baseline in hip bone mineral density at week 48, percentage change from baseline in spine bone mineral density at week 48, change from baseline in serum creatinine at week 48, treatment-emergent proteinuria through week 48, proportion of participants with HIV-1 RNA lower than 20 per mL at week 48, change from baseline in CD4 cell count at week 48, percentage change from baseline in urine retinol binding protein to creatinine ratio at week 48, percentage change from baseline in urine β2-microglobulin to creatinine ratio at week 48, percentage change from baseline in urine protein to creatinine ratio at week 48, and percentage change from baseline in urine albumin to creatinine ratio. Safety was assessed by physical examinations, laboratory tests, 12-lead electrocardiogram, and recording of adverse events. The pharmacokinetics of tenofovir alafenamide and its metabolite, tenofovir was assessed through an intensive pharmacokinetic substudy done on a non-randomised subset of patients at week 4 or 8, which included plasma sampling for tenofovir alafenamide and tenofovir and peripheral blood mononuclear cell sampling for intracellular tenofovir-diphosphate

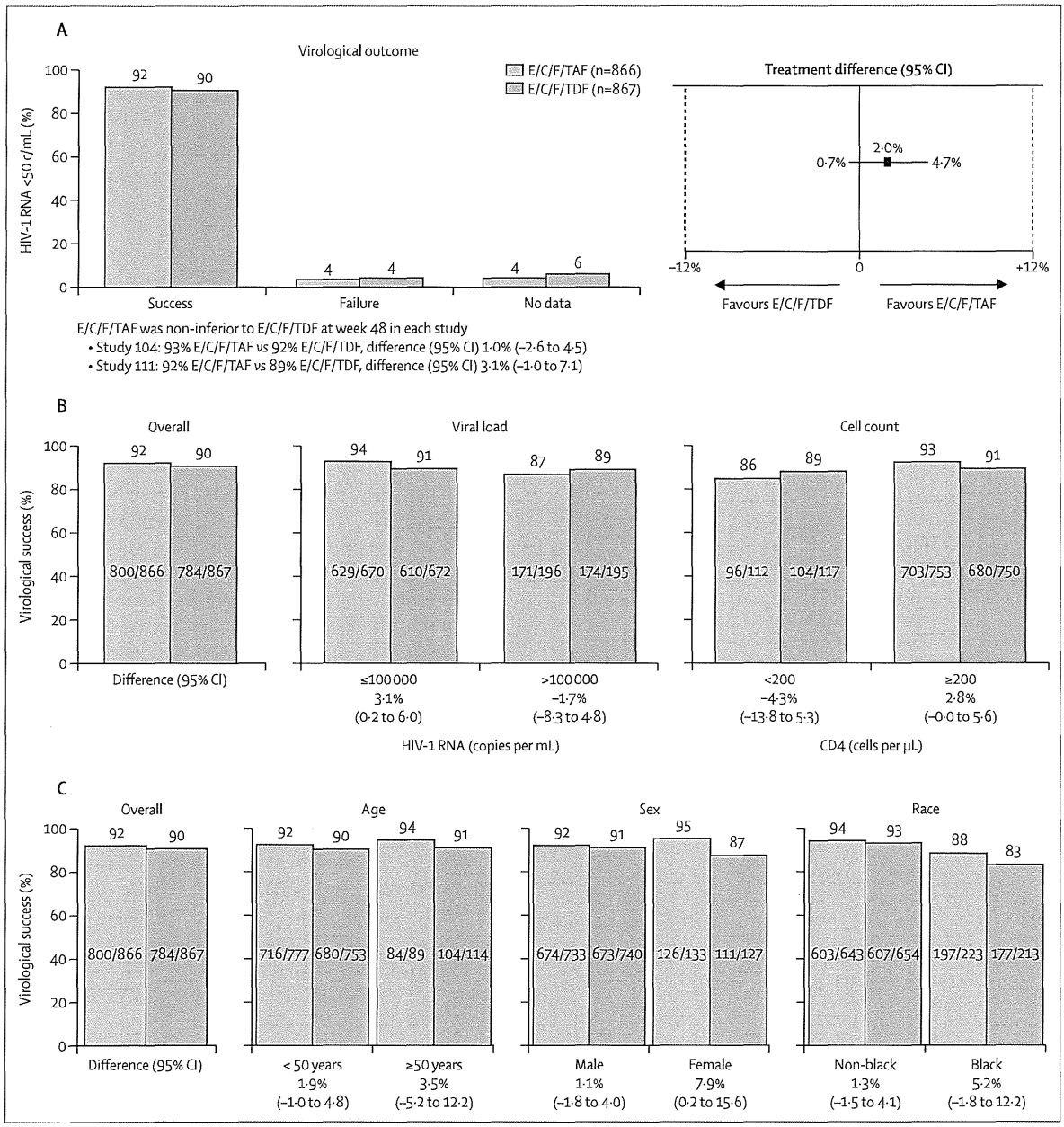

Figure 2: **(A) Primary endpoint: HIV-1 RNA <50 copies/mL at week 48, (B) efficacy in baseline HIV-RNA and CD4 subgroups, and (C) efficacy in selected subgroups**

E/C/F/TAF=elvitegravir, cobicistat, emtricitabine, tenofovir alafenamide. E/C/F/TDF=elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate.

concentrations. Bioanalytical analyses of drug concentrations of tenofovir alafenamide and tenofovir in plasma and tenofovir-diphosphate in peripheral blood mononuclear cells were done by QPS (Newark, DE, USA).

Role of the funding source

The funder designed the study, collected and analysed data, interpreted the results, and helped write the report. PES and DW are investigators who had access to the analyzed data, independently interpreted the results, and helped write the report. All authors had access to the analysed data and could assess the results and conclusions. Additional information or analyses were available to any author upon request. PES, DW, SM, MWF, and AKC made the decision to submit the report.

See Online for appendix

	Elvitegravir, cobicistat, emtricitabine, tenofovir alafenamide (n=866)	Elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate (n=867)
Diarrhoea	147 (17%)	164 (19%)
Nausea	132 (15%)	151 (17%)
Headache	124 (14%)	108 (13%)
Upper respiratory tract infection	99 (11%)	109 (13%)
Nasopharyngitis	78 (9%)	80 (9%)
Fatigue	71 (8%)	71 (8%)
Cough	67 (8%)	60 (7%)
Vomiting	62 (7%)	54 (6%)
Arthralgia	61 (7%)	39 (5%)
Back pain	60 (7%)	57 (7%)
Insomnia	57 (7%)	48 (6%)
Rash	55 (6%)	46 (5%)
Pyrexia	45 (5%)	41 (5%)
Dizziness	44 (5%)	37 (4%)

Data are n (%).

Table 2: **Common adverse events (all grades) in ≥5% of patients**

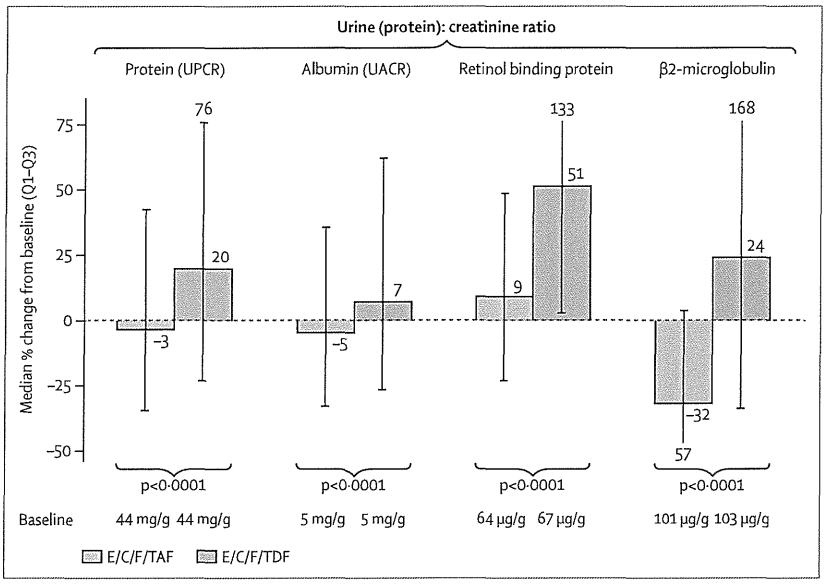

Figure 3: **Changes in quantitative proteinuria at week 48**
UPCR=urine protein to creatinine ratio. UACR=urine albumin to creatinine ratio. E/C/F/TAF=elvitegravir, cobicistat, emtricitabine, tenofovir alafenamide. E/C/F/TDF=elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate.

Results

2175 patients were screened for both studies, of whom 1744 were randomly assigned to receive treatment. 1733 received at least one dose of study drug; 866 received E/C/F/tenofovir alafenamide and 867 received E/C/F/tenofovir disoproxil fumarate (figure 1). Table 1 shows baseline characteristics of participants. E/C/F/tenofovir alafenamide was non-inferior to E/C/F/tenofovir disoproxil fumarate for the combined primary outcome (800 patients [92%] *vs* 784 patients [90%], adjusted difference 2·0%, 95% CI −0·7% to 4·7%) and for each study (figure 2). With a cutoff of fewer than 20 copies per mL, virological outcome at week 48 by FDA snapshot algorithm was 84·4% for the E/C/F/tenofovir alafenamide group and 84·0% for the E/C/F/tenofovir disoproxil fumarate group (difference in percentages 0·4%, 95% CI −3·0% to 3·8%, p=0·83). Viral suppression was high in both treatment groups for per-protocol analysis (781 [98%] of 801 for E/C/F/tenofovir alafenamide group and 763 [97%] of 789 patients for E/C/F/tenofovir disoproxil fumarate group, adjusted difference 0·8%, 95% CI −1·0% to 2·5%) and the other the secondary efficacy endpoints (appendix) and for various subgroups (figure 2). We noted significant differences in efficacy for those with fewer than 100 000 copies per mL baseline HIV-1 RNA (94% for E/C/F/tenofovir alafenamide *vs* 91% for tenofovir disoproxil fumarate, difference in percentage 3·1%, 95% CI 0·2–6·0) and for women (95% for tenofovir alafenamide and 87% for tenofovir disoproxil fumarate, difference in percentage 7·9%, 95% CI 0·2–15·6). The mean increases from baseline in CD4 cell counts were higher for the E/C/F/tenofovir alafenamide group through week 48 (observed data), as follows: E/C/F/tenofovir alafenamide 230 (SD 177·3) cells per mL; E/C/F/tenofovir disoproxil fumarate 211 (170·7) cells per mL; difference in LSM 19 cells per mL, 95% CI: 3 to 36 cells per mL; p=0·024.

