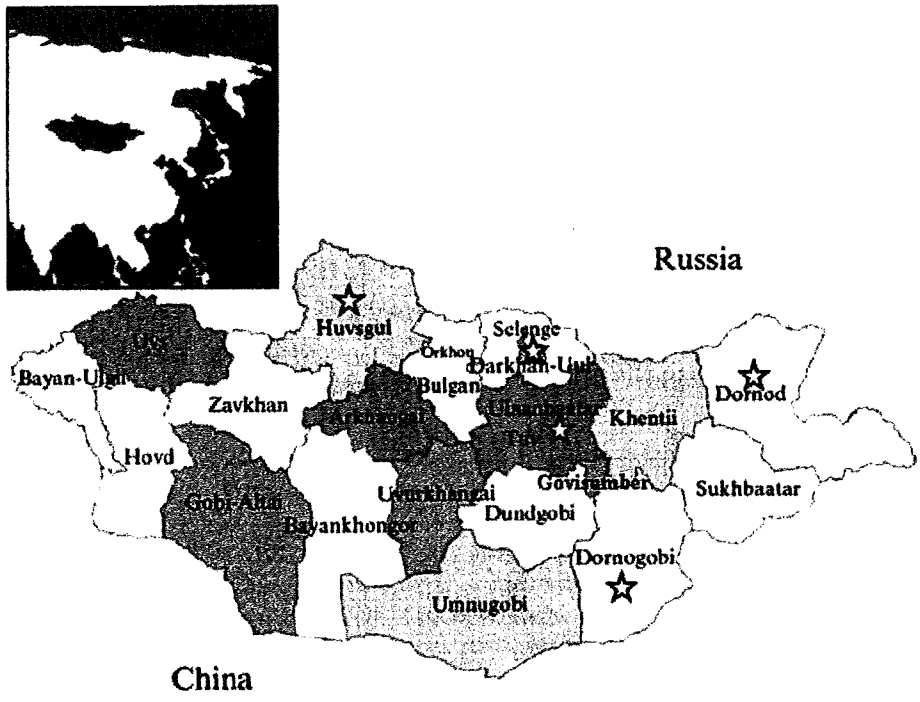

Figure 1 A map of Mongolia. Blood samples were collected from the capital city Ulaanbaatar and four aimags (provinces), such as Darkhan-Uul, Huvsgul, Dornod and Dornogobi. Asterisks indicate the sites where blood samples were obtained

collected anonymously from both high-risk and healthy control populations in Ulaanbaatar (the capital city of Mongolia) and four aimags including Dornod, Huvsgul (borders of Russia), Dornogobi (a border of China) and Darkhan-Uul (Figure 1). A total of 2465 samples were collected: 1415 samples from high-risk populations and 1050 samples from healthy control populations. The high-risk populations included FSWs, MSM, mobile men, tuberculosis (TB) patients and male STI clinic clients. The number of samples in each population and demographic characteristics are listed in Table 1.

Cluster sampling was used for FSWs in locations such as bars, nightclubs, sauna and massage parlours serving as clusters. MSM were sampled only from Ulaanbaatar city, due to the limited data on MSM in other areas of the country. Mobile men were sampled from Ulaanbaatar city and Domogobi aimag (province), along major road and rail networks and areas such as truck stops and checkpoints at borders. As for TB patients, those who were diagnosed with TB for the first time during the sampling period were enrolled. A male STI clinic client was defined as one who attended public STI clinics during the sampling period. A healthy control group included youth and blood donors. Youth was defined as unmarried, 15–35 years old students in college or university of both sexes. The blood donors were selected in health facilities during the sampling period.

Specimen collection and serology

All sera were stored below −20°C until use. Sera were tested for antibodies to HIV-1/2 (anti-HIV-1/2), *Treponema pallidum* (anti-TP), hepatitis B surface antiboby (HBs Ab), and hepatitis C virus (anti-HCV) and HBs Ag by using the chemiluminescent enzyme immunoassay (CLEIA) (Lumipalus, Fujirebio, Tokyo, Japan) according to the instructions provided by the manufacturer. Seropositive samples for anti-HIV-1/2 by CLEIA were further confirmed by chemiluminescent immunoassay (Architect, Abbott Laboratories, Abbott Park, IL, USA) and a Western blot for the final diagnosis. All laboratory analyses were performed at the AIDS Clinical Center, International Medical Center of Japan.

Statistical analyses

Differences among high-risk and/or healthy control populations were examined by the Fisher's exact test. Univariate logistic analyses were used to determine the odds ratios (OR) with corresponding 95% confidence intervals (CI). All analyses

Table 1 Demographic characteristics of persons who gave blood samples

Populations	No.	Sex M:F	Age Range	Age Mean ± SD
High risk				
FSWs	410	0:410	17–52	25.3 ± 6.8
MSM	50	50:0	19–48	28.5 ± 6.3
Male STI clients	545	545:0	15–64	29.2 ± 7.9
TB patients	110	41:69	16–70	34.8 ± 12.5
Mobile men	300	300:0	17–57	30.9 ± 8.8
Subtotal	1415	936:479	15–70	28.8 ± 8.7
Healthy controls				
Blood donors	150	101:49	18–49	28.2 ± 9.3
Youth	900	450:450	15–35	19.9 ± 2.6
Subtotal	1050	551:499	17–49	21.0 ± 4.9

FSW = female commercial sex worker; MSM = men who have sex with men; TB = tuberculosis; STI = sexually transmitted infection

Table 2 **Seroprevalence between high-risk and healthy controls in Mongolia**

	% Positive in each high-risk group					High risk (%)	Healthy controls (%)	OR (95% CI)	P value
	FSWs	MSM	Male STI clients	TB patients	Mobile men				
Anti-HIV-1/2	0	0	0	0	0	0	0	–	–
HBs Ag	11.5	18	15.4	16.4	20.7	15.5	14.7	1.1 (0.9–1.3)	0.570
HBs Ab	48.5	42	48.9	50.9	46.7	48.2	44.4	1.2 (1.0–1.4)	0.060
Anti-HCV	6	18	7.5	15.4	7	8	4.4	1.9 (1.3–2.7)	<0.001
Anti-TP	39.5	30	17.2	10.9	14.7	23.1	3.1	9.3 (6.4–13.4)	<0.0001

FSW = female commercial sex worker; MSM = men who have sex with men; TB= tuberculosis; STI = sexually transmitted infection; OR = odds ratio; CI = confidence interval; HBs Ag = hepatitis B surface antigen; HBs Ab = hepatitis B surface antibody; TP = *Treponema pallidum*; HCV = hepatitis C virus

were conducted using the *Stat View* software version 5.0 (SAS Institute, Cary, NC, USA). A P value of <0.05 was considered statistically significant.

RESULTS

The seroprevalences of anti-HIV-1/2, HBs Ab and Ag, anti-HCV and anti-TP of each group of the high-risk and healthy populations are presented in Table 2. None of the anti-HIV-1/2-positive samples was detected in this study. The prevalences of HBs Ag and HBs Ab in the high-risk population, including among each high-risk group, were not different compared with those in the healthy control. In contrast, the prevalences of anti-HCV (8%) and anti-TP (23.1%) in the high-risk population were significantly higher than those in the healthy control. The ORs of anti-HCV and anti-TP comparing between the high-risk population and the healthy control were 1.9 (95% CI: 1.3–2.7, $P < 0.001$) and 9.3 (95% CI: 6.4–13.4, $P < 0.0001$), respectively. The prevalences of anti-HCV in MSM and TB patients were higher than those of other risk groups. The prevalences of anti-TP in FSWs (39.5%) and MSM (30%) were surprisingly high.

Geographical differences of seroprevalence are shown in Table 3. Again, there were no significant differences of the prevalence of HBs Ab and Ag in different regions of specific high-risk groups. However, incidences of anti-HCV and anti-TP had some differences in different regions of the specific groups. A striking feature was that the prevalence of anti-TP in Ulaanbaatar FSWs was 54.7%.

The prevalence of HBs Ab was high. However, there were no differences in the prevalence between high-risk and healthy control populations over the country. One reason was that a hepatitis B virus (HBV) vaccination programme in childhood has been implemented 18 years ago. Therefore, we divided the subjects into two age-related groups (below 18 years and over 20 years) and analysed the seroprevalence of HBs Ab (Figure 2). There were no differences between the high-risk and healthy control populations in both age-related groups. However, in both high-risk and healthy control populations, higher age groups had significantly higher prevalence.

DISCUSSION

Since 1992 when the first case of HIV-1 infection was reported in Mongolia, the number of reported cases remained low until 2005. However, the number has been increasing sharply since 2005, and 36 cases have been reported as of February 2008 (Ministry of Health, Mongolia, unpublished data). By the estimated report of the Global Fund for AIDS, Tuberculoses and Malaria ('Impact of AIDS in Mongolia' 2004), without prevention measures, Mongolian HIV/AIDS prevalence will be doubled every two years and 2500 people will die of AIDS by 2014. Our result supports this estimation. A current prevalence of HIV-1 infection is still low but the risk status of HIV-1 infection must be high because of the very high prevalence of syphilis in FSWs (39.5%), especially in Ulaanbaatar (54.7%). Another report also presented similar prevalence among low-income FSWs in Mongolia (43%).[9] Schwebke *et al.*[7] reported the prevalence rate (8.6%) of syphilis among 137 male STI clients in

Table 3 **Seroprevalence of HBV, HCV and syphilis among a high-risk population by residence**

	% positive for					
	No	Anti-HIV-1/2	HBs Ag	HBs Ab	Anti-HCV	Anti-TP
Ulaanbaatar						
FSWs	150	0	8.7	48.7	8.7	54.7
MSM	50	0	18	42	18	30
Male STI clients	200	0	10	51.5	5.5	16.5
TB patients	50	0	18	56	12	12
Mobile men	150	0	22	48	5.3	14.7
Subtotal	600	0	14	49.5	7.8	26.3
Darkhan – Uul						
FSWs	200	0	14.5	47	4	31.5
Male STI clients	100	0	28	44	7	26
TP patients	30	0	13.3	46.7	16.7	13.3
Subtotal	330	0	18.5	46	6	28.2
Dornogobi						
FSWs	20	0	5	55	5	30
Male STI clients	45	0	26.7	48.9	13.3	15.6
TB patients	10	0	20	70	10	10
Mobile men	150	0	19.3	45.3	8.7	14.7
Subtotal	225	0	19.6	48	9.3	16
Dornod						
FSWs	10	0	10	40	10	20
Male STI clients	100	0	15	46	5	17
TP patients	10	0	10	40	10	0
Subtotal	120	0	14.2	45	5.8	15.8
Huvsgul						
FSWs	30	0	10	56.7	6.7	30
Male STI clients	100	0	9	51	12	11
TP patients	10	0	20	30	40	10
Subtotal	140	0	10	50.7	12.9	15

HBV= hepatitis B virus; HCV = hepatitis C virus; TP= *Treponema pallidum*; FSW = female commercial sex worker; MSM = men who have sex with men; TB = tuberculosis; STI = sexually transmitted infection; OR = odds ratio; CI = confidence interval; HBs Ag = hepatitis B surface antigen; HBs Ab = hepatitis B surface antibody

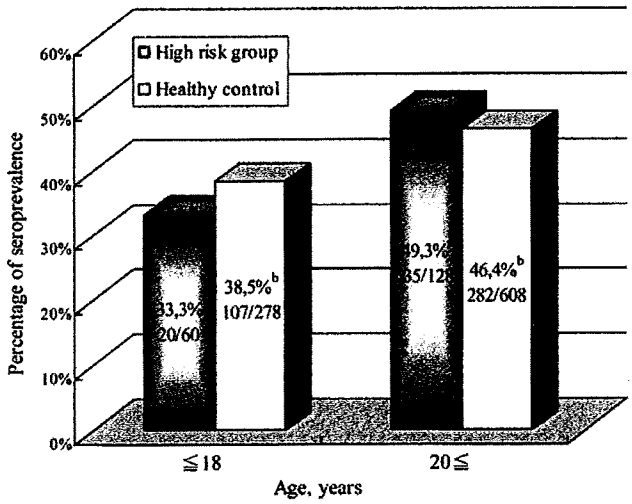

Figure 2 Age-related seroprevalence of hepatitis B surface antibody (HBs Ab). *N-positive for HBs Ab/N-tested. [a]$P < 0.05$. [b]$P < 0.05$

Ulaanbaatar in 1998. In our study conducted in 2007, this rate in Ulaanbaatar was 16.5%, suggesting that the prevalence of syphilis is increasing. It is true that these rates were anti-TP. Therefore, it did not mean active syphilis. However, these rates document that the exposure to syphilis is very high. A 100% condom programme is strongly recommended.

According to the unpublished data by NCCD, another risk factor for HIV-1 acquisition is that the predominant route of HIV-1 transmission in Mongolia is through sexual intercourse in MSM. The present study showed higher rates of anti-TP (30%) and anti-HCV (18%) in MSM than those in neighbouring countries: for example, 7% in Beijing (China) and 10% in St Petersburg (Russia) for syphilis and 0.8% or 5.2% in Beijing (China) for HCV.[14-16] These results indicate active high-risk sexual intercourse in Mongolian MSM. There is strong prejudice and discrimination against MSM in Mongolia. Hence, access to the MSM group was very difficult in this study. This barrier makes the delivery of information to MSM difficult. A quick countermeasure to MSM is crucial and a larger serological survey is necessary to grasp the actual prevalence of HIV-1 in Mongolian MSM.

Compared with other STIs, evaluation of hepatitis B was not simple because of the high-prevalence rate in the general population. A hepatitis B vaccination programme has been conducted 18 years ago. Around 35% of people below 18 years have HBs Ab. In contrast, those over 20 years had a significantly higher rate of HBs Ab in both high-risk and healthy control populations. Analysis of HBc-Ab could make it possible to discriminate between HBV-vaccinated and HBV-exposed individuals, which unfortunately we could not perform in this study. This result also suggests the frequent exposure to hepatitis B in Mongolians. Takahashi *et al.*[10] reported a comparable rate of HBs Ab prevalence, indicating a low selection bias of subjects in this study except for MSM and drug abusers.

The present study demonstrates that HIV prevalence is currently low. However, according to the high prevalence of syphilis and HCV in high-risk populations and the social stigma against MSM, the risk status for HIV-1 infection is estimated to be high. Close monitoring of the HIV epidemic is important in order to take quick measures for the high-risk populations and consequently keep the prevalence of HIV low in Mongolia.

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Favourable use of non-boosted fosamprenavir in patients treated with warfarin

Sir: Warfarin is metabolized by cytochrome P450 2C9 (CYP2C9).[1,2] Due to drug interactions, warfarin dose modification is required when it is combined with other drugs. For example, antiretroviral drugs, nevirapine and lopinavir-ritonavir, reduce the serum concentration of warfarin,[3,4] while efavirenz increases the concentration,[4] probably via CYP2C9 induction and inhibition, respectively. No clinical data are currently available for other antiretrovirals. We describe three HIV-1-infected patients in whom the use of non-boosted fosamprenavir had a favourable outcome. Case 1 was a 60-year-old Japanese man who had been treated with a stable dose of warfarin (mean daily dose, 3 mg) for chronic atrial flutter. The international normalized ratio (INR) was maintained within the optimal range (1.5-2.5). One year later, antiretroviral therapy (ART) was started with abacavir, lamivudine and non-boosted fosamprenavir (1400 mg twice daily). In this patient, dose modification of warfarin was not necessary because INR was controlled within 1.46-3.01. Case 2 was a 33-year-old Japanese man who had been treated with abacavir, lamivudine and lopinavir/ritonavir. He developed deep vein thrombosis followed by pulmonary embolism. Warfarin was given initially at 7 mg/day to maintain INR within the optimal range (2-3). After several months, the control of INR became difficult; at 9 mg of warfarin, INR was 1.38-1.75. Therefore, ART was changed to abacavir, lamivudine and fosamprenavir (1400 mg twice daily), and warfarin dose was decreased to 4.25-5.00 mg/day. The new treatment allowed maintenance of INR within the optimal range. Case 3 was a 49-year-old Japanese man who had been treated with abacavir, lamivudine and fosamprenavir (1400 mg twice daily). He underwent mitral mechanical valve replacement for mitral incompetence and heart failure. After surgery, warfarin was given initially at 2-2.5 mg/day to almost maintain INR within the optimal range (2.5-3.5); at 2.5 mg of warfarin, INR was 2.85-3.65.

Amprenavir is mainly metabolized by CYP3A4 and to a lesser extent by CYP2D6, CYP2C19 and CYP2C9.[5] Interaction between fosamprenavir and warfarin is theoretically rare. Actually in our cases, non-boosted fosamprenavir showed favourable results. Along with the long-term use of ART, cardiovascular events are increasing. When warfarin co-administration is indispensable, non-boosted fosamprenavir can be an antiretroviral agent of choice.

H Honda*, H Gatanaga*, J Matsumura*†, M Kamimura*, K Goto*‡, K Tsukada*, M Honda*, K Teruya*, Y Kikuchi* and S Oka*

*AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan; †Department of Microbiology and Immunology, Nippon Medical School, Tokyo, Japan; ‡Department of Infectious Diseases, Graduate School of Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan
Correspondence to: H Honda, AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan
Email: hhonda@imcj.acc.go.jp
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Retrospective review of *Pneumocystis jirovecii* pneumonia in a French intensive care unit (1994-2000)

Sir: We read with great interest the paper by Travis and colleagues about outcomes of *Pneumocystis jirovecii* pneumonia (PJP) in HIV patients managed in the intensive care unit (ICU).[1,2] Our experience with severe PJP provides both support and nuance to their conclusions.

We also performed a retrospective study, although we included only patients with severe PJP, in 1994-2000, at our 18-bed ICU, to compare outcomes with those in the same ICU in 1989-1993.[3] We identified 76 patients with severe PJP, including 25 (33%) before and 51 during the highly active antiretroviral therapy (HAART) era; only nine (9/51; 18%) were taking HAART at the diagnosis of severe PJP. Median age was 40 years, and 59 (59/76; 78%) patients were men. Only 25 (33%) patients were on PJP prophylaxis, and 34 (45%) patients were not known to be HIV positive before the PJP episode. At ICU admission, median PaO_2 on room air was 49 mmHg (interquartile range [IQR], 43-57). CD4 was 15 cells/L (IQR, 7-51), viral load was 2.1×10^5 copies/mL (IQR, 1.0-4.2), serum lactate dehydrogenase was 1052 IU/L (IQR, 724-1392) and the Simplified Acute Physiology Score II was 32 (IQR, 27-40). Endotracheal mechanical ventilation (MV) was required in 23 (31%) patients, at admission ($n = 12$) or after failure of another method (continuous positive airway pressure, $n = 6$ among 35 who had this treatment; or non-invasive ventilation, $n = 5$ among six who had this treatment). Initial treatment was with cotrimoxazole in 63 (83%) patients, pentamidine in 12 and atovaquone in one. Steroids were used in 66 (87%) patients. Overall ICU mortality was 24% ($n = 18$); 13

BRIEF REPORT

Serum (1→3) β-D-Glucan as a Noninvasive Adjunct Marker for the Diagnosis of *Pneumocystis* Pneumonia in Patients with AIDS

Tamayo Watanabe,[1,2] Akira Yasuoka,[3] Junko Tanuma,[1] Hirohisa Yazaki,[1,2] Haruhito Honda,[1] Kunihisa Tsukada,[1] Miwako Honda,[1] Hiroyuki Gatanaga,[1,2] Katsuji Teruya,[1] Yoshimi Kikuchi,[1] and Shinichi Oka[1,2]

[1]AIDS Clinical Center, International Medical Center of Japan, Tokyo, [2]Division of Infectious Disease, Center for AIDS Research, Kumamoto University, Kumamoto, and [3]Infection Control and Education Center, Nagasaki University, Nagasaki, Japan

High serum (1→3) β-D-glucan levels are described in patients with *Pneumocystis* pneumonia (PCP). We evaluated the diagnostic value of β-D-glucan in 111 patients with AIDS who had PCP and confirmed its usefulness. However, it does not correlate with disease severity and is not suitable for monitoring response to treatment.

Pneumocystis pneumonia (PCP) is associated with significant morbidity and mortality in patients with human immnuodeficiency virus type 1 (HIV-1) infection [1, 2]. PCP is usually diagnosed microscopically by identifying *Pneumocystis jirovecii* in bronchoalveolar lavage fluid (BALF) or bronchoscopically obtained lung tissue [3]. Bronchoscopy, however, is invasive, especially in patients with hypoxemia associated with PCP. Therefore, a minimally invasive method is desirable for diagnosis.