We noted virological failure with resistance in seven (0·8%) of 866 patients in the E/C/F/tenofovir alafenamide group versus five (0·6%) of 867 patients in the E/C/F/tenofovir disoproxil fumarate group (appendix). Resistance mutation development was similar between treatment groups (appendix). All patients with emergent resistance developed the reverse transcriptase mutation, Met184Val/Ile. One patient in the E/C/F/tenofovir alafenamide group and in two patients in E/C/F/tenofovir disoproxil fumarate developed the Lys65Arg reverse transcriptase mutation. Eight of 12 patients (five in the E/C/F/tenofovir alafenamide group and three in the E/C/F/tenofovir disoproxil fumarate group) developed primary INSTI-R, all of which were genotypically

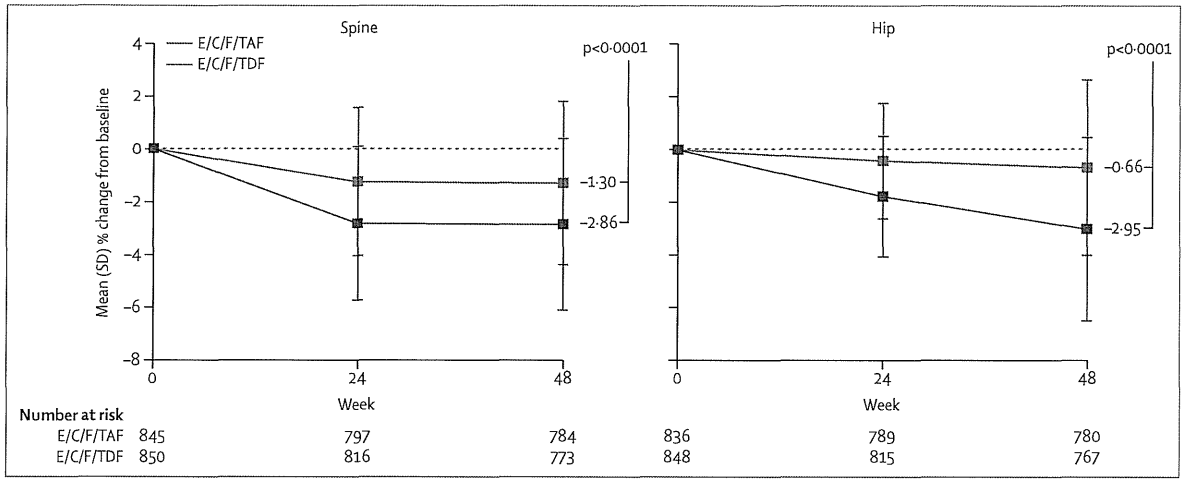

Figure 4: **Changes in spine and hip bone mineral density through week 48**
E/C/F/TAF=elvitegravir, cobicistat, emtricitabine, tenofovir alafenamide. E/C/F/TDF=elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate.

susceptible to dolutegravir. We did not record any novel tenofovir resistance mutations in any of the patients given E/C/F/tenofovir alafenamide.

36 participants in the tenofovir alafenamide group and 29 in the E/C/F/tenofovir disoproxil fumarate group participated in the intensive pharmacokinetic substudy; five of those enrolled were women. Of those, 21 patients who received E/C/F/tenofovir alafenamide and 14 who received E/C/F/tenofovir disoproxil fumarate participated in the PBMC substudy. Plasma tenofovir exposure (AUC_{tau}) after administration of E/C/F/tenofovir alafenamide was 91% lower than tenofovir exposure achieved with administration of E/C/F/tenofovir disoproxil fumarate (appendix). The PBMC tenofovir-diphosphate AUC_{tau} was 4·1 times higher in participants receiving E/C/F/tenofovir alafenamide than in those receiving E/C/F/tenofovir disoproxil fumarate.

Both treatments were well tolerated, with most adverse events reported as mild or moderate in severity (appendix). Adverse events leading to study drug discontinuation were uncommon: E/C/F/tenofovir alafenamide 8 (0·9%) and E/C/F/tenofovir disoproxil fumarate 13 (1·5%); adverse events leading to study drug discontinuation deemed related to study drugs were similar: E/C/F/tenofovir alafenamide 7 (0·8%) and E/C/F/tenofovir disoproxil fumarate 11 (1·3%). Table 2 shows adverse events reported by 5% or more of patients in either treatment group. Roughly 20% of patients in either group had a grade 3 or 4 laboratory abnormality (appendix). Five patients died (E/C/F/tenofovir alafenamide two patients, embolic stroke, and alcohol poisoning; E/C/F/tenofovir disoproxil fumarate three patients, cardiac arrest, multiple drug overdose, and myocardial infarction). None of the serious adverse events that resulted in the deaths were deemed related to study drugs by the investigator.

There were no discontinuations due to renal adverse events in the E/C/F/tenofovir alafenamide group. Four patients in the E/C/F/tenofovir disoproxil fumarate group discontinued study drug because of renal adverse events. Three patients had decreased glomerular filtration rate and another patient developed nephropathy, all believed to be related to study drug. We noted no cases of proximal renal tubulopathy (including Fanconi syndrome) in either treatment group. We recorded decreases from baseline in mean estimated glomerular filtration rate by week 2 with no further change thereafter. We noted significantly smaller decreases in estimated glomerular filtration rate in the E/C/F/tenofovir alafenamide group than in the E/C/F/tenofovir disoproxil fumarate group (appendix). At 48 weeks, quantitative proteinuria (total urinary protein, albumin, retinol binding protein and β2-microglobulin to urine creatinine ratios) increased from baseline in the E/C/F/tenofovir disoproxil fumarate group; reductions or significantly smaller increases in these urinary proteins were noted in the E/C/F/tenofovir alafenamide group (figure 3). Other measures of proximal renal tubular function (fractional excretion of phosphate and uric acid) showed significantly less change in patients receiving E/C/F/tenofovir alafenamide compared with the E/C/F/tenofovir disoproxil fumarate group (data not shown).

Fractures were uncommon in both treatment groups (one in the E/C/F/tenofovir alafenamide group and seven in the E/C/F/tenofovir disoproxil fumarate group), and deemed by the investigator to be the result of trauma and unrelated to the study drugs; none resulted in permanent discontinuation of study drugs. Patients in the E/C/F/tenofovir alafenamide group had significantly less reduction in bone mineral density than those in the E/C/F/tenofovir disoproxil fumarate group through 48 weeks (figure 4). Decrease in bone mineral density was significantly lower in the E/C/F/tenofovir alafenamide group for both lumbar spine (mean –1·30% [SD 3·08] *vs* –2·86 [3·25]; p<0·0001) and total hip (–0·66 [3·26] *vs*

−2·95 [3·41], p<0·0001; figure 3). Roughly one-third as many patients in the E/C/F/tenofovir alafenamide had more than 3% bone loss at the hip (E/C/F/tenofovir alafenamide 131/780 [16·8%]; E/C/F/tenofovir disoproxil fumarate 384/767 [50·1%]), and about half as many patients in the E/C/F/tenofovir alafenamide group had more than 3% bone loss at the spine (E/C/F/tenofovir alafenamide 208/784 [26·5%]; E/C/F/tenofovir disoproxil fumarate 354/773 [45·8%]; appendix).

We recorded greater increases in the fasting lipid parameters total cholesterol, direct low-density lipoprotein, high-density lipoprotein, and triglycerides, but identical changes in total cholesterol to high-density lipoprotein ratio, in patients given E/C/F/tenofovir alafenamide compared with those given E/C/F/tenofovir disoproxil fumarate at week 48 (appendix). 31 (3·6%) of 866 of patients given E/C/F/tenofovir alafenamide and 25 (2·9%) of 867 of participants given E/C/F/tenofovir disoproxil fumarate started lipid-lowering drugs (p=0·42).

Discussion

In these two randomised phase 3 clinical trials, we show that the novel tenofovir prodrug, tenofovir alafenamide achieved a high rate of virological suppression when given as part of a coformulated tablet that included emtricitabine, elvitegravir, and cobicistat. The response was non-inferior to the control group, which consisted of the approved single tablet regimen of elvitegravir, cobicistat, emtricitabine, and tenofovir disoproxil fumarate. The results were mostly non-inferior between the two groups irrespective of baseline demographic or clinical characteristics, although outcome was significantly better for tenofovir alafenamide in women and in those who had baseline viral loads lower than 100 000 copies per mL. CD4 cell count increases at week 48 were significantly greater in the tenofovir alafenamide group than in the tenofovir disoproxil fumarate group. Both coformulations were well tolerated and discontinuations for drug-related adverse events were rare in both study groups.

The high rates of successful treatment (92% in the E/C/F/tenofovir alafenamide group and and 90% in the E/C/F/tenofovir disoproxil fumarate group) marks the first time that both treatment groups in a fully powered comparative clinical trial exceeded the 90% threshold for virological suppression (plasma HIV-1 RNA <50 copies per mL) using the snapshot analysis at 48 weeks. Virological failure was infrequent in both groups, arising in 3·6% of paitents given E/C/F/tenofovir alafenamide and 4·0% of patients given E/C/F/tenofovir disoproxil fumarate. Although resistance to study treatment was not recorded in the E/C/F/tenofovir alafenamide group of the phase 2 trial, in these two larger studies a small percentage of patients (<1% in both groups) did develop drug resistance to some of the treatments, most commonly the nucleoside reverse transcriptase inhibitor mutation Met184Val selected by emtricitabine.

The high virological suppression recorded in these studies reinforces the extraordinary effectiveness of contemporary HIV treatment. With prolonged virological suppression, improved clinical outcomes, and longer survival,[16] patients will potentially be exposed to antiretroviral agents for decades. As a result, maximising the safety of drugs used for HIV remains a high priority, and long-term renal and bone safety are important considerations. Although generally well tolerated as initial treatment, findings of several studies have shown an association between tenofovir disoproxil fumarate and kidney disease. A meta-analysis[17] of prospective studies of HIV treatments showed a significantly greater loss of kidney function in patients receiving tenofovir disoproxil fumarate-based treatments versus non-tenofovir disoproxil fumarate regimens; a higher risk of acute renal failure was also noted. In a large cohort analysis from the Veterans Health Administration, tenofovir exposure was independently associated with proteinuria, rapid estimated glomerular filtration rate decrease, and the development estimated glomerular filtration rate less than 60 mL per min.[18] Commonly cited risk factors for tenofovir disoproxil fumarate-related nephrotoxicity include older age, co-administration with ritonavir-boosted protease inhibitors (which further increase tenofovir plasma levels), and other comorbidities associated with renal disease.[4]

There is an increased prevalence of osteopenia and osteoporosis in patients with HIV infection.[19] The cause is multifactorial, with both HIV disease-specific and treatment-specific effects observed. Generally, initiation of antiretroviral therapy leads to a reduction in bone mineral density,[20] possibly related to immune reconstitution.[21] This effect is larger in patients receiving tenofovir disoproxil fumarate and certain protease-inhibitor based regimens.[5,22] The mechanism of tenofovir disoproxil fumarate-related reductions in bone mineral density is poorly understood but might include osteomalacia as a result of increased urinary phosphate loss.[23]

In these two clinical trials, protocol-specified renal and bone endpoints confirmed the favourable safety and tolerability profile of tenofovir alafenamide reported in earlier studies. Although no particpants had overt renal failure or clinically significant tubulopathy, patients given tenofovir alafenamide had smaller reductions in estimated glomerular filtration rate and more favourable changes in urine protein to creatinine and urine albumin to creatinine ratios. Specific markers of proximal renal tubular dysfunction, including urinary retinol binding protein, urinary β2-microglobulin, fractional excretion of uric acid, and fractional excretion of phosphate all significantly favoured the tenofovir alafenamide over the tenofovir disoproxil fumarate group, suggesting a lower potential for nephrotoxicity with tenofovir alafenamide than with tenofovir disoproxil fumarate.

The present studies represent the largest bone mineral density dataset in patients with HIV up to now. Treatment

with E/C/F/tenofovir alafenamide resulted in significantly smaller reductions in bone mineral density at both the hip and the lumbar spine at week 48. The magnitude of bone mineral density decline recorded in the tenofovir alafenamide group at the hip (0·7%) similar to that seen in randomised studies of treatment-naive patients on nucleoside or nucleotide-sparing regimens.[24,25] Furthermore, with a 3% threshold for the least significant change to account for the imprecision of repeat dual energy x-ray absorptiometry measures,[26] 27% of patients in the tenofovir alafenamide group versus 46% in the tenofovir disoproxil fumarate group exceeded this threshold at the spine, and 17% versus 50% at the hip.

Treatment with tenofovir disoproxil fumarate has consistently been associated with less increase in lipids compared with other regimens in treatment-naive patients. The independent effect of tenofovir on lipids was most clearly shown in a study that added tenofovir disoproxil fumarate to stable background treatment in virologically suppressed patients;[27] findings showed a significant reduction in total, LDL, and non-HDL cholesterol levels. In both the phase 2 comparative study of tenofovir alafenamide *vs* tenofovir disoproxil fumarate and the larger phase 3 studies presented here, increases in total, LDL, and HDL cholesterol, and triglycerides, were greater in the tenofovir alafenamide than the tenofovir disoproxil fumarate group. However, the difference in total cholesterol to HDL ratio at week 48 was not significantly different between treatment groups, and a small and similar proportion of participants (<4%) initiating lipid-modifying agents.

The net favourable effects on renal and bone parameters for tenofovir alafenamide almost certainly relates to the lower plasma levels of tenofovir recorded in those receiving tenofovir alafenamide instead of tenofovir disoproxil fumarate. In a pharmacokinetic substudy, plasma tenofovir exposure was 90% lower in the tenofovir alafenamide than in the tenofovir disoproxil fumarate group. Conversely, the intracellular concentration of the active metabolite, tenofovir diphosphate, was four times higher. The ability to achieve higher intracellular concentrations enables a markedly lower daily dose of tenofovir alafenamide (10 mg with ritonavir or cobicistat) versus tenofovir disoproxil fumarate (300 mg) while achieving a similar or greater antiviral effect.[11] This lower dose of tenofovir alafenamide will help with both a broader range of coformulations and reduce the cost of manufacturing of the compound, the latter an important consideration in resource-limited settings. In addition to the coformulation E/C/F/tenofovir alafenamide, tenofovir alafenamide is being studied in various fixed dose combinations for HIV (with emtricitabine, with rilpivirine and emtricitabine, and with darunavir, cobicistat, and emtricitabine), and as a single agent for hepatitis B virus.

Strengths of these two studies include the large overall sample size, the randomised blinded study design with one variable of tenofovir alafenamide verus tenofovir disoproxil fumarate, and protocol-specified renal and bone endpoints. Additionally, study sites were geographically diverse, as was the ethnic origin of the participants enrolled. Limitations include a low power to assess rare clinical safety events such as renal adverse events and fractures in patients with limited baseline risk factors for kidney and bone disease, a small proportion of study participants with advanced HIV disease, a small proportion of women participants, and the exclusion of patients with chronic hepatitis B virus infection. The efficacy of tenofovir alafenamide in the treatment of chronic hepatitis B monoinfection, as well as HIV and hepatitis B virus co-infection, is currently being studied. Additionally, a clinical trial of women (NCT01705574) will give substantially more information about the efficacy, tolerability, and pharmacokinetic parameters of tenofovir alafenamide in women with HIV. Importantly, the efficacy of tenofovir alafenamide alone, or in combination with emtricitabine, for prevention, such as pre-exposure prophylaxis, is unknown and currently being explored.

In summary, in these two randomised clinical trials, treatment with a coformulated tablet of E/C/F/tenofovir alafenamide provided non-inferior virological suppression to an already approved and guidelines-recommended tablet of E/C/F/tenofovir disoproxil fumarate. Compared with tenofovir disoproxil fumarate, the nucleotide reverse transcriptase inhibitor tenofovir alafenamide showed significantly more favourable effects on renal and bone parameters. All these effects were probably related to the markedly lower plasma concentrations of tenofovir reported with tenofovir alafenamide compared with tenofovir disoproxil fumarate. Although the long-term clinical significance of these findings is unknown, it is reasonable to expect that these results will translate into improved safety of tenofovir alafenamide-based antiretroviral therapy over years of treatment while maintaining a similarly high efficacy rate.

Contributors

PES and DW enrolled patients, and edited and approved the report. MY, FP, ED, MS, AP, MT, DP, JMM, SO, EK, BT, JA-V, GC enrolled patients, reviewed and interpreted analyses of data, and edited the draft report. JMC, AP, LZ, HC, HM, CC, AKC, MWF, and SM designed the study. AP, HC, HM, MWF, SM, and AKC oversaw data collection. JMC, LZ, HC, HM, CC did data analyses, which were reviewed and interpreted by AKC, MWF, and SM. The first draft was written by PES, HM, and MWF. The manuscript was edited by PES, DW, MTY, FP, ED, MS, AP, MT, DP, JMM, SO, EK, BT, JA-V, GC, JMC, LZ, HC, HM, CC, AKC, MWF, and SM.

Declaration of interests

PES has received research support from Bristol-Myers Squibb, Gilead Sciences, GlaxoSmithKline, and Merck Laboratories; consulting fees from AbbVie, Bristol-Myers Squibb, Gilead Sciences, GlaxoSmithKline, Merck Laboratories, and Janssen. DW has received research grant support from Merck and GlaxoSmithKline, and receives consulting fees from Janssen Therapeutics and Gilead Sciences. MTY has received consulting fees as a member of advisory boards for Gilead Sciences and Abbvie. FP has received research grant support from Gilead Sciences; and consulting fees as a member of advisory boards for Gilead Sciences, ViiV, MSD, and Abbvie. ED has received research grant support from Abbott Laboratories,

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MAJOR ARTICLE

High-Dose Oral Amoxicillin Plus Probenecid Is Highly Effective for Syphilis in Patients With HIV Infection

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Background. Intramuscular benzathine penicillin G (BPG) is widely used for the treatment of syphilis. However, BPG is not available in some countries. This study examined the effectiveness and safety of high-dose oral amoxicillin plus probenecid for the treatment of syphilis in patients with human immunodeficiency virus type 1 (HIV-1).

Methods. This retrospective observational study included 286 HIV-infected male patients with syphilis (median age, 36 years; median CD4 count, 389 cells/μL) who were treated with oral amoxicillin 3 g plus probenecid. Syphilis was diagnosed by both serum rapid plasma reagin (RPR) titers ≥8 and positive *Treponema pallidum* hemagglutination test. Patients with neurosyphilis diagnosed by cerebrospinal fluid examination were excluded. Successful treatment was defined as a at least 4-fold decrement in RPR titer.

Results. The overall treatment efficacy was 95.5% (95% confidence interval [CI], 92.4%–97.7%; 273/286 patients), and efficacy for primary, secondary, early latent, late latent, and unknown duration syphilis was 93.8% (95% CI, 68.1%–99.8%; 15/16), 97.3% (95% CI, 92.9%–99.2%; 142/146), 100% (95% CI, 90.5%–100%; 37/37), 85.7% (95% CI, 58.6%–96.4%; 18/21), and 92.4% (95% CI, 81.9%–97.3%; 61/66), respectively. Treatment duration was mostly 14–16 days (49.7%) or 28–30 days (34.3%), with efficacy of 94.4% (134/142) and 95.9% (94/98), respectively; 96.3% of successfully treated patients achieved a ≥4-fold decrement in RPR titer within 12 months. Adverse events were noted in 28 (9.8%) patients, and 25 of these (89.3%) were successfully treated. Only 6% of patients underwent lumbar puncture.

Conclusions. The combination of oral amoxicillin 3 g plus probenecid was highly effective and tolerable for the treatment of syphilis in patients with HIV-1 infection.

Keywords. syphilis; amoxicillin; HIV; MSM; treatment.

Syphilis is a common sexually transmitted infection caused by *Treponema pallidum*, and in recent years, an increase in the number of cases with syphilis has been reported among men who have sex with men (MSM), particularly among those with human immunodeficiency virus type 1 (HIV-1) infection in resource-rich settings [1, 2]. A recent study of syphilis [3] in HIV-1–infected men, comprised mostly of MSM, showed that syphilis is associated with an increase in HIV-1 RNA load and with a small decrement in CD4 cell count, suggesting that syphilis may increase a risk of HIV-1 transmission. Furthermore, progression to neurosyphilis may be faster in HIV-1–infected patients than patients without HIV-1 infection [4–6], and HIV-1 infection may exacerbate the clinical symptoms of neurosyphilis [7]. For the above-mentioned reasons, early diagnosis and treatment of syphilis are important in HIV-infected patients.

One-shot intramuscular benzathine penicillin G (BPG) injection is widely used for the treatment of primary, secondary, and early latent syphilis based on its high efficacy and convenience [8–10]; however, intramuscular injection is painful and, for the treatment of late latent syphilis and syphilis of unknown duration,

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3 injections at 1-week intervals (3 hospital visits in total) is required. BPG is not available in some countries, including Japan [11, 12], and oral amoxicillin plus probenecid has been used as an alternative for BPG in the treatment of syphilis.

However, there is no evidence available on the efficacy and safety of oral amoxicillin plus probenecid for the treatment of syphilis; the only available evidence is from pharmacokinetic studies published in the 1970s–1980s [13, 14].