Serum (1→3) β-D-glucan (hereafter, β-D-glucan) is a common component of the cell wall of most fungi and is the major component of the cyst of *P. jirovecii*. Therefore, it is measured in patients who are suspected to have PCP, as well as in those with deep-seated mycotic infections [4]. Although β-D-glucan has been used as an adjunct test for the diagnosis of PCP [5], only a few reports have evaluated its level [5–7] and its correlation with other parameters (such as lactate dehydrogenase [LDH] level) in mixed populations that included a small number of HIV-infected patients [6]. For this purpose, we analyzed the correlation between β-D-glucan levels and other parameters among patients with AIDS who have PCP.

Methods. We evaluated data from 111 consecutive HIV-1–infected patients with PCP at the International Medical Center of Japan, an 885-bed tertiary care hospital in Tokyo, from April 1997 through July 2007. This study was approved by the Ethics Review Committee of the hospital (IMCJ-H20-569). Patients who did not undergo diagnostic bronchoscopy were excluded from the study.

Medical records were reviewed, and the following data were collected: age; sex; mode of infection; $CD4^+$ cell count; serum levels of LDH, β-D-glucan, and C-reactive protein (CRP); and alveolar-arterial oxygen tension gradient ($AaDO_2$). Serum β-D-glucan levels were measured using the Fungitec G MK test (Seikagaku). Manipulation was performed described elsewhere [4, 5], in accordance with the manufacturer's instructions. Serum β-D-glucan levels in HIV-1–infected patients without PCP determined during the same period were used as a control. If serum β-D-glucan levels had been determined several times for the same patient, only the first measurement was included. Although oral and esophageal candidiasis are superficial infections, they were included as an independent factor and analyzed. In this report, the term *candidiasis* refers to oral and/or esophageal candidiasis.

The diagnosis of PCP was established by identification of *P. jirovecii* in BALF. Each BALF specimen (100 μL) was centrifuged at 900 *g* for 2 min by means of a Shandon Cytospin III device, and a monolayer of deposited cells were stained using Diff-Quik (Dade Behring) and examined microscopically for the presence of *P. jirovecii*.

Data were expressed as means ± standard deviations (SDs) or as medians. Differences in categorical variables between patients with PCP and control patients were assessed using the Mann-Whitney *U* test. The Mann-Whitney *U* test (for comparison of 2 groups) and the Kruskal-Wallis test (for comparison of 3 groups) were used for analysis of differences in serum β-D-glucan levels. A receiver-operating-characteristic (ROC) curve was constructed to illustrate the cutoff value for β-D-glucan. The relationships were analyzed by linear regression analysis. Differences were considered significant at $P < .05$. Statistical analyses were performed using SPSS, version 17.0 (SPSS).

Results. A total of 111 patients had a definite diagnosis of PCP, and serum β-D-glucan level was measured in each. Of

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Reprints or correspondence: Dr. Shinichi Oka, AIDS Clinical Center, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan (oka@imcj.hosp.go.jp).

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these patients, 67 also had candidiasis at admission. Of the control group (425 patients who did not have PCP), 28 had candidiasis, 3 had cryptococcal infection, and 394 had neither.

The patients with PCP were older than the control patients (mean ± SD, 42.3 ± 11.9 vs 38.7 ± 11.7 years; $P < .01$), and $CD4^+$ cell counts were significantly higher in the control patients than in the patients with PCP (mean ± SD, 178.6 ± 155.6 vs 49.1 ± 63.1 cells/μL; $P < .001$). Sex and mode of transmission of HIV were similar in both groups ($P = .81$ and $P = .53$, respectively). All patients with PCP received treatment, and 6 patients died of PCP.

Of the patients with PCP, 67 had candidiasis and 44 did not; of the control patients, 28 had candidiasis, 3 had cryptococcal infection, and 394 did not have any fungal infection. The median (range) serum β-D-glucan level in each group was 171.2 (14.9–2966), 209.6 (2.4–2469), 7.40 (1.0–73.0), 22.7 (9.3–69.7), and 8.25 (1.0–310) pg/mL, respectively (Figure 1). The median serum level of β-D-glucan among all patients with PCP (174.8 [2.4–2966] pg/mL) was significantly higher than that among the control patients (8.2 [1.0–310.1] pg/mL) ($P < .001$). The presence of candidiasis in both the PCP group and the control group and of cryptococcal infection in the control group did not significantly influence serum levels of β-D-glucan ($P = .53$, $P = .83$, and $P = .08$, respectively).

With respect to the diagnostic value of β-D-glucan, the area under the ROC curve for β-D-glucan level was 0.964 (95% confidence interval, 0.945–0.984) (Figure 2). A β-D-glucan cutoff value of 23.2 pg/mL (which represented the technique's threshold of detection) had a sensitivity of 96.4% and a specificity of 87.8%.

There was no correlation between serum levels of β-D-glucan and $AaDO_2$ at room air ($r = 0.125$; $P = .30$), LDH ($r = .030$; $P = .76$), or CRP ($r = .002$; $P = .62$). In 42 instances, serum β-D-glucan levels were measured before and after treatment. On the basis of a cutoff value of 23.2 pg/mL, normalization of serum β-D-glucan levels was noted in 7 patients. In contrast, serum β-D-glucan levels slightly increased in 9 patients despite clinical improvement being noted at week 3. This finding indicates that β-D-glucan levels reflected the clinical course in only 16.7% of patients (7 of 42) within 3 weeks of treatment.

Discussion. The present study has reported 3 major findings. The first major finding is the usefulness of quantitative measurement of serum β-D-glucan levels for the diagnosis of PCP. With a cutoff value of 23.2 pg/mL, β-D-glucan level had

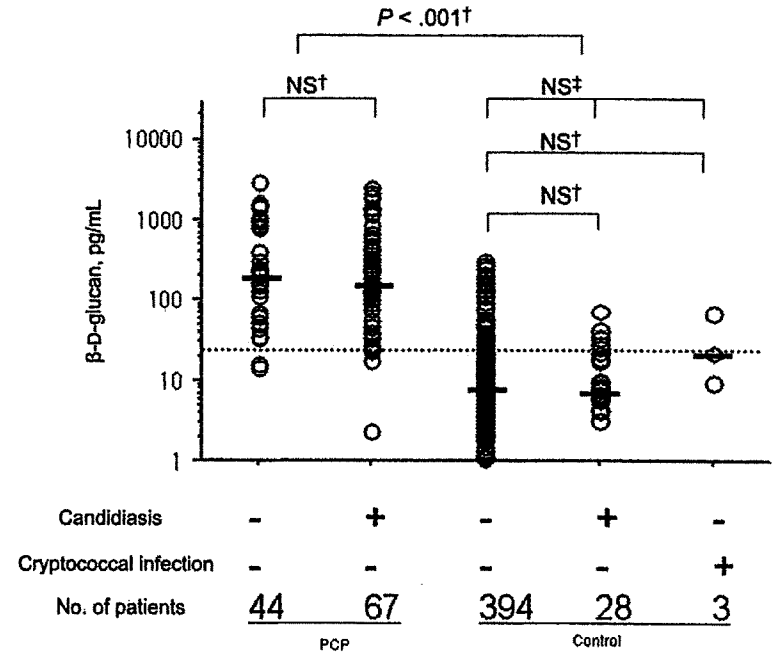

Figure 1. Serum levels of (1→3) β-D-glucan. Levels of β-D-glucan in serum were examined before treatment of *Pneumocystis* pneumonia (PCP), candidiasis, and cryptococcal infection. The Mann-Whitney *U* test (†) and the Kruskal-Wallis test (‡) were used for comparison of serum β-D-glucan levels. Individual values are plotted, and horizontal bars represents medians. The presence of candidiasis in both the PCP group and the control group and of cryptococcal infection in the control group did not significantly influence serum β-D-glucan levels ($P = .53$, $P = .83$, and $P = .08$, respectively). Serum β-D-glucan levels were significantly higher in patients with PCP than in those without PCP, despite the presence of candidiasis and cryptococcal infection ($P < .001$). NS, not significant.

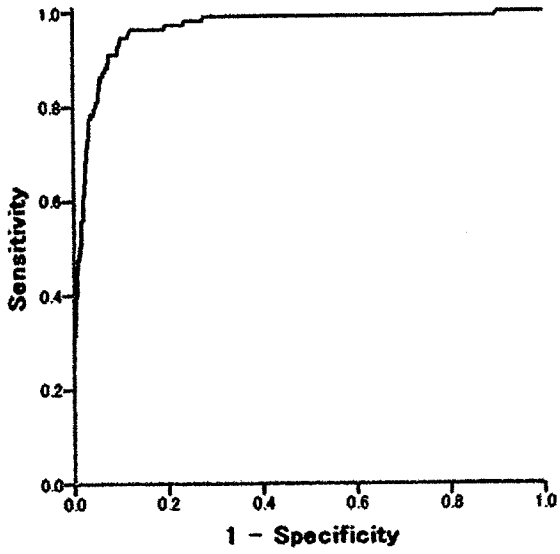

Figure 2. Receiver-operating-characteristic (ROC) curve for the (1→3) β-D-glucan cutoff. The area under the ROC curve for β-D-glucan was 0.964 (95% confidence interval, 0.945–0.984). A β-D-glucan cutoff value of 23.2 pg/mL (which represented the technique's threshold of detection) had a sensitivity of 96.4% and a specificity of 87.8%.

a high sensitivity (96.4%) and specificity (87.8%) for the diagnosis of PCP. Interestingly, serum β-D-glucan levels among those with PCP were not affected by the presence of superficial fungal infection (ie, oral and/or esophageal candidiasis). Deep-seated mycosis other than PCP and cryptococcal infection are quite rare in Japan, and no patients were suspected to have aspergillosis in this study. Hence, we could not analyze the effect of aspergillosis. According to our data and those of others [4], β-D-glucan level increases during cryptococcal infection, but the level is significantly lower than that observed during PCP. The number of *P. jirovecii* organisms in the lungs of patients with AIDS may be significantly higher than that in patients without AIDS [8]. In a meta-analysis of 7 reports in which PCP was diagnosed by staining, the average sensitivity of induced sputum was 56%, whereas that of BALF was >95% [9]. To eliminate false-positive and false-negative results, we analyzed data obtained only from patients who underwent BALF analysis and had a definite diagnosis of PCP.

The second major finding was that the serum level of β-D-glucan does not reflect the severity of PCP in patients with AIDS. Although Shimizu et al [10] reported that β-D-glucan is a negative prognostic marker for PCP in patients with connective tissue diseases, there was no significant difference in β-D-glucan level between survivors and nonsurvivors in our study. Furthermore, Tasaka et al [6] reported that serum levels of LDH correlated with those of β-D-glucan in patients with PCP, whereas our data showed no such relationship. These differences are probably the result of differences in the patient populations studied, especially regarding whether the patients have HIV-1 infection. Considered collectively, these results emphasize the need for further studies to define the exact relationship between β-D-glucan and prognosis as well as LDH.

The third major finding of the present study was that β-D-glucan level did not reflect the effectiveness of therapy. In nearly 85% patients, serum β-D-glucan levels did not decrease to normal despite clinical improvement. Furthermore, 20% of patients had increased levels of β-D-glucan during the early phase of treatment. However, β-D-glucan levels normalized several months or years after treatment in all patients. These results mean that β-D-glucan levels increase transiently early during treatment and decrease thereafter but do not always return to normal during treatment. The transient increase in β-D-glucan level is probably due to lysis of *P. jirovecii* shortly after treatment.

PCP is usually suspected on the basis of chest radiographic findings, clinical symptoms, and low $CD4^+$ cell counts in HIV-infected patients. In the present study, a high serum level of β-D-glucan (especially >23.2 pg/mL by the MK test) was found to be highly indicative of PCP in practically all patients with AIDS. Therefore, the β-D-glucan test is useful for the diagnosis of PCP, especially in HIV-infected patients who are unable to undergo bronchoscopy owing to severe hypoxemia. In conclusion, the present study has demonstrated that β-D-glucan is a useful, noninvasive adjunct marker for the diagnosis of PCP in patients with AIDS. However, its serum levels do not reflect the severity of the disease, and it is not suitable for monitoring response to treatment.

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Correspondence

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The impact of the 0–0 cell on measures of amino acid covariation

The covariation between the amino acids expressed at different residues on the HIV genome has been examined in numerous studies [1–6]. In particular, the phi statistic (which is equivalent to the Pearson correlation coefficient for 0/1 data) has been widely utilized as a measure of covariation. Studies of samples from antiretroviral therapy (ART)-naive patients may provide insights into fitness epistasis, although this cannot be easily disentangled from the effect of evolutionary relationships between sequences [2,6]. Studies of samples from ART-experienced patients may identify mutations that lie on a common mutational pathway under specific selective drug pressure (positive association) or mutations that are biologically antagonistic (negative association), such as K65R and T215F/Y [7].

We recently examined the covariation between the N348I connection domain mutation and key reverse transcriptase (RT) mutations, based on a case–control analysis of ART-experienced patients in a large clinical database [8]. Cases were defined as the first sample per patient in which N348I was detected ($n = 198$). The control series consisted of 10 N348N samples that were closest in calendar time to the sampling date of each of the cases ($n = 1980$). Associations were quantified by the odds ratio (OR), the only estimable measure of association in case–control studies.

The initial analysis indicated a strong positive association between N348I and virtually all key RT mutations. For example, the OR between N348I and T215F/Y was 2.72 [95% confidence interval (CI) 2.00–3.69, $P < 0.0001$] (Table 1). However, an increasing proportion of samples from ART-experienced patients lack any major resistance mutations (25% in 2001, 56% in 2007) (http://www.hivrdb.org/public/surveillance.asp); this is presumed to largely reflect changes in the clinical indication for conducting an HIV resistance test [10]. As the detection of wild-type virus suggests an absence of antiretroviral selection pressure, we, therefore, repeated our analysis excluding such samples to better approximate the target population of 'ART-experienced' patients.

By definition, the re-analysis results in a reduction in the number of samples in the bottom-left cell of the table (from 1462 to 654) while leaving the other cells unchanged. The effect of this is to nullify the previously observed strong association between N348I and T215F/Y (OR = 1.22, 95% CI 0.89–1.67, $P = 0.21$). A large attenuation in the strength of the associations for other key mutations was similarly observed, although some remained statistically significant. Thus, the conclusions reached on covariation between amino acids are seen to depend critically on analytical assumptions.

The Jaccard coefficient, which ignores samples that lack both the mutations being assessed, has recently been proposed as a measure of covariation [3,6]. It has been argued that this coefficient '...does not inflate the correlation between two mutations that may appear correlated by other measures when both mutations are nearly always absent' [3]. Unfortunately, however, the statistic to test whether the observed Jaccard coefficient differs significantly from the expected value under the null hypothesis of zero covariation fundamentally depends on the number of samples harboring neither mutation.

Many subtle biases underlie analyses of amino acid covariation. It is important to note that although the procedure we adopted is an improvement on the initial analysis, it does not provide a definitive solution. Another generally overlooked point is that observed mutational associations are a function of the patients' treatment histories, which are often highly variable, especially in observational studies. Analyses that do not stratify by drug exposure provide only limited biological insights.

David Dunn[a], Viktor von Wyl[b], Huw Price[c] and David Asboe[c], *[a]Medical Research Council Clinical Trials Unit, London, UK, [b]University Hospital Zurich, University of*

Table 1. Association between N348I and T215F/Y.

	All samples			Excluding wild-type samples[a]		
	T215T	T215F/Y	Total	T215T	T215F/Y	Total
Cases (N348I)	103 (52%)	95 (48%)	198 (100%)	103 (52%)	95 (48%)	198 (100%)
Controls (N348N)	1462 (74%)	518 (26%)	1980 (100%)	654 (56%)	518 (44%)	1172 (100%)
	OR = 2.72 (95% CI 2.00–3.69), $P < 0.0001$			OR = 1.22 (95% CI 0.89–1.67), $P = 0.21$		

Odds ratios estimated by conditional logistic regression. CI, confidence interval; OR, odds ratio.
[a]Samples lacking any major reverse transcriptase (RT) mutation according to IAS-USA 2008 list [9] and N348I.

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Zurich, Zurich, Switzerland, and [c]Chelsea and Westminster NHS Foundation Trust, London, UK.

Correspondence to David Dunn, MRC Clinical Trials Unit, 222 Euston Road, London NW1 2DA, UK. Tel: +44 207 670 4700; fax: +44 207 670 4818; e-mail: d.dunn@ctu.mrc.ac.uk

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Raltegravir-associated perihepatitis and peritonitis: a single case report

Raltegravir, the first approved HIV integrase inhibitor, has demonstrated an excellent safety and tolerability profile in several clinical trials [1] and is currently used widely as one of the key components of salvage regimens. However, the duration of clinical use is relatively short, and unknown adverse effect may occur. Here, we report one case of peritonitis associated with use of raltegravir. Abdominal symptoms appeared within 2 weeks of commencement of treatment, and raltegravir had to be stopped due to worsening of clinical condition.

Case report

The patient was a 49-year-old Japanese hemophiliac coinfected with HIV and hepatitis C virus (HCV). HIV-RNA was undetectable, and $CD4^+$ cell count was above 500 cells/μl for more than 5 years under the combination of abacavir, nevirapine and lopinavir/ritonavir. In January 2009, lopinavir/ritonavir was replaced with raltegravir because of bleeding tendency related to the use of a protease inhibitor. Abacavir and nevirapine were continued, and no other drugs were modified. The patient visited the hospital on day 18 after the use of raltegravir, complaining of a gradually worsening pain in the right upper abdomen and lower chest wall for 3 days. A nonsteroidal anti-inflammatory drug was not effective, and a computed tomography (CT) scan performed 11 days after the onset of the symptom revealed contrast enhancement of the liver surface (Fig. 1a) and fatty stranding of the greater omentum (Fig. 1b), which are findings compatible with perihepatitis and peritonitis. Oral prednisone (60 mg/day for 3 days, then 30 mg/day for 3 days) was prescribed, and all the symptoms resolved immediately. However, abdominal symptoms developed again after withdrawal of prednisone, necessitating its reintroduction on day 31 at 30 mg/day. Attempts to taper prednisone led to worsening of abdominal pain and development of stomatitis, resulting in continuation of treatment at 20 mg/day. Raltegravir was switched to lopinavir/ritonavir 11 weeks after the onset of abdominal pain and, finally, all antiretroviral drugs were terminated 4 days later because of diarrhea and bleeding related to lopinavir/ritonavir. Abdominal symptoms gradually improved, and prednisone could be tapered to 10 mg/day within 2 weeks. A CT scan performed 10 days after cessation of antiretroviral therapy showed an improvement of perihepatic enhancement. C-reactive protein levels increased to 1.42 mg/dl during raltegravir use and fell to normal levels 6 days after discontinuation of raltegravir. Other laboratory data including transaminase levels showed no changes, and $CD4^+$ cell count and HIV-RNA were stable throughout the course.

This is the first reported case of severe peritonitis associated with raltegravir use. Although not described here, we have experienced several other cases with similar abdominal symptoms that disappeared after raltegravir termination. Several case reports have recently described previously unknown adverse effects related to raltegravir, such as rhabdomyolysis [2] and exacerbation of depression [3]. However, to our knowledge, raltegravir-associated peritonitis has not been reported. In the BENCHMRK

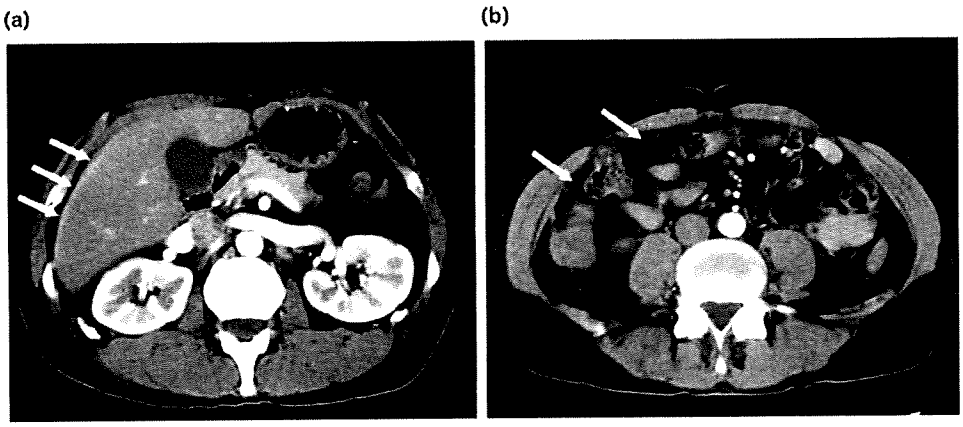

Fig. 1. A computed tomography scan performed 11 days after the onset of the symptoms. A computed tomography scan shows contrast enhancement around the liver surface (a) and fatty stranding of the greater omentum (b).