We investigated the efficacy and safety of high-dose oral amoxicillin (3 g) plus probenecid for the treatment of syphilis (excluding neurosyphilis) in patients with HIV-1 infection in an observational setting.

METHODS

Patients and Study Design

We conducted a retrospective cohort study of HIV-1–infected patients with syphilis to investigate the efficacy and safety of oral amoxicillin plus probenecid for the treatment of syphilis at the AIDS Clinical Center, National Center for Global Health and Medicine (NCGM), Tokyo, Japan. The enrollment criteria were HIV-1–infected patients who were diagnosed with syphilis and started treatment with 3 g oral amoxicillin plus probenecid at our center between January 2000 and June 2014. We included all patients treated with the combination of 3 g amoxicillin and probenecid, irrespective of the dose of the latter. The diagnosis of syphilis was based on both serum rapid plasma regain (RPR) titers ≥8 and positive *T. pallidum* hemagglutination (TPHA) result [15]. The following exclusion criteria were applied: (1) lack of follow-up tests, (2) patients with neurosyphilis diagnosed based on the findings of cerebrospinal fluid (CSF) [16], or ocular or auditory syphilis, (3) patients who started treatment with antibiotics other than 3 g amoxicillin plus probenecid, (4) patients with clinical symptoms compatible with primary or secondary syphilis but RPR titers <8, (5) patients suspected of reinfection after initiation of syphilis treatment (≥4-fold rise in RPR titer after 4-fold decrement with or without symptoms) [15, 17].

Data Collection

Data on the following parameters were collected at the time of treatment of syphilis: stage of syphilis infection, age, sex, race, route of HIV-1 infection, antiretroviral therapy (ART) use, history of syphilis treatment, status of hepatitis B and C infection, RPR titer, CD4 count, and HIV-1 RNA load. The stage of syphilis was classified into early syphilis (including primary, secondary, and early latent syphilis) and late syphilis (including late latent syphilis and syphilis of unknown duration) [8, 9]. Early latent syphilis was defined as asymptomatic syphilis that was confirmed to be infected within a year from the day of diagnosis, and late latent syphilis was defined as asymptomatic syphilis confirmed to be infected more than a year before diagnosis [8, 9]. Syphilis with unknown duration was defined as asymptomatic syphilis that could not be classified into either early latent or late latent syphilis [8, 9]. The methods of amoxicillin and probenecid administration, treatment duration, treatment efficacy, adverse events during treatment, and changes to doxycycline from amoxicillin were also collected from the medical records. Among the adverse events, the presence of fever and/or acute exacerbation of maculopapular skin rash within 24 hours of administration of amoxicillin was defined as Jarisch-Herxheimer reaction for syphilis and was not regarded as drug-related adverse events [18].

Successful treatment of syphilis was defined as at least 4-fold decrement in RPR titer within 24 months after initiation of treatment. Follow-up serum RPR titer was examined at the discretion of the attending physician. Because at our clinic, written informed consent was obtained from each patient to store serum samples at the first and subsequent visits [19], the RPR data based on stored samples were also used to supplement the RPR data from daily clinical practice order to determine treatment response and the syphilis staging. RPR Test "Sankoh" (EIDIA Co, Ltd, Tokyo, Japan) was used for the measurement of RPR titer both in clinical practice and with stored samples.

Patients visited our clinic at least once every 3 months for monitoring and prescription, as the prescription period under the Japanese healthcare system is limited to 3 months [20]. The study was approved by the Human Research Ethics Committee of NCGM, and was conducted according to the principles expressed in the Declaration of Helsinki.

Statistical Analysis

The study patients were classified according to the results of the combination treatment into the success group (patients with successful treatment of syphilis) and failure group (failure of treatment). The baseline characteristics were compared between the 2 groups using the Student *t* test or χ^2 test (Fisher exact test when appropriate) for continuous or categorical variables, respectively. To estimate risk factors for treatment failure, univariate logistic regression model was constructed. Because the number of patients in the failure group was small (n = 13) in this study, the multivariate analysis was not performed. Statistical significance was defined as 2-sided *P* values <.05. We used odds ratios (ORs) with 95% confidence intervals (CIs) for logistic regression analysis. All statistical analyses were performed with Stata 11 (Stata Corp, College Station, Texas).

RESULTS

During the study period, 403 HIV-1–infected patients were diagnosed with syphilis. One hundred seventeen patients were excluded from the study based on the inclusion and exclusion criteria set for this study (Figure 1), and data of the remaining

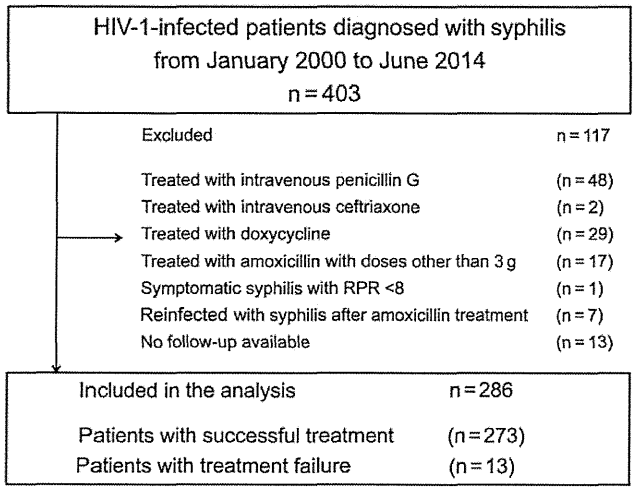

Figure 1. Patient enrollment process. Abbreviations: HIV-1, human immunodeficiency virus type 1; RPR, rapid plasma reagin.

286 patients were analyzed. All study patients were men with a median age of 36 years (interquartile range [IQR], 30–42 years). They were mostly Asians who were infected with HIV-1 through homosexual contact. The median CD4 count was 389 cells/μL (IQR, 276–538 cells/μL), ART had been started in 156 (54.5%) patients, and 170 patients (59.4%) had a history of syphilis treatment. Primary syphilis, secondary syphilis, early latent syphilis, late latent syphilis, and syphilis with unknown duration were diagnosed in 16 (5.6%), 146 (51.0%), 37 (12.9%), 21 (7.3%), and 66 (23.1%) patients, respectively. Furthermore, 199 (69.6%) patients were categorized with early syphilis, which included primary, secondary, and early latent syphilis, and 87 (30.4%) with late syphilis, which included late latent syphilis and syphilis of unknown duration.

Treatment with 3 g oral amoxicillin and probenecid decreased RPR titer by 4-fold and was thus regarded successful in 273 (95.5% [95% CI, 92.4%–97.7%]) patients (success

Table 1. Baseline Characteristics of the Study Patients

Characteristic	All Patients (N = 286)	Patients With Successful Treatment (n = 273)	Patients With Treatment Failure (n = 13)	*P* Value
Age, y[a]	36 (30–42)	36 (30–42)	30 (24–37)	.003
Male sex	286 (100)	273 (100)	13 (100)	
Asian race	281 (98.3)	268 (98.2)	13 (100)	
Route of HIV-1 transmission				
Homosexual	274 (95.8)	262 (96.0)	11 (84.6)	
Heterosexual	7 (2.4)	6 (2.2)	1 (7.7)	
Injection drug user	3 (1.0)	2 (0.7)	1 (7.7)	
Unknown	2 (0.7)	2 (0.7)	0 (0)	
ART use	156 (54.5)	150 (54.9)	6 (46.2)	.579
History of syphilis treatment	170 (59.4)	163 (59.7)	7 (53.8)	.775
Stage of syphilis				
Early syphilis	199 (69.6)	194 (71.1)	5 (38.5)	.025[b]
Primary	16 (5.6)	15 (5.5)	1 (7.7)	
Secondary	146 (51.0)	142 (52.0)	4 (30.8)	
Early latent	37 (12.9)	37 (13.6)	0 (0)	
Late syphilis	87 (30.4)	79 (28.9)	8 (61.5)	
Late latent	21 (7.3)	18 (6.6)	3 (23.1)	
Unknown duration	66 (23.1)	61 (22.3)	5 (38.5)	
Baseline RPR titer[a]	96 (32–128)	128 (32–128)	64 (32–128)	.510
CD4 count, cells/μL[a]	389 (276–538)	393 (285–542)	286 (180–369)	.003
HIV-1 load, $\log_{10}$ copies/mL[a]	2.06 (1.70–4.49)	1.91 (1.70–4.45)	4.26 (3.04–4.70)	.048
Hepatitis	35 (12.2)	35 (12.8)	0 (0)	
Positive HBsAg	30 (10.5)	30 (11.0)	0 (0)	
Positive HCV antibody	5 (1.7)	5 (1.8)	0 (0)	
Lumbar puncture performed	17 (5.9)	12 (4.4)	5 (38.5)	<.001

Data are presented as No. (%) unless otherwise specified.

Abbreviations: ART, antiretroviral therapy; HBsAg, hepatitis B surface antigen; HCV, hepatitis C; HIV-1, human immunodeficiency virus type 1; RPR, rapid plasma regain.

[a] Median (interquartile range).

[b] By 2 × 2 table for early syphilis vs late syphilis and by Fisher exact test for successful treatment vs treatment failure.

group). Among 13 patients with treatment failure (failure group), none showed any evidence of clinical failure. The baseline HIV-1 load was lower in the success group than the failure group, whereas the baseline CD4 count was higher in the success group. Lumbar puncture for the examination of CSF was performed before treatment initiation in only 17 (5.9%) patients, including 13 patients with late syphilis, and neurosyphilis was ruled out in all 17 patients based on negative TPHA in the CSF [16]. The treatment efficacy of primary, secondary, early latent, late latent, and unknown duration syphilis was 93.8% (95% CI, 68.1%–99.8%; 15/16 patients), 97.3% (95% CI, 92.9%–99.2%; 142/146), 100% (95% CI, 90.5%–100%; 37/37), 85.7% (95% CI, 58.6%–96.4%; 18/21), and 92.4% (95% CI, 81.9%–97.3%; 61/66), respectively (Table 1). Also, the treatment efficacy for early syphilis (including primary, secondary, and early latent syphilis) and late syphilis (including late latent syphilis and syphilis of unknown duration) was 97.5% (95% CI, 94.1%–99.2%) and 90.8% (95% CI, 82.6%–96.4%), respectively. Treatment duration was mostly 14–16 days (49.7%) or 28–30 days (34.3%), with 94.4% (134/142) and 95.9% (94/98) efficacy, respectively. Among patients with early syphilis, treatment efficacy did not differ between the 2-week treatment and 4-week treatment (105 of 107 [98.1%] vs 109 of 113 [96.5%]; $P = .49$ by Fisher exact test). However, among patients with late syphilis, treatment efficacy tended to be lower among patients treated for 2 weeks than those treated for 4 weeks, although the difference was not statistically significant (29 of 35 [82.9%] vs 34 of 36 [94.4%]; $P = .15$). The frequency of oral amoxicillin administration was mainly 3 times a day (80.8%) with 96.1% (222/231) efficacy. The dosage of probenecid was 0.75 g/day in the majority of patients (60.1%), 1.0 g/day (23.4%), and 1.5 g/day (13.3%), with success rate of 96.5% (166/172), 92.5% (62/67), and 94.7% (36/38), respectively (Table 2). For patients with successful treatment, the median time for the documentation of ≥4-fold decrement in RPR titer after treatment was 4 months (IQR, 3–6 months), and 96.3% of the success group achieved the ≥4-fold decrease in RPR titers within 12 months (Figure 2). The median number of follow-up RPRs for the success group and the failure group was 1 (IQR, 1–2) and 4 (IQR, 2–6), respectively, and follow-up RPRs were more frequently measured for the failure group than for the success group ($P < .001$).