(Blocking integrase in treatment Experienced patients with a Novel Compound against HIV: MeRcK, MK-0518) study [1], abdominal symptoms, such as diarrhea and nausea, were noted in patients on raltegravir, and some of which might be associated with mild peritonitis.

Fortunately, raltegravir-associated peritonitis seemed reversible, at least to some extent. However, the longer use of raltegravir after onset of symptoms may lead to irreversible and lethal sequelae. Cessation of antiretroviral therapy as a result of severe abdominal symptoms is a potential risk for re-emergence of acute retroviral syndrome or the further accumulation of HIV-resistant mutations.

Whether the described side effects are universal or related to Asians, hemophiliacs or those who have underlying liver disease is unknown at present. Careful monitoring of abdominal symptoms and the consideration of an appropriate radiographic examination are warranted after commencement of raltegravir-containing regimens.

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Kunihisa Tsukada, Katsuji Teruya, Daisuke Tasato, Hiroyuki Gatanaga, Yoshimi Kikuchi and Shinichi Oka, *AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan.*

Correspondence to Kunihisa Tsukada, MD, AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. Tel: +81 3 3202 7181; fax: +81 3 3202 7198; e-mail: ktsukada@imcj.acc.go.jp

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Synthesis and biological evaluation of novel allophenylnorstatine-based HIV-1 protease inhibitors incorporating high affinity P2-ligands

Arun K. Ghosh [a,*], Sandra Gemma [a], Elena Simoni [a], Abigail Baldridge [a], D. Eric Walters [b], Kazuhiko Ide [c], Yasushi Tojo [c], Yasuhiro Koh [c], Masayuki Amano [c], Hiroaki Mitsuya [c,d]

[a] Departments of Chemistry and Medicinal Chemistry, Purdue University, West Lafayette, IN 47907, United States
[b] Departments of Biochemistry and Molecular Biology, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064, United States
[c] Departments of Hematology and Infectious Diseases, Kumamoto University School of Medicine, Kumamoto 860-8556, Japan
[d] Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, Bethesda, MD 20892, United States

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ABSTRACT

A series of stereochemically defined cyclic ethers as P2-ligands were incorporated in an allophenylnorstatine-based isostere to provide a new series of HIV-1 protease inhibitors. Inhibitors **3b** and **3c**, containing conformationally constrained cyclic ethers, displayed impressive enzymatic and antiviral properties and represent promising lead compounds for further optimization.

The introduction of protease inhibitors into highly active antiretroviral treatment (HAART) regimens with reverse transcriptase inhibitors represented a major breakthrough in AIDS chemotherapy.[1] This combination therapy has significantly increased life expectancy, and greatly improved the course of HIV management. Therapeutic inhibition of HIV-1 protease leads to morphologically immature and noninfectious viral particles.[2] However, under the selective pressure of chemotherapeutics, rapid adaptation of viral enzymes generates strains resistant to one or more antiviral agents.[3] As a consequence, a growing number of HIV/AIDS patients harbor multidrug-resistant HIV strains. There is ample evidence that such strains can be readily transmitted.[4] Therefore, one of the major current therapeutic objectives has been to develop novel protease inhibitors (PIs) with broad-spectrum activity against multidrug-resistant HIV-1 variants. In our continuing interest in developing concepts and strategies to combat drug-resistance, we have reported a series of novel PIs including Darunavir, TMC-126, GRL-06579, and GRL-02031.[5–8] These inhibitors have shown exceedingly potent enzyme inhibitory and antiviral activity as well as exceptional broad spectrum activity against highly cross-resistant mutants. Darunavir, which incorporates a (*R*)-(hydroxymethyl)-sulfonamide isostere and a stereochemically defined bis-tetrahydrofuran (bis-THF) as the P2-ligand, was initially approved for the treatment of patients with drug-resistant HIV and more recently, it has been approved for all HIV/AIDS patients including pediatrics[9] (Fig. 1).

Darunavir was designed based upon the 'backbone binding' concept developed in our laboratories. Darunavir-bound X-ray structure revealed extensive hydrogen bonding with the protease backbone throughout the enzyme active site.[10] The P2-bis-THF ligand is responsible for its superior drug-resistance properties. The bis-THF ligand has been documented as a privileged ligand for the S2-subsite. Incorporation of this ligand into other transition-state isosteres also resulted in significant potency enhancement.[11] Besides 3(*S*)-THF, and [3a*S*,5*S*,6*R*]-bis-THF, we have designed a number of other novel cyclic ether-based high affinity ligands. Incorporation of these ligands in (*R*)-(hydroxyethyl)-sulfonamide isosteres provided PIs with excellent potency and drug-resistance properties.[6–8] We then investigated the potential of these structure-based designed P2-ligands in a KNI-764-derived isostere designed by Mimoto and coworkers.[12] This PI incorporates an allophenylnorstatine isostere. Interestingly, KNI-764 has maintained good activity against HIV-1 clinical strains resistant to several FDA-approved PIs. The flexible *N*-(2-methyl benzyl) amide P2′-ligand may have been responsible for its activity against drug-resistant HIV-1 strains as the flexible chain allows better adaptability to mutations.[12,13] The bis-THF and other structure-based designed P2-ligands, make several critical

* Corresponding author. Tel.: +1 765 494 5323; fax: +1 765 496 1612.
E-mail address: akghosh@purdue.edu (A.K. Ghosh).

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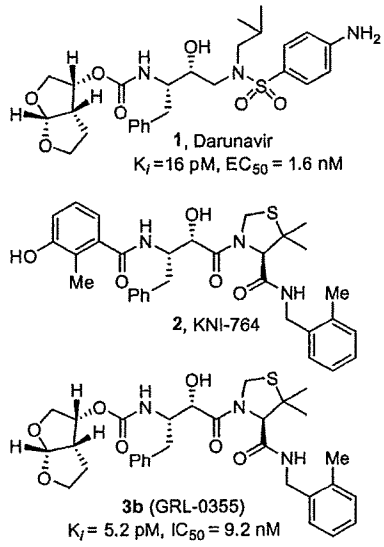
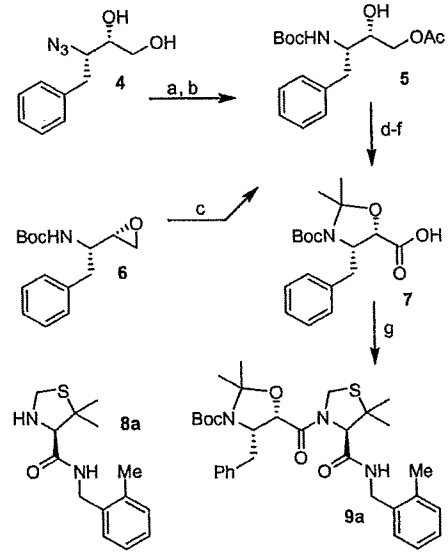


Figure 1. Structures of inhibitors **1**, **2**, and **3b**.

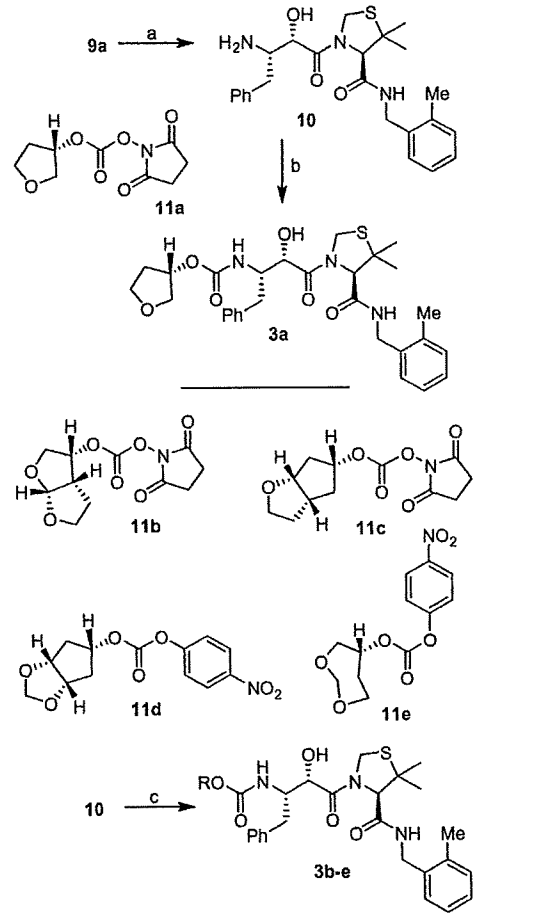
hydrogen bonds with the protein backbone, particularly with Asp-29 and Asp-30 NH's.[11] Therefore, incorporation of these ligands into the KNI-764-derived isostere, may lead to novel PIs with improved potency and efficacy against multidrug-resistant HIV-1 variants. Furthermore, substitution of the P2-phenolic derivative in KNI-764 with a cyclic ether-based ligand could result in improved metabolic stability and pharmacological properties since phenol glucuronide is readily formed when KNI-764 is exposed to human hepatocytes in vitro.[12]

The synthesis of target compounds **3a–e** was accomplished as described in Scheme 1. Our synthetic plan for carboxylic acid **7** (Scheme 1) involved the preparation of the key intermediate **5** through two different synthetic pathways. In the first approach, known optically active azidodiol **4**[14] was first hydrogenated in the presence of Boc_2O. The resulting diol was converted to **5** by selective acylation of the primary alcohol with acetic anhydride in the presence of pyridine and a catalytic amount of DMAP at 0 °C for 4 h to provide **5** in 77% overall yield. As an alternative approach, commercially available optically active epoxide **6** was exposed to lithium acetate, formed in situ from lithium carbonate and acetic acid in DMF. This resulted in the regioselective opening[15] of the epoxide ring and afforded compound **5** in 62% yield. The alcohol **5** thus obtained was protected as the corresponding acetonide by treatment with 2-methoxypropene in the presence of a catalytic amount of CSA. The acetate group was subsequently hydrolyzed in the presence of potassium carbonate in methanol to afford the corresponding alcohol. This was subjected to an oxidation reaction using ruthenium chloride hydrate and sodium periodate in a mixture of aqueous acetonitrile and CCl_4 at 23 °C for 10 h. This resulted in the formation of the target carboxylic acid **7** in 61% yield. Amide **9a** was prepared by activation of carboxylic acid **7** into the corresponding mixed anhydride by treatment with isobutylchloroformate followed by reaction with amine **8a**.[16,17]

Synthesis of various inhibitors was carried out as shown in Scheme 2. Deprotection of the Boc and acetonide groups was carried out by exposure of **9** to a 1 M solution of hydrochloric acid in methanol at 23 °C for 8 h. This provided amine **10** in quantitative



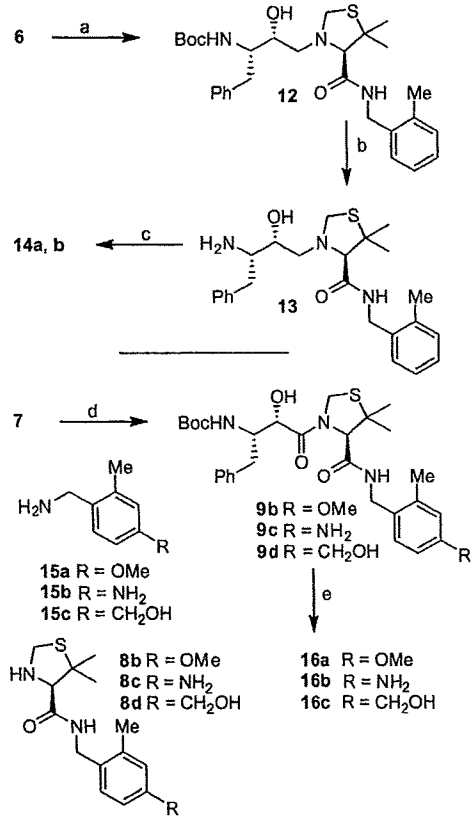
Scheme 1. Reagents: (a) H_2, Pd/C, Boc_2O, EtOAc; (b) Ac_2O, Pyr, DMAP; (c) $LiCO_3$, AcOH, DMF; (d) 2-methoxypropene, CSA, DCM; (e) K_2CO_3, MeOH; (f) $RuCl_3$, $NaIO_4$, CCl_4–MeCN–H_2O (2:2:3); (g) *N*-methylmorpholine, iBuOCOCl, **8a**, THF.



Scheme 2. Reagents: (a) 1 M HCl, MeOH; (b) **11a**, Et_3N, CH_2Cl_2; (c) **11b**,**c**, Et_3N, CH_2Cl_2; or, **11d**,**e**, DIPEA, THF.

yield. Reaction of **11a** with amine **10** in CH_2Cl_2 in the presence of Et_3N at 23 °C for 6 h, provided inhibitor **3a** in 62% yield. The 3(*S*)-tetrahydrofuranyl carbonate **11a** was prepared as described previously.[18] Similarly, allophenylnorstatine-based inhibitors **3b–e** were synthesized. As shown, carbonates **11b**,[19] **11c**,[7] and **11d–e**[19] were prepared as previously described. Reaction of these carbonates with amine **10** furnished the desired inhibitors **3b–e** in 45–62% yield.

The syntheses of inhibitors **14a,b** and **16a–c** were carried out as shown in Scheme 3. Inhibitors **14a,b**, containing hydroxyethylamine isostere were prepared by opening epoxide **6** with amine **8a** in the presence of lithium perchlorate in diethyl ether at 23 °C for 5 h to provide amino alcohol **12** in 64% yield. Removal of the Boc-group by exposure to 1 M HCl in MeOH at 23 °C for 12 h afforded amine **13**. Reactions of amine **13** with activated carbonates **11a** and **11b** afforded urethane **14a** and **14b** in 44% and 59% yields, respectively. For the synthesis of inhibitors **16a–c**, commercially available (*R*)-5,5-dimethyl-thiazolidine-4-carboxylic acid was protected as its Boc-derivative. The resulting acid was coupled with amines **15a–c** in the presence of DCC and DMAP in CH_2Cl_2 to provide the corresponding amides. Removal of the Boc-group by exposure to 30% trifluoroacetic acid afforded **8b–d**. Coupling of these amines with acid **7** as described in Scheme 1, provided the corresponding products **9b–d**. Removal of Boc-group and reactions of the resulting amines with activated carbonate **11b** furnished inhibitors **16a–c** in good yields (55–60%).



Scheme 3. Reagents: (a) **8a**, $Li(ClO_4)$, Et_2O; (b) CF_3CO_2H, CH_2Cl_2; (c) **11a** or, **11b**, Et_3N, CH_2Cl_2; (d) *N*-methylmorpholine, isobutylchloroformate, **8b-d**, THF; (e) CF_3CO_2H, CH_2Cl_2, then **11b**, Et_3N, CH_2Cl_2.

Inhibitors **3a–e** were first evaluated in enzyme inhibitory assay utilizing the protocol described by Toth and Marshall.[20] Compounds that showed potent enzymatic K_i values were then further evaluated in antiviral assay. The inhibitor structure and potency are shown in Table 1. As shown, incorporation of a stereochemically defined 3(*S*)-tetrahydrofuran ring as the P2-ligand provided inhibitor **3a**, which displayed an enzyme inhibitory potency of 0.2 nM and antiviral IC_{50} value of 20 nM. The corresponding derivative **14a** with a hydroxyethylamine isostere exhibited over 400-fold reduction in enzyme inhibitory activity. Introduction of a stereochemically defined bis-THF as the P2-ligand, resulted in inhibitor **3b**, which displayed over 40-fold potency enhancement with respect to **3a**. Inhibitor **3b** displayed a K_i of 5.2 pM in the enzyme inhibitory assay. Furthermore, compound **3b** has shown an impressive antiviral activity with an IC_{50} value of 9 nM. Inhibitor **14b** with hydroxyethylamine isostere is significantly less potent than the corresponding norstatine-derived inhibitor **3b**. Inhibitor **3c** with a (3a*S*, 5*R*, 6a*R*)-5-hydroxy-hexahydrocyclopenta[*b*]furan as the P2-ligand has displayed excellent inhibitory activity, and particularly, antiviral activity, showing an IC_{50} value of 13 nM. Other structure-based designed ligands in inhibitors **3d** and **3e** have shown subnanomolar enzyme inhibitory activity. However, inhibitor **3b** with a bis-THF ligand has shown the most impressive activity.

To obtain molecular insight into the possible ligand-binding site interactions, we have created energy-minimized models of a number of inhibitors based upon protein-ligand X-ray structure of KNI-764 (**2**).[21] An overlayed model of **3b** with the X-ray structure of **2**-bound HIV-1 protease is shown in Figure 2. This model for inhibitor **3b** was created from the X-ray crystal structure of KNI-764 (**2**)-bound HIV-1 protease (KNI-764, pdb code 1MSM[21]) and the X-ray crystal structure of darunavir (pdb code 2IEN[22]), by combining the P2-end of the darunavir structure with the P2′-end of the KNI-764 structure, followed by 1000 cycles of energy minimization. It appears that both oxygens of the bis-THF ligand are suitably located to form hydrogen bonds with the backbone atoms of Asp-29 and Asp-30 NH's, similar to darunavir-bound HIV-1 protease.[10] Furthermore, the KNI-764-X-ray structure-derived model of **3b** suggested that the incorporation of appropriate substituents on the phenyl ring could interact with Asp-29′ and Asp-30′ in the S2′-subsite. In particular, it appears that a 4-hydroxymethyl substituent on the P2′-phenyl ring could conceivably interact with backbone Asp-30′ NH in the S2′-subsite. Other substituents such as a methoxy group or an amine functionality also appears to be within proximity to Asp-29′ and Asp-30′ backbone NHs. Based upon these speculations, we incorporated *p*-MeO, *p*-NH_2 and *p*-CH_2OH substituents on the P2′-phenyl ring of inhibitor **3b**. As shown in Table 1, neither *p*-MeO nor *p*-NH_2 groups improved enzyme inhibitory potency compared to inhibitor **3b**. Of particular note, compound **16a**, displayed a good antiviral potency, possibly suggesting a better penetration through the cell membrane. Inhibitor **16c** with a hydroxymethyl substituent showed sub-nanomolar enzyme inhibitory potency but its antiviral activity was moderate compared to unsubstituted derivative **3b**. As it turned out, inhibitor **3b** is the most potent inhibitor in the series. We subsequently examined its activity against a clinical wild-type X_4-HIV-1 isolate (HIV-$1_{ERS104pre}$) along with various multidrug-resistant clinical X_4- and R_5- HIV-1 isolates using PBMCs as target cells.[5b] As can be seen in Table 2, the potency of **3b** against HIV-$1_{ER104pre}$ (IC_{50} = 31 nM) was comparable to the FDA approved PI, amprenavir with an IC_{50} value of 45 nM. Darunavir and atazanavir on the other hand, are significantly more potent with IC_{50} values of 5 nM and 3 nM, respectively. Inhibitor **3b**, while less potent than darunavir, maintained 5-fold or better potency over amprenavir against HIV-$1_{MDR/C}$, HIV-$1_{MDR/G}$, HIV-$1_{MDR/TM}$ and HIV-$1_{MDR/MM}$. It maintained over a 2-fold potency against HIV-$1_{MDR/JSL}$. In fact, inhibitor **3b** maintained comparable potency to atazanavir against all

Table 1
Enzymatic inhibitory and antiviral activity of allophenylnorstatine-derived inhibitors