Adverse events related to treatment of syphilis were recorded in 28 (9.8%) patients, with skin rash being the most common symptom (n = 21), followed by fever (n = 9), diarrhea (n = 2),

Table 2. Frequency of Amoxicillin Administration, Probenecid Dosing, Treatment Duration, and Variables Related to Adverse Events

Variable	All Patients (N = 286)	Early Syphilis (n = 199)	Late Syphilis (n = 87)	Successfully Treated Patients (n = 273)	Patients With Treatment Failure (n = 13)	*P* Value*
Frequency of amoxicillin administration						
4 times daily	44 (15.4)	26 (13.7)	18 (20.7)	40 (14.7)	4 (30.8)	
3 times daily	231 (80.8)	165 (82.9)	66 (75.9)	222 (81.3)	9 (69.2)	.241[a]
Twice daily	11 (3.8)	8 (4.0)	3 (3.5)	11 (4.0)	0 (0)	
Dose of probenecid, g, median (IQR)	0.75 (0.75–1.0)	0.75 (0.75–1.0)	0.75 (0.75–1.0)	0.75 (0.75–1.0)	1.0 (0.75–1.0)	.213
1.5 g/d	38 (13.3)	26 (13.7)	12 (13.8)	36 (13.2)	2 (15.4)	
1.0 g/d	67 (23.4)	47 (23.6)	20 (23.0)	62 (22.7)	5 (38.5)	
0.75 g/d	172 (60.1)	119 (59.8)	53 (60.9)	166 (60.8)	6 (46.2)	.693[b]
0.5 g/d	8 (2.8)	6 (3.0)	2 (2.3)	8 (2.9)	0 (0)	
0.25 g/d	1 (0.3)	1 (0.5)	0 (0)	1 (0.4)	0 (0)	
Treatment duration, d, median (IQR)	14 (14–28)	14 (14–28)	21 (14–28)	14 (14–28)	14 (14–28)	.362
<14 d	18 (6.3)	12 (6.0)	6 (6.9)	17 (6.2)	1 (7.7)	
14–16 d	142 (49.7)	107 (53.8)	35 (40.2)	134 (49.1)	8 (61.5)	
17–27 d	7 (2.4)	3 (1.5)	4 (4.6)	7 (2.6)	0 (0)	.770[c]
28–30 d	98 (34.3)	62 (31.2)	36 (41.4)	94 (34.4)	4 (30.8)	
>30 d	21 (7.3)	15 (7.5)	6 (6.9)	21 (7.7)	0 (0)	
Adverse events	28 (9.8)	15 (7.5)	13 (14.9)	25 (9.2)	3 (23.1)	.123
Switched to doxycycline	21 (7.3)	13 (6.5)	10 (11.5)	19 (7.0)	2 (15.4)	.246

Data are presented as No. (%) unless otherwise specified.

Abbreviation: IQR, interquartile range.

[a] By use of 3 × 2 table, Fisher exact test.

[b] By use of 5 × 2 table, Fisher exact test.

[c] By use of 5 × 2 table, Fisher exact test.

* For comparison of patients with successful treatment and those with treatment failure.

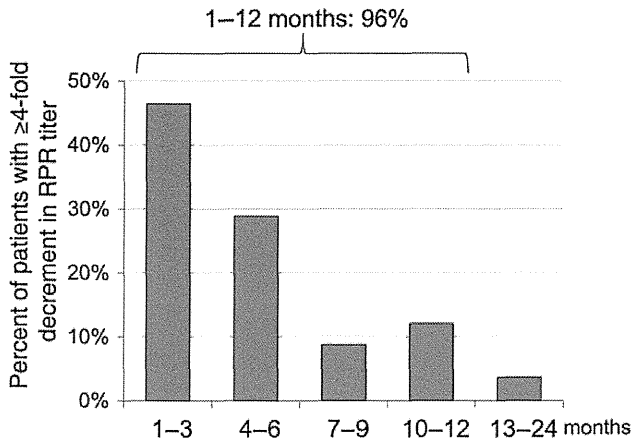

Figure 2. Time until 4-fold decrement in rapid plasma reagin (RPR) titer after initiation of syphilis treatment for successfully treated patients. The median time until ≥4-fold decrement in RPR titer was 4 months (interquartile range, 3–6 months). The ≥4-fold decrement in RPR titer within 12 months was accomplished in 96.3% of the patients. The number and proportion of patients with ≥4-fold decrement in RPR titer at 1–3, 4–6, 7–9, 10–12, and 13–24 months after treatment initiation were 127 (46.5%), 79 (28.9%), 24 (8.8%), 33 (12.1%), and 10 (3.7%), respectively.

and elevated liver enzymes (n = 1). Furthermore, 5 patients presented with both rash and fever. Analysis of the medical records showed that treatment with amoxicillin was changed to doxycycline in 21 (75%) patients due to the side effects. Despite the adverse events, treatment was considered successful in 25 of the 28 (89.3%) patients. Among the 7 patients who showed adverse events but did not change amoxicillin to doxycycline, treatment was successful in 6, although amoxicillin was administered for the median of only 10 days (IQR, 10–18 days). None of the patients discontinued amoxicillin due to Jarisch-Herxheimer reaction.

Logistic regression analysis was performed to identify the risk factors associated with treatment failure. Univariate analysis demonstrated that late syphilis (OR, 3.9 [95% CI, 1.25–12.4]; $P = .019$) and high HIV-1 load (per 1 $\log_{10}$ copies/mL: OR, 1.5 [95% CI, 1.03–2.26]; $P = .033$) before treatment were associated with treatment failure (Table 3). On the other hand, older age was associated with successful treatment (per 1 year: OR, 0.9 [95% CI, .84–.99]; $P = .025$), and higher CD4 count was also marginally associated (per 1 cell/μL: OR, 1.0 [95% CI, .99–1.00]; $P = .053$).

DISCUSSION

The present study investigated the treatment efficacy of 3 g oral amoxicillin plus probenecid for early and late syphilis among HIV-1–infected patients in an observational setting. The results showed that 95.5% of the study patients were successfully

Table 3. Results of Univariate Analysis to Estimate Risk Factors for Treatment Failure With Oral Amoxicillin Plus Probenecid for Syphilis

Variable	OR (95% CI)	*P* Value
Age (per 1 y)	0.9 (.84–.99)	.025
Late syphilis vs early syphilis	3.9 (1.25–12.4)	.019
HIV-1 load (per 1 $\log_{10}$ copies/mL)	1.5 (1.03–2.26)	.033
CD4 count (per 1 cell/μL)	1.0 (.99–1.00)	.053
ART use	0.7 (.23–2.15)	.536
History of syphilis treatment	0.8 (.26–2.41)	.675
Adverse events	3.0 (.77–11.5)	.114

Abbreviations: ART, antiretroviral therapy; CI, confidence interval; HIV-1, human immunodeficiency virus type 1; OR, odds ratio.

treated based on 4-fold decrement in RPR titer. Treatment efficacy was 97.5% in patients with early syphilis (including primary, secondary, and early latent syphilis) and 90.8% in patients with late syphilis (including late latent syphilis and syphilis with unknown duration). This high treatment efficacy is surprising considering that our study population could have included asymptomatic neurosyphilis, because neurosyphilis in HIV-infected patients could occur even in early syphilis without clinical symptoms [5, 6], and most study patients (94.1%) did not undergo lumbar puncture for CSF examination. Furthermore, because increased rate of treatment failure has been reported in HIV-1–infected patients compared with noninfected patients [21, 22], the treatment efficacy shown in this study can be generalized to or could be even better among patients without HIV-1 infection. The regimen of 3 g oral amoxicillin plus probenecid was highly tolerable as well; only 28 (9.8%) patients experienced adverse events, and amoxicillin was switched to doxycycline in only 21 (7.3%) patients. It is also noteworthy that treatment of syphilis was successful in 89.3% of the patients who developed adverse events. Thus, high-dose oral amoxicillin (3 g) plus probenecid can be considered the treatment of choice for early syphilis, late latent syphilis, and syphilis of unknown duration where intramuscular BPG is not available or 3 injections of intramuscular BPG at 1-week intervals is not feasible or is inconvenient to patients with late syphilis.

In the present study, the treatment duration for most patients was either for 14–16 days (49.7%) or 28–30 days (34.3%). Comparison of treatment efficacy between early and late syphilis according to treatment duration showed that treatment efficacy was similar for both 2-week and 4-week treatment in early syphilis, whereas it tended to be lower for 2-week than 4-week treatment in late syphilis, albeit statistically insignificant ($P = .15$). Based on these results and considering that the majority of the study patients used 750 mg of probenecid, we recommend 2 weeks of treatment with 3 g oral amoxicillin plus 750 mg

probenecid for patients with early syphilis and 4 weeks of treatment using the same doses for patients with late latent syphilis and syphilis of unknown duration.

To our knowledge, this is the first study to report the treatment efficacy of high-dose oral amoxicillin plus probenecid for syphilis, regardless of HIV-1 infection status. Although the treatment of oral amoxicillin plus probenecid is described as an alternative syphilis treatment in the UK national guidelines on the management of syphilis [10], previous studies were either only pharmacokinetic studies that examined amoxicillin level in the CSF, or anecdotal, and all of these studies were published in the 1970s and 1980s [13, 14, 23, 24]. It is also noteworthy that amoxicillin, similar to aqueous penicillin, crosses the blood-brain barrier to reach the causative bacteria in the CSF [13, 25], whereas BPG, the preferred choice for early and late syphilis, does not [26]. This might be particularly important for patients with HIV-1 infection because these patients likely present with asymptomatic neurosyphilis, and progression to neurosyphilis is faster in HIV-1–infected patients than in noninfected patients [4–6]. Doxycycline and azithromycin are alternative choices for oral treatment of syphilis for patients with penicillin allergy listed in the guidelines [10]; however, evidence for treatment efficacy of these regimens is limited, particularly among patients with HIV-1 infection [27], and unfortunately, emerging resistance of azithromycin for *T. pallidum* has been reported [28, 29].