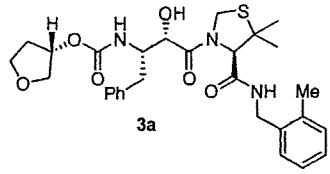
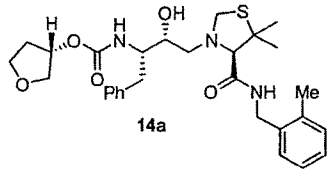
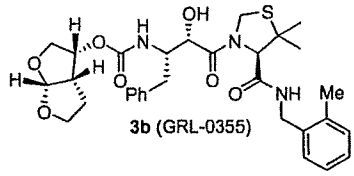
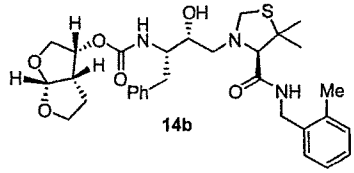
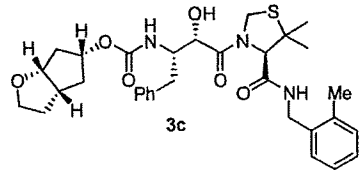
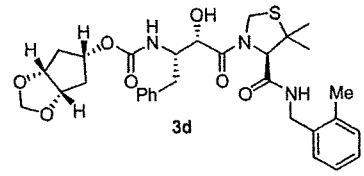
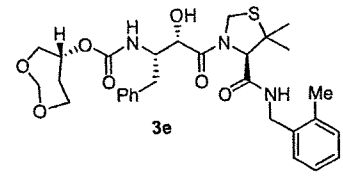
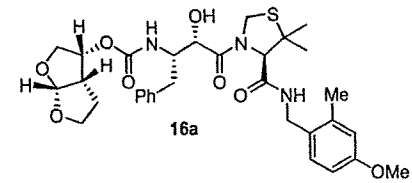
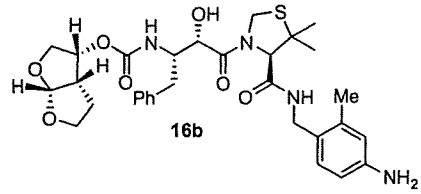
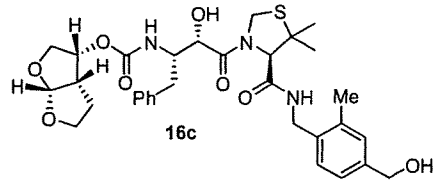
Entry	Inhibitor	K_i (nM)	IC_{50}[a,b] (μM)
1	 **3a**	0.21	0.02
2	 **14a**	86.2	nt
3	 **3b (GRL-0355)**	0.0052	0.009
4	 **14b**	2.6	nt
5	 **3c**	0.29	0.013
6	 **3d**	0.65	nt
7	 **3e**	0.78	nt
8	 **16a**	2.03	0.051

Table 1 (*continued*)

Entry	Inhibitor	K_i (nM)	IC_{50}[a,b] (μM)
9	 16b	1.01	0.53
10	 16c	0.31	0.23

[a] Values are means of at least three experiments.
[b] Human lymphoid (MT-2) cells were exposed to 100 $TCID_{50}$ values of HIV-1_{LAI} and cultured in the presence of each PI, and IC_{50} values were determined using MTT assay. Darunavir exhibited K_i = 16 pM, IC_{50} = 1.6 nM.

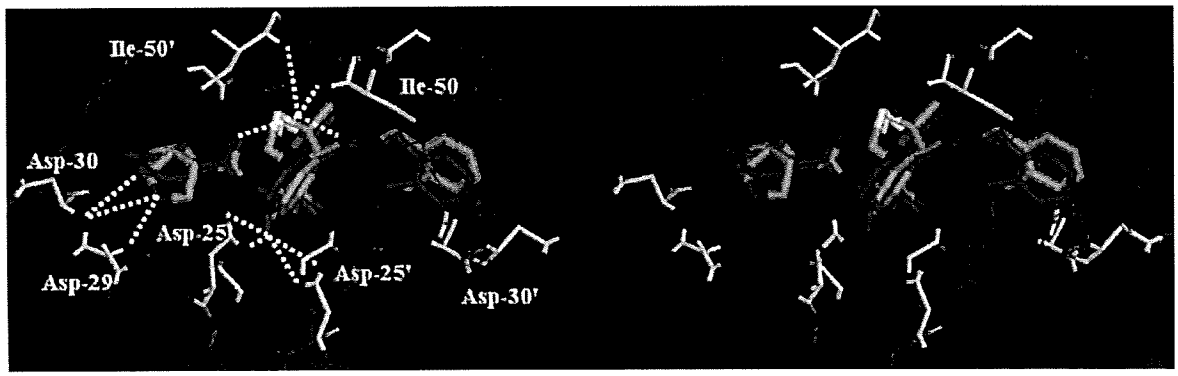


Figure 2. Structure of inhibitor **3b**, modeled into the active site of HIV-1 protease, superimposed on the X-ray crystal structure of KNI-764. Inhibitor **3b** carbons are shown in green and KNI-764 carbons are shown in magenta.

Table 2
Antiviral activity of **3b** (GRL-0355) against multidrug-resistant clinical isolates in PHA-PBMs.

Virus	IC_{50} (μM)			
	3b (GRL-0355)	APV	ATV	DRV
HIV-$1_{ERS104pre}$ (wild-type: X4)	0.031 ± 0.002	0.045 ± 0.014	0.003 ± 0.003	0.005 ± 0.001
HIV-$1_{MDR/C}$ (X4)	0.061 ± 0.005 (2)	0.346 ± 0.071 (8)	0.045 ± 0.026 (15)	0.010 ± 0.006 (2)
HIV-$1_{MDR/G}$ (X4)	0.029 ± 0.002 (1)	0.392 ± 0.037 (9)	0.029 ± 0.020 (10)	0.019 ± 0.005 (4)
HIV-$1_{MDR/TM}$ (X4)	0.064 ± 0.032 (2)	0.406 ± 0.082 (9)	0.047 ± 0.009 (16)	0.007 ± 0.003 (1)
HIV-$1_{MDR/MM}$ (R5)	0.042 ± 0.001 (1)	0.313 ± 0.022 (7)	0.040 ± 0.002 (13)	0.027 ± 0.008 (5)
HIV-$1_{MDR/JSL}$ (R5)	0.235 ± 0.032 (8)	0.531 ± 0.069 (12)	0.635 ± 0.065 (212)	0.028 ± 0.008 (6)

The amino acid substitutions identified in the protease-encoding region of HIV-$1_{ERS104pre}$, HIV-1_C, HIV-1_G, HIV-1_{MM}, HIV-1_{JSL} compared to the consensus type B sequence cited from the Los Alamos database include L63P; L10I, I15V, K20R, L24I, M36I, M46L, I54V, I62V, L63P, K70Q,V82A, L89M; L10I, V11I, T12E, I15V, L19I, R41K, M46L, L63P, A71T, V82A, L90M; L10I, K14R, R41K, M46L, I54V, L63P, A71V, V82A, L90M; L10I, K43T, M46L, I54V, L63P, A71V, V82A, L90M, Q92K; and L10I, L24I, I33F, E35D, M36I, N37S, M46L, I54V, R57K, I62V, L63P, A71V, G73S, V82A, respectively. HIV-$1_{ERS104pre}$ served as a source of wild-type HIV-1. The IC_{50} values were determined by using PHA-PBMs as target cells and the inhibition of p24 Gag protein production by each drug was used as an endpoint. The numbers in parentheses represent the fold changes of IC_{50} values for each isolate compared to the IC_{50} values for wild-type HIV-$1_{ERS104pre}$. All assays were conducted in duplicate, and the data shown represent mean values (±1 standard deviations) derived from the results of two or three independent experiments. Amprenavir = APV; Atazanavir = ATV; Darunavir = DRV.

multidrug-resistant clinical isolates tested. The reason for its impressive potency against multidrug-resistant clinical isolates is possibly due to its ability to make extensive hydrogen-bonds with the protease backbone in the S2 subsite and its ability to fill in the hydrophobic pockets in the S1′–S2′ subsites effectively.

In conclusion, incorporation of stereochemically defined and conformationally constrained cyclic ethers into the allophenylnorstatine resulted in a series of potent protease inhibitors. The promising inhibitors **3b** and **3c** are currently being subjected to further in-depth biological studies. Design and synthesis of new

classes of inhibitors based upon above molecular insight are currently ongoing in our laboratories.

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9. (a) FDA approved Darunavir on June 23, 2006: FDA approved new HIV treatment for patients who do not respond to existing drugs. Please see: http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2006/ucm108676.htm (b) On October 21, 2008, FDA granted traditional approval to Prezista (darunavir), co-administered with ritonavir and with other antiretroviral agents, for the treatment of HIV-1 infection in treatment-experienced adult patients. In addition to the traditional approval, a new dosing regimen for treatment-naïve patients was approved.
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Design of HIV-1 Protease Inhibitors with Pyrrolidinones and Oxazolidinones as Novel P1′-Ligands To Enhance Backbone-Binding Interactions with Protease: Synthesis, Biological Evaluation, and Protein–Ligand X-ray Studies[∞]

Arun K. Ghosh,*[,†] Sofiya Leshchenko-Yashchuk,[†] David D. Anderson,[†] Abigail Baldridge,[†] Marcus Noetzel,[†] Heather B. Miller,[†] Yunfeng Tie,[‡] Yuan-Fang Wang,[‡] Yasuhiro Koh,[⊥] Irene T. Weber,[‡] and Hiroaki Mitsuya[∥,⊥]

Departments of Chemistry and Medicinal Chemistry, Purdue University, West Lafayette, Indiana 47907, Department of Biology, Molecular Basis of Disease, Georgia State University, Atlanta, Georgia 30303, Departments of Hematology and Infectious Diseases, Kumamoto University School of Medicine, Kumamoto 860-8556, Japan, and Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, Bethesda, Maryland 20892

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Structure-based design, synthesis, and biological evaluation of a series of novel HIV-1 protease inhibitors are described. In an effort to enhance interactions with protease backbone atoms, we have incorporated stereochemically defined methyl-2-pyrrolidinone and methyl oxazolidinone as the P1′-ligands. These ligands are designed to interact with Gly-27′ carbonyl and Arg-8 side chain in the S1′-subsite of the HIV protease. We have investigated the potential of these ligands in combination with our previously developed bis-tetrahydrofuran (bis-THF) and cyclopentanyltetrahydrofuran (Cp-THF) as the P2-ligands. Inhibitor **19b** with a (*R*)-aminomethyl-2-pyrrolidinone and a Cp-THF was shown to be the most potent compound. This inhibitor maintained near full potency against multi-PI-resistant clinical HIV-1 variants. A high resolution protein–ligand X-ray crystal structure of **19b**-bound HIV-1 protease revealed that the P1′-pyrrolidinone heterocycle and the P2-Cp-ligand are involved in several critical interactions with the backbone atoms in the S1′ and S2 subsites of HIV-1 protease.

Introduction

Advances in the treatment of HIV[a]/AIDS with HIV-1 protease inhibitors in combination with reverse transcriptase inhibitors have been widely documented.[1] The combination therapy, also known as highly active antiretroviral therapy (HAART), blocks critical viral replication at two different stages of the replication cycle.[2] The HAART regimens have resulted in dramatic reduction of blood plasma viral load levels, increased CD_4^+ lymphocyte counts, and improved life expectancy and significantly reduced HIV/AIDS-related mortality in the developed world.[3] Despite these important advances, effective long-term suppression of HIV infection with HAART regimens is a complex issue in medicine for a number of reasons. These include drug side effects, poor penetration into protected HIV reservoir sites, poor oral bioavailability, and interactions between drugs.[4] Perhaps one of the most daunting problems in future management of HIV is the emergence of drug-resistant HIV-1 variants and the transmission of these viral strains.[5,6] Thus, development of antiretroviral therapy with broad-spectrum activity and minimal drug side effects is critical for an effective management of current and future HIV/AIDS treatment. We recently reported the design and development of a number of exceedingly potent nonpeptidic HIV-1 protease inhibitors (PIs) **1**–**3** (Figure 1).[7–9] One of those PIs is darunavir (**1**, TMC-114), which was approved by the FDA in 2006 for the treatment of HIV/AIDS patients who are harboring drug-resistant HIV and do not respond to other therapies.[10] More recently, darunavir has received full approval for all HIV/AIDS patients.[11]

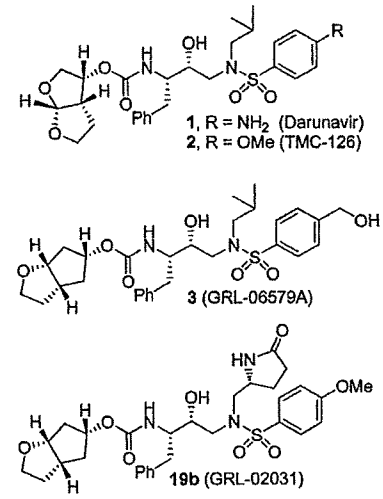

Figure 1. Structures of inhibitors **1**–**3** and **19b**.

To combat drug resistance, our structure-based design strategies are to maximize the protease active-site interactions with the inhibitor and particularly to promote extensive hydrogen bonding with the protein backbone atoms.[12] It is evident that active site backbone conformation of mutant proteases is only minimally distorted compared to that of the wild-type HIV-1 protease.[13,14] Therefore, the "backbone binding" strategy may be important to combat drug resistance.[12] Using high resolution protein–ligand X-ray structures of **1**- and **3**-bound HIV-1

[∞] The PDB accession code for **19b**-bound HIV-1 protease X-ray structure is 3H5B.

* To whom correspondence should be addressed. Phone: (765) 494-5323. Fax: (765) 496-1612. E-mail: akghosh@purdue.edu.

[†] Purdue University.

[‡] Georgia State University.

[⊥] Kumamoto University School of Medicine.

[∥] National Cancer Institute.

[a] Abbreviations: HIV, human immunodeficiency virus; bis-THF, bis-tetrahydrofuran; Cp-THF, cyclopentanyltetrahydrofuran; PI, protease inhibitor; HAART, highly active antiretroviral therapy; APV, amprenavir; DRV, darunavir; SQV, saquinavir; IDV, indinavir; LPV, lopinavir; RTV, ritonavir.

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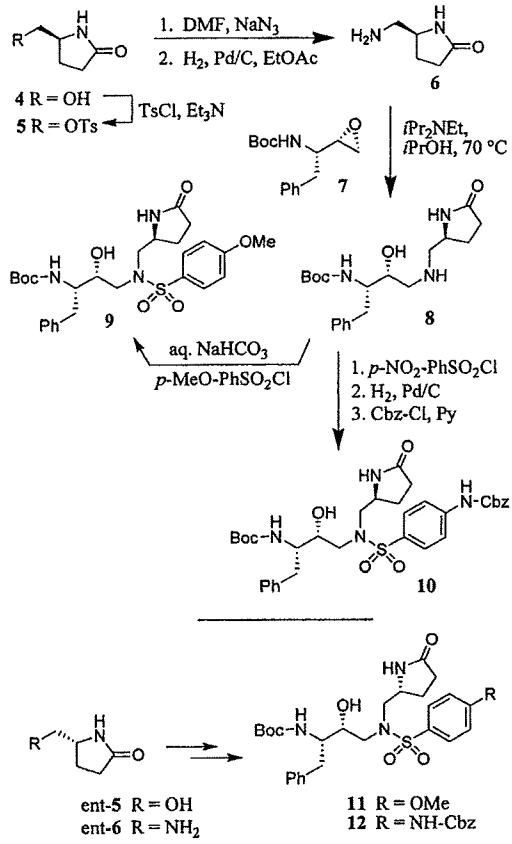
Scheme 1. Synthesis of Lactam Containing Sulfonamide Isosteres

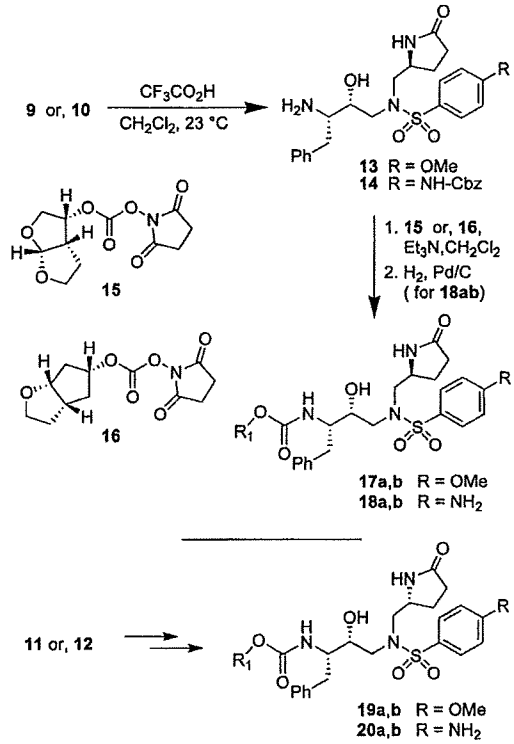


protease, we have shown that these PIs were engaged in extensive hydrogen bonding interactions with the backbone atoms throughout the active site cavity from the S2 to S2′ regions.[9,15] To further enhance "backbone binding" interactions, we became interested in designing an appropriately functionalized P1′-ligand that could interact with the backbone atoms, particularly with the Gly-27′ and Arg-8 in the S1′-subsite. This enhancement of "backbone binding" interaction may lead to inhibitors with improved drug-resistance profiles. Herein, we report the design, synthesis, and biological evaluation of a series of potent HIV-1 protease inhibitors that incorporated structure-based designed stereochemically defined lactam and oxazolidinone derivatives as the P1′-ligands in combination with the bis-THF or Cp-THF as the P2-ligands. Inhibitor **19b** incorporating a (*R*)-5-aminomethyl-2-pyrrolidinone as the P1′-ligand and Cp-THF as the P2-ligand is the most potent PI in the series. Interestingly, this PI has retained full potency against a range of multidrug-resistant HIV-1 variants. The protein−ligand X-ray structure of **19b**-bound HIV-1 protease revealed important molecular insight into the ligand-binding site interactions.

Chemistry

The optically active synthesis of the requisite 5-aminomethyl-2-pyrrolidinone for P1-ligands and their conversion to respective sulfonamide isostere are shown in Scheme 1. Commercially available 5-(*S*)-hydroxymethyl-2-pyrrolidinone **4** was reacted with tosyl chloride and triethylamine to provide tosylate **5**. Displacement of the tosylate with sodium azide in DMF at 55 °C for 9 h provided the azide derivative in 92% yield over two

Scheme 2. Synthesis of Lactam Containing PIs



steps. Catalytic hydrogenation of the azide over 10% Pd−C in ethyl acetate afforded optically active amine **6** in quantitative yield. 5-(*R*)-Hydroxymethyl-2-pyrrolidinone (*ent*-**5**) was similarly converted to optically active amine *ent*-**6** in comparable yield. Amine **6** was reacted with commercially available epoxide **7** in the presence of *i*-Pr$_2$NEt (DIPEA) in 2-propanol at 70 °C for 36 h to provide epoxide-opened product **8** in 85% yield.[16] Amine **8** was converted to *p*-methoxybenezenesulfonamide derivative **9** by reaction with *p*-methoxybenzenesulfonyl chloride in the presence of aqueous NaHCO$_3$ in quantitative yield. Treatment of amine **8** with *p*-nitrobenzenesulfonyl chloride afforded the corresponding nitrosulfonamide. Catalytic hydrogenation over 10% Pd−C gave the corresponding aniline derivative, which was reacted with benzyl chloroformate in the presence of pyridine to furnish Cbz-derivative **10** in 63% yield for three steps. Enantiomeric amine (*ent*-**6**) was converted to the respective methoxy and Cbz-derived **11** and **12** by analogous procedures.

The synthesis of various PIs incorporating methylpyrrolidinones as the P1′-ligand is shown in Scheme 2. Exposure of Boc-derivatives **9** and **10** to 30% CF_3CO_2H in CH_2Cl_2 at 23 °C for 40 min resulted in the respective amines **13** and **14**. Alkoxycarbonylation of amine **13** with activated mixed carbonates **15**[16] and **16**[9] in the presence of Et_3N in CH_2Cl_2 furnished inhibitors **17a** and **17b** in 98% and 87% yields, respectively.[17] Alkoxycarbonylation of amine **14** with activated carbonates **15** and **16** afforded the corresponding Cbz-protected urethanes. Removal of the Cbz-group by catalytic hydrogenation over 10% Pd−C in ethyl acetate provided inhibitor **18a** and **18b** in 58% and 62% yields, respectively. Sulfonamide derivatives **11** and **12** containing enantiomeric P1′-ligands were converted to inhibitors **19a,b** and **20a,b** by following analogous procedures.

The synthesis of sulfonamide isosteres incorporating methyloxazolidinone as the P1′-ligand is shown in Scheme 3. Optically