Despite several strengths of our study, such as novelty and large number of patients treated with the same regimen of oral amoxicillin plus probenecid, we acknowledge several limitations. First, cases of early latent syphilis might have been included among those with syphilis of unknown duration, because blood samples 1 year before the diagnosis of syphilis were not necessarily available for all patients. Thus, the treatment efficacy for syphilis of unknown duration might be overestimated. However, because only 5.9% of the study patients underwent lumbar puncture, neurosyphilis might be included in this group as well, suggesting that the treatment efficacy might be underestimated. Second, this study defined successful treatment as a ≥4-fold decrement in RPR titer by 24 months after starting treatment. This criterion was chosen because the serologic response after treatment in HIV-infected patients is slower than that in noninfected patients [21, 30]. Although the long observation period might increase the risk of unexpected antibiotic exposure and might contribute to overestimated treatment efficacy, in this study, 96.3% of patients with successful treatment against syphilis achieved a ≥4-fold decrement in RPR titer within 12 months after treatment (Figure 2). Third, the retrospective nature of the study could have introduced some selection and information biases. In fact, although all study patients were treated with 3 g amoxicillin plus probenecid, the duration of treatment, frequency of drug administration, and probenecid dose were not identical among the study patients. However, as described above, most patients were treated for either 2 weeks or 4 weeks, and the treatment efficacy was equally high (the efficacy of 2 weeks' treatment tended to be lower than that of 4 weeks among patients with late syphilis, though not statistically significant). Similarly, most (80.8%) patients were treated with amoxicillin 3 times a day, and 60.1% were treated with probenecid 750 mg/day. These are the reasons for recommending 2 weeks of 3 g oral amoxicillin plus 750 mg probenecid for patients with early syphilis and 4 weeks of treatment for patients with late syphilis.

In conclusion, the efficacy of 3 g oral amoxicillin plus 750 mg probenecid daily was very high in HIV-1–infected patients with early and late syphilis. This regimen was also highly tolerable and required only a single hospital visit. Two weeks of this regimen for patients with primary, secondary, and early latent syphilis, and 4 weeks of treatment for late latent syphilis and syphilis of unknown duration are suggested. Three grams of amoxicillin plus probenecid may be an acceptable replacement for intramuscular BPG. Additional prospective studies are warranted.

Notes

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Ultrasensitive method to quantify intracellular zidovudine mono-, di- and triphosphate concentrations in peripheral blood mononuclear cells by liquid chromatography–tandem mass spectrometry

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Although zidovudine (AZT) is not the preferred antiretroviral drug for adult HIV-infected patients, it is still widely used in infants for both prevention of mother-to-infant HIV-1 transmission and treatment of HIV-infected children. However, it is difficult to measure intracellular concentrations of AZT metabolites in small blood samples due to their extremely low concentrations in peripheral blood mononuclear cells and interference by endogenous nucleotide triphosphates, residual plasma phosphates and electrolytes. We developed an ultrasensitive assay using liquid chromatography–tandem mass spectrometry (LC–MS/MS) for measurement of intracellular concentrations of zidovudine (AZT)-monophosphate (AZT-MP), -diphosphate (AZT-DP) and -triphosphate (AZT-TP). The high sensitivity was due to the improvement of peripheral blood mononuclear cells extraction for complete removal of plasma and electrolytes, alkalization of LC buffer and use of alkaline-stable high performance liquid chromatography column and tetrabutylammonium hydroxide as the ion pair. Using this method, the lower limits of quantification of AZT, AZT-MP, -DP and -TP were 6, 6, 10 and 10 fmol per sample, respectively. Accuracy ranged 89–115% and precision was lower than 15% in the quantification range of 6–6000 fmol/sample for plasma AZT and intracellular AZT-MP and 10–10 000 fmol/sample for AZT-DP and -TP. The validation parameters met the international requirements. Among nine AZT-treated HIV-infected adult patients, five had low AZT-TP levels (<10 fmol/10^6 cells). Our assay has high sensitivity and is advantageous for evaluation of AZT phosphates in children and infants based on minimum blood sampling requirement. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: LC–MS/MS; ultrasensitive; zidovudine; phosphates; intracellular

Introduction

At present, zidovudine (AZT) is not the preferred antiretroviral drug for adult HIV-infected patients,[1] although it remains one of the main drugs used for prevention of mother-to-infant HIV-1 transmission and also for the treatment of HIV-infected children. Although serious concerns on AZT-associated mitochondrial toxicity have been raised,[2,3] there is little information on the pharmacokinetics of AZT and metabolites in infants,[4] mainly due to limitation of blood sampling volume.

Despite the recent developments in liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for measurement of intracellular concentrations of metabolites of nucleoside reverse transcriptase inhibitors (NRTI) used for the treatment of HIV infection,[5–7] it is still difficult to measure intracellular concentrations of AZT phosphates because of the extremely low concentrations in peripheral blood mononuclear cells (PBMC). Intracellular AZT phosphate levels can be determined by indirect and direct measurements. The indirect measurement involves several complex steps: (1) serial isolation of AZT-monophosphate (MP), -diphosphates (DP) and -triphosphates (TP) using an ion-exchange cartridge; (2) cleavage of the phosphate groups by acid phosphatase; and (3) determination of AZT concentration isolated from each type of AZT phosphate using radioimmunoassay[8] or LC–MS/MS.[9–13] These assays are quite sensitive and useful for evaluation of intracellular AZT metabolites in AZT-treated patients. However, there is concern about the stability of the different AZT phosphates and reproducibility of the measurement related to the complexity of the aforementioned procedures. Furthermore, because these methods do not directly measure AZT phosphate but rather dephosphorylated products of AZT phosphate, it is difficult to confirm whether this method can precisely discriminate each type of AZT phosphates.

A direct measurement assay of AZT phosphates has been described recently, which is based on the use of the LC–MS/MS system without the dephosphorylation step.[14] However, it was concluded that such LC–MS/MS assay lacks sufficiently high sensitivity for extremely lower concentrations of intracellular AZT

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phosphates, mainly due to interference by endogenous nucleotides triphosphates and other unknown plasma phosphates and electrolytes.[14]

We developed a highly sensitive method for determination of plasma AZT concentration and intracellular AZT-MP, -DP and -TP concentrations using LC–MS/MS. We describe here the validation data of the assay and the clinical data in adult HIV-infected and AZT-treated patients.

Materials and methods

Chemicals and reagents

AZT (>98% purity) and 3′-azido-2′, 3′-dideoxyuridine (AZdU) (>98%), as an internal standard (IS), were obtained from Sigma-Aldrich (St. Quentin-Fallavier, France); AZT-MP (>95%), -DP (>90%) and -TP (>95%), adenosine triphosphate (ATP) (>95%) and 2′-deoxyguanosine-5′-triphosphate (dGTP) (>95%) were obtained from Moravek Biochemicals Inc. (Brea, CA, USA). 5-Methyluridine-5′-triphosphate (5-Methyl-UTP) (>90%), used as an IS, was obtained from TriLink Biotechnologies (San Diego, CA, USA). Tetrabutylammonium hydroxide (TBAH), ammonium formate, ammonium hydroxide, acetonitrle LC gradient grade and methanol LC gradient grade were obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Instrumentation

The LCMS 8030 triple-quadrupole LC/MS/MS system (Shimadzu Corp. Kyoto, Japan) was used in this study. It was monitored using Labsolutions LCMS software (Shimadzu Corp. Kyoto, Japan).

Standard solutions

Standard solutions of AZT, AZT-MP, -DP and -TP were prepared daily from three separate master stock solutions (1 mmol/L of AZT, AZT-MP, -DP and -TP in water) of each compound. Identical stock solutions of AZdU and 5-methyl-UTP were prepared in water from the respective high-concentration master stock solutions (1 mmol/L of AZdU and 5-Methyl-UTP in water). All master solutions were stored at −80 °C.

Preparation of spiked plasma and PBMC samples

PBMCs were prepared as described previously.[15] Briefly, anticoagulated whole blood diluted with phosphate-buffered saline was layered gently on 5 mL of a Ficoll-Paque Plus solution (GE Healthcare, Piscataway, NJ, USA) in a centrifuge tube and centrifuged at 600 × *g* for 25 min. After centrifugation, the plasma portion, which formed above the PBMCs layer, was collected gently and stored at −80 °C. PBMCs, which were sandwiched between the plasma and Ficoll-Paque Plus solution, were collected and counted using a cell counter (Celltac, Nihon Kohden, Tokyo, Japan) after washing twice with phosphate-buffered saline (PBS) containing 2% fetal calf serum. After centrifugation, 1–10 × 10^6 cells of the PBMCs were collected and suspended with 100 μL saline. PBMCs were separated from plasma using the following procedure: (i) a mixture of silicone oil (catalog no. SH-550; Nacalai, Kyoto, Japan) and *n*-hexadecane (Nacalai) (800 μl; 90:10 [vol/vol]) was added into the outer tube of a 1.5 ml microcentrifuge tube; (ii) the inner tube (comprising 0.5 ml microcentrifuge tube) with a hole at the bottom, was sunk in the silicone oil/*n*-hexadecane mixture in the surrounding outer tube; (iii) PBMCs suspended in 100 μl of saline were layered on the silicone oil mixture in the inner tube; (iv) the lid of the inner tube was pressed down; (v) the assembled 'double tube' was centrifuged at 16 000 × *g* for 1 min, which resulted in the rapid sedimentation of PBMCs at the bottom of the outer tube; (vi) the inner tube containing plasma and silicone oil was removed, with the lid kept closed to prevent the passage of plasma into the outer tube; and (vii) the silicone was removed from the outer tube while leaving the pelleted PBMCs in place; any remaining silicone oil on the outer tube inner surface was removed after a brief centrifugation. The pelleted PBMCs were suspended in 30 μl of pure water (suspension of cells in PBS is not recommended because the presence of phosphate ions in samples affects ionization of molecules in mass spectrometry and could even sometimes damage the analyzer). Finally, 70 μl of ethanol was added to 30 μl of the cell suspension, and the PBMC solution was stored at −80 °C.

On the day of the analysis, IS (5-Methyl-UTP) and AZT-MP, -DP and -TP standards were added to the PBMC solution for calibration (final spiked amount 6 fmol, 60 fmol, 600 fmol, and 6 pmol of AZT-MP, and 10 fmol, 100 fmol, 1 pmol and 10 pmol of AZT-DP and -TP per each sample). Specifically, AZT and AZdU, and 70 μl of ethanol were added to 30 μl of plasma for calibration (final spiked amount 6 fmol, 60 fmol, 600 fmol and 6 pmol of AZT per each sample, and 20 fmol of AZdU in total volume of 100 μl of solution).

Cellular debris was removed by centrifugation at 18 000 × *g* for 2 min at 4 °C, and the supernatant was transferred to a centrifugation tube. Samples were evaporated to dryness using Savant SpeedVac (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The dried samples were re-suspended with 20 μl of Buffer A, and the remaining debris was thoroughly removed by spinning at 18 000 × *g* for 2 min followed by the transfer of the supernatant to a fresh tube for LC–MS/MS analysis.

Samples from adult patients

PBMCs collected from the whole blood of adult patients as described earlier were suspended with 70% ethanol solution and stored at −80 °C. On the day of the analysis, the PBMC solution was spiked with 200 fmol of 5-Methyl-UTP per sample. Thirty microliter of plasma collected from whole blood was diluted with 70 μl of ethanol and stored at −80 °C. On the day of the analysis, the plasma solutions were spiked with 10 fmol of AZdU per each run.

Chromatographic and mass spectrometric settings

Assay for plasma AZT concentration

Chromatography was conducted using InnertSil ODS-30, 5 μm, 50 x 1.5 mm (GL Science, Tokyo, Japan). The mobile phase was delivered at a flow rate of 0.3 mL/min.

In the assay for plasma AZT, the mobile phase comprised a mixture of solutions A and B. Solution A was composed of 5 mM of acetate (pH = 5.0) in water, while solution B was composed of the same solute in methanol. A linear gradient was set from 0% to 50% B in 10 min (total run time: 20 min).

Assay for intracellular AZT-MP, -DP and -TP concentrations

Chromatography was conducted using InnertSustain (GL Science), 5 μm, 50 × 1.5 mm, which can be applied for a wide range of pH (pH 2–10). The mobile phase was delivered at a flow rate of 0.3 mL/min. In the assays for intracellular AZT-MP, -DP and -

TP, the mobile phase comprised a mixture of solutions A and B. Solution A was composed of 5 mM of ammonium formate, 2.5 mM ammonium hydroxide and 0.01 mM of TBAH (pH = 8.5) in water, and solution B was composed of the same solute in acetonitrile/methanol (90/10 v/v). A linear gradient was set from 0% to 50% B in 10 min (total run time: 20 min). The instrument was operated in the positive mode under MS/MS conditions using multiple reaction monitoring. Fragmentation was achieved with nitrogen. Ion transitions monitored by *m/z* were 268.25/127.30 for AZT, 254.10/113.05 for AZdU, 365.10/81.10 for AZT-MP, 445.10/81.15 for AZT-DP, 525.10/81.20 for AZT-TP and 498.85/96.95 for 5-methyl-UTP. Collision energy was set at −15 V for AZT, −10 V for AZdU, −15 V for AZT-MP, −10 V for AZT-DP, −10 V for AZT-TP and −15 V for 5-methyl-UTP.

Validation of the LC–MS/MS method

Selectivity and specificity

To investigate whether endogenous compounds ATP and dGTP interfered with the assays, we analyzed 5 pmol of ATP, dGTP and also blank PBMC taken from six healthy subjects and six HIV-infected naïve-to-treatment with AZT or d4T subjects.

Accuracy and precision

The accuracy and precision of the method were assessed by analyzing the intraday and interday accuracies and precisions at concentrations 6–6000 (6, 60, 600 and 6000) fmol per sample of AZT and AZT-MP, and 10–10 000 (10, 100, 1000 and 10 000) fmol per sample of AZT-DP and -TP using spiked plasma (for AZT) and PBMC (for AZT-MP, -DP and -TP) samples together with standard solutions at the same concentrations. For intraday precision and accuracy, five replicates at the four concentrations were assayed in the same run. For both intraday and interday experiments, the mean, accuracy and coefficient of variation were calculated at all tested concentrations. Accuracy was expressed as the absolute percentage of the theoretically determined concentration, and precision was evaluated as the coefficient of variation (CV). Intra-run accuracy was required to be within ±15%, and precision was required not to exceed 15%, except for the lower limit of quantification (LLOQ) in which accuracy was to be within ±20%, and precision was not to exceed 20%. Inter-run accuracy was required to be within ±15%, and precision was required not to exceed 15%, except for the LLOQ in which accuracy was to be within ±20% and precision not to exceed 20%.

Matrix effect

To evaluate the matrix effect, six lots of spiked plasma samples with various dilutions by PBS from 25% to 100% and six lots of spiked PBMC samples with various numbers of cells from 3× to 15×10^6 cells at low and high concentrations. Low level was set at three times the LLOQ, and high level was set at the upper limit of quantification (HQ). The mean and the standard deviation of the area ratio between analytes and internal standard for each spiked sample contain 3, 6, 9, 12 and 15×10^6 cells. Precision was required to be below 15%.

Pharmacokinetics of intracellular AZT-MP, -DP and -TP concentrations in AZT-exposed PBMC

We studied the association between AZT exposure levels and intracellular AZT metabolites using activated PBMC, according to the standard procedures for phenotypic assay.[16] Briefly, HIV-negative donor PBMC were prepared by Ficoll-Paque plus solution density gradient centrifugation. PBMC were suspended at 1–2 × 10^6/mL in 24-well microtiter culture plates in 2 ml of culture medium (RPMI 1640 medium supplemented with 15% heat-inactivated FCS, 5% purified human interleukin-2, 50U of penicillin per ml, 50 μg of streptomycin and 4 mM L-glutamine) were stimulated with phytohemagglutinin (PHA) (3 μg/ml) for 3 days. First, PHA-stimulated PBMC were incubated with different concentrations of AZT (1, 3.2, 10, 100, 1000 and 10 000 nmol/L) for 24 h. Second, PHA-simulated PBMC were incubated with 10 μmol/L of AZT for different time intervals (0.5, 1, 2, 6, 12 and 24 h). These AZT-exposed PBMC were immediately washed twice with PBS, extracted as described earlier, and analyzed to determine the concentrations of AZT metabolites.

Determination of plasma AZT and intracellular AZT-MP,-DP and -TP concentrations in adult AZT-treated patients

We determined the plasma AZT and intracellular AZT-MP, -DP and -TP concentrations in 13 blood samples from nine adult patients. Among 3650 HIV-infected patients who had been treated at our hospital, only nine patients were treated with AZT-containing antiretroviral therapy. A written informed consent was obtained from all the subjects. Two serial blood samples were obtained from two patients, respectively: the first was obtained immediately before taking AZT and the second was at 4 h after taking AZT. Whole blood was collected into ethylenediaminetetraacetic acid 2 K tube, and plasma and PBMC were extracted immediately and stored. Plasma AZT and intracellular AZT-MP, -DP and -TP concentrations were determined subsequently.

Results and discussion

Validation of the LC–MS/MS method

The method used in the present analysis markedly improved the sensitivity of measurement of intracellular AZT-MP, -DP and -TP through several technical improvements. First, there is a need to overcome interference by endogenous nucleotide phosphates by detecting specific precursor-product ions and positive ionization mode.[7] We succeeded in discriminating AZT phosphates from ATP and dGTP by adopting a positive ionization mode. The fragmentation patterns of the precursor ions of AZT, AZT-MP, -DP, -TP, AZdU, 5-Methyl-UTP, ATP, and dGTP obtained in the positive ionization mode are shown in Figure 1. Relatively little attention has been paid to the problem of unknown phosphate compounds and electrolytes in residual plasma or PBS used for washing PBMC, which also can inhibit ionization of AZT phosphates.[7] Even double washing with saline does not completely remove residual plasma phosphates or electrolytes. To remove residual plasma phosphates and sodium ions, we used a double tube filled with silicon oil for PBMC extraction.[15] Furthermore, for complete elimination of sodium ions, the extracted PBMC aliquot was suspended not in saline but pure water. These improvements of PBMC extraction procedures markedly eliminated various factors known to interfere with ionization. Second, our mobile phase was set at a high pH (around 8.5), and it contained low concentrations of ion-pairing agent. Because NRTI phosphates are highly hydrophilic negative ions, a high pH buffer (pH 8.5) and a positive ion pair are necessary for separation in LC. Because a conventional HPLC column is vulnerable to exposure to a high pH buffer, we used a highly stable HPLC column with a high pH buffer. Several studies employing NRTI phosphates assay

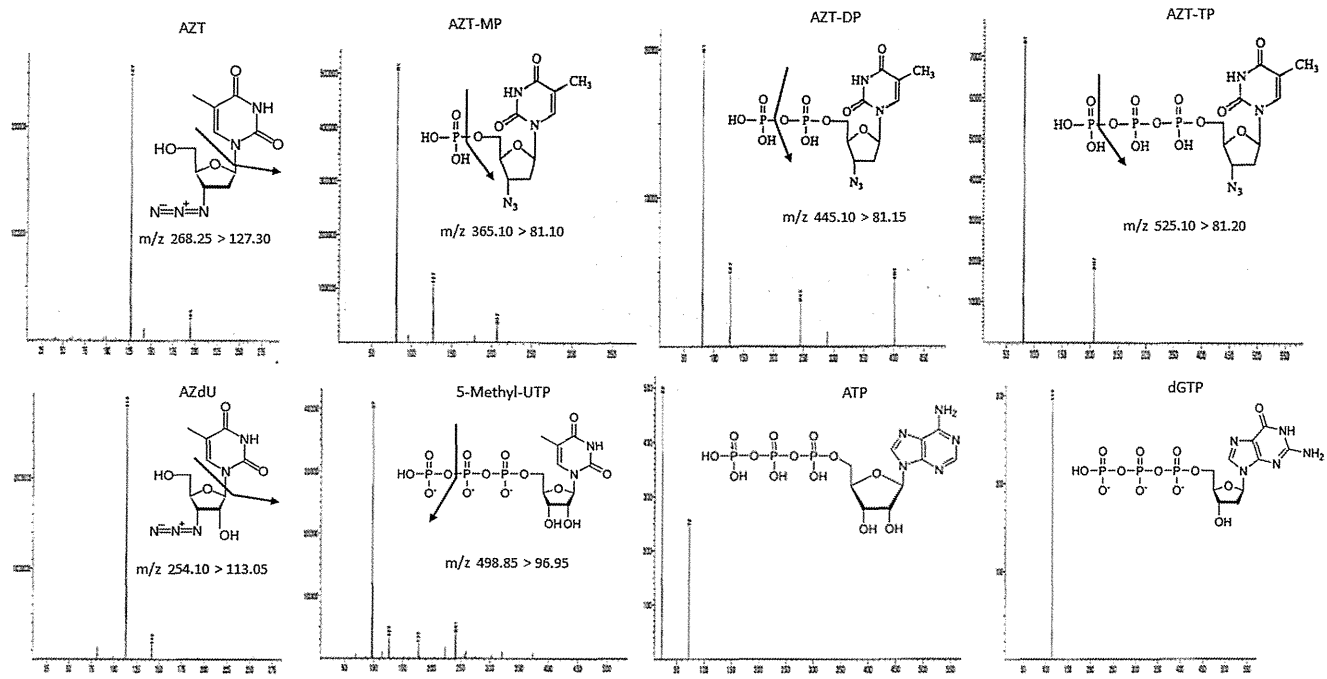

Figure 1. Chemical structures, precursor/product ions used in MRM and MS/MS spectra of fragmentation patterns of AZT, AZT-MP, AZT-DP, AZT-TP, AZdU, 5-Methyl-UTP and chemical structures and MS/MS spectra of fragmentation patterns of ATP and dGTP.

used 1,5-dimethylhexylamine as an ion pair.[5–7,14] However, while high concentrations of 1,5-dimethylhexylamine can increase the pH of buffers, they can inhibit the ionization of NRTI-TPs. To improve this procedure, we used the combination of ammonium hydrate, as an alkalization agent, and low concentrations of TBAH, as an ion pair, which allowed clear separation and increased the sensitivity. Furthermore, the low concentration of an ion pair interferes less with the ionization of AZT metabolites and can be easily washed from LC lines, which contributed to the stability of this assay (Fig. 1).

The chromatogram of each of the compounds analyzed in this study is shown in Fig. 2. The total runtime of the two assays were 20 min, respectively. The retention times were about 10, 11 for AZT, AZdU (IS), and 5, 6, 6.5 and 6 min for AZT-MP, AZT-DP, AZT-TP and 5-Methyl-UTP (IS), respectively.

Selectivity and specificity

For the blank PBMCs prepared from six different patients, there was no interference at the retention time of the nucleotides and IS. Two endogenous nucleotides triphosphates, ATP and dGTP, can

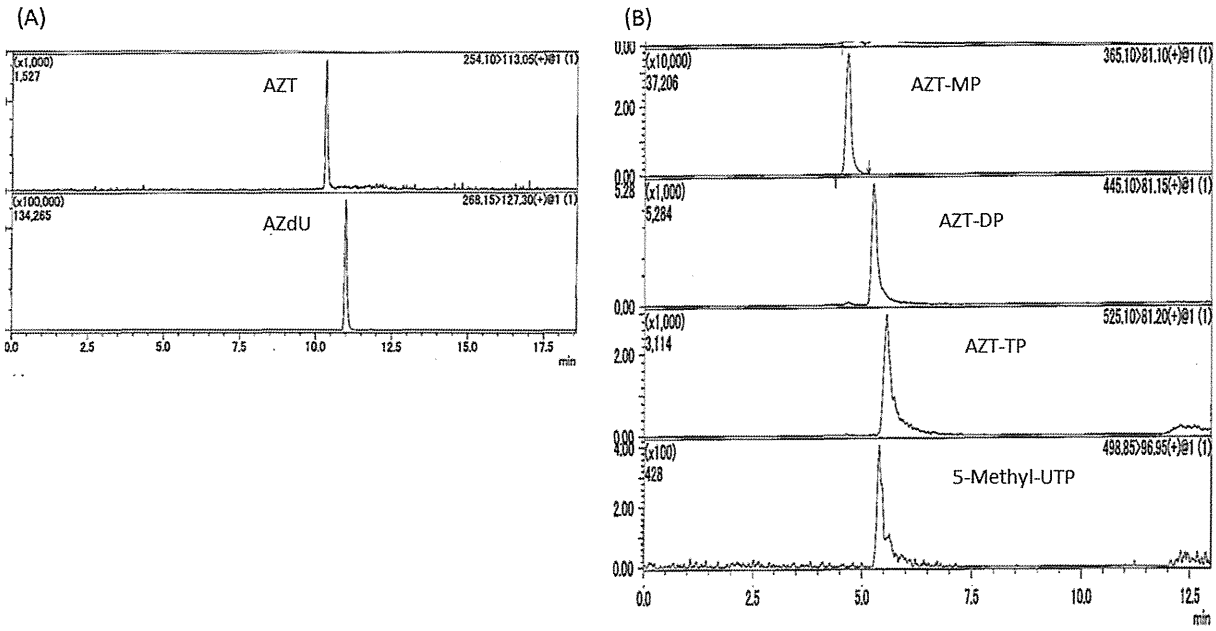

Figure 2. Multiple reaction monitoring ion chromatograms for determination of (A) AZT and AZdU (IS) in spiked plasma and (B) AZT-MP, AZT-DP, AZT-TP and 5-Methyl-UTP (IS) in spiked human peripheral blood mononuclear cells.

interfere with the detection of AZT-TP due to the similarity in molecular weights (ATP: 507, dGTP: 506, AZT-TP: 507) and almost identical fragmentation patterns, especially in the negative electrospray ionization mode. In our method, although both ATP and dGTP can be detected in the same precursor-product ion pattern as AZT-TP, with comparable retention time (4.7 min for AZT-TP, 4.5 min for ATP and 4.0 min for dGTP), they were discriminated by the large differences in the peaks of the large amounts of these compounds (areas under curve for 5 pmol of ATP and dGTP versus AZT-TP were 60 and 400 versus 1,00 000. Fig. 3).

Quantification range and lower limit of quantification

Intraday precision (CV) was less than 15%, and intraday accuracy ranged from 89% to 109% for AZT-MP, -DP and -TP at quantification range (Table 1). Interday precision was less than 15%, and interday accuracy was between 89% and 115% for all analytes. Figure 4 shows typical chromatograms of each analyte in standard solution and spiked samples at LLOQ and HQ.

Despite recent advances in LC–MS/MS technology for measurement of NRTI-TP,[1–3] it is difficult to determine intracellular AZT metabolites (especially AZT-DP and -TP) due to their low concentrations in AZT-treated patients (reported to be around 10–40 fmol/10^6 cell).[4,12] In the present assay, the LLOQ for each run was 6 fmol per sample for plasma AZT, and 6, 10 and 10 fmol per sample for intracellular AZT-MP, -DP and -TP, respectively. These values can be translated into 0.4 nmol/L of plasma and 0.6, 1.0 and 1.0 fmol/10^6 cells containing 10×10^6 cells, for intracellular AZT-MP, -DP and -TP, respectively. In comparison with previously reported LLOQ of AZT-MP (300 fmol per sample) and -TP (150 fmol per sample),[14] the LLOQ of our assay (6 and 10 fmol per sample for AZT-MP and -TP) improved the sensitivity by 50 and 15 times, respectively. These LLOQ were low for the measurement of intracellular AZT metabolites concentrations in AZT-treated adult patients.

Matrix effect

Data of the matrix effect are summarized in Table 2. The precision of area ratios between AZT and AZdU (IS) in spiked plasma with 25%, 50% and 100% of plasma were 7.9% at three times of LLOQ and 2.5% at HQ. The precision of area ratios between AZT phosphates and 5-Methyl-UTP (IS) in spiked PBMC with 3×, 6×, 9×, 12× and 15×10^6 cells ranged from 6.9% to 11.2% at LQ and 3.8% to 6.9% at HQ, respectively.

In vitro pharmacokinetics of intracellular AZT-MP, -DP and -TP concentrations in PBMC from AZT-treated patients

Figure 5(A) shows the dose-dependent relationship between the AZT exposure level and intracellular AZT metabolites in activated PBMC. Interestingly, AZT-MP level increased proportionally with the increases in AZT level, whereas the increase in AZT-DP and -TP levels blunted at high AZT levels. AZT-TP increased only 94-fold

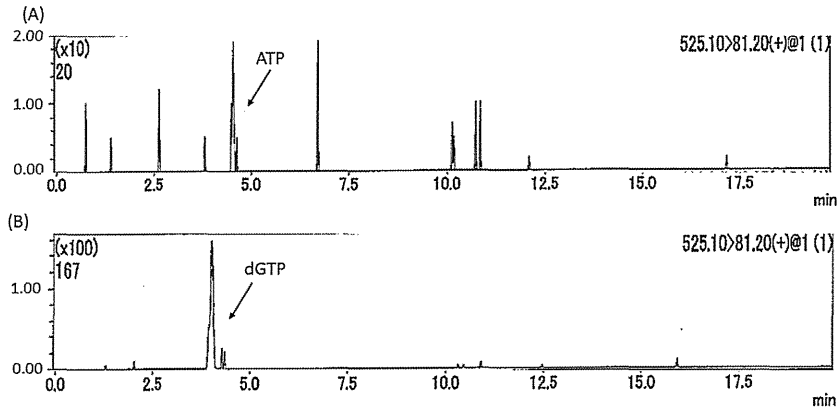

Figure 3. Multiple reaction monitoring (MRM) ion chromatograms of 5 pmol of ATP and dGTP using MRM setting for determination of AZT-TP.

Table 1. Intraday and interday assay precision and accuracy for AZT and AZT-MP, -DP and -TP in human plasma and human PBMC

	Theoretical Concentration (fmol/sample)															
	LLOQ				Low				Middle				High			
	6	6	10	10	60	60	100	100	600	600	1000	1000	6000	6000	10 000	10 000
	AZT	AZT-MP	AZT-DP	AZT-TP	AZT	AZT-MP	AZT-DP	AZT-TP	AZT	AZT-MP	AZT-DP	AZT-TP	AZT	AZT-MP	AZT-DP	AZT-TP
Intraday ($n = 5$)																
Precision	8.9	10.2	8.0	8.0	7.4	2.2	5.8	7.5	0.5	2.5	3.9	5.6	2.2	1.1	2.1	1.9
Accuracy	93.8	108.8	92.8	91.6	102.9	98.4	102.2	110.3	91	100.7	96.4	97.5	89	101.2	100.1	101.7
Interday ($n = 5$)																
Precision	8.9	10.9	8.3	9.7	2.6	6.2	9.9	9.1	0.7	2.4	4.9	4.5	1.5	1.3	1.8	1.7
Accuracy	93.8	103.6	104.6	114.9	107.7	97.3	96.7	91.1	91.0	103.1	102.3	96.0	89.3	102.7	102.3	100.3

AZT, zidovudine; MP, monophosphate; DP, diphosphate; TP, triphosphate; PBMC, peripheral blood mononuclear cells; LLOQ. lower limit of quantification.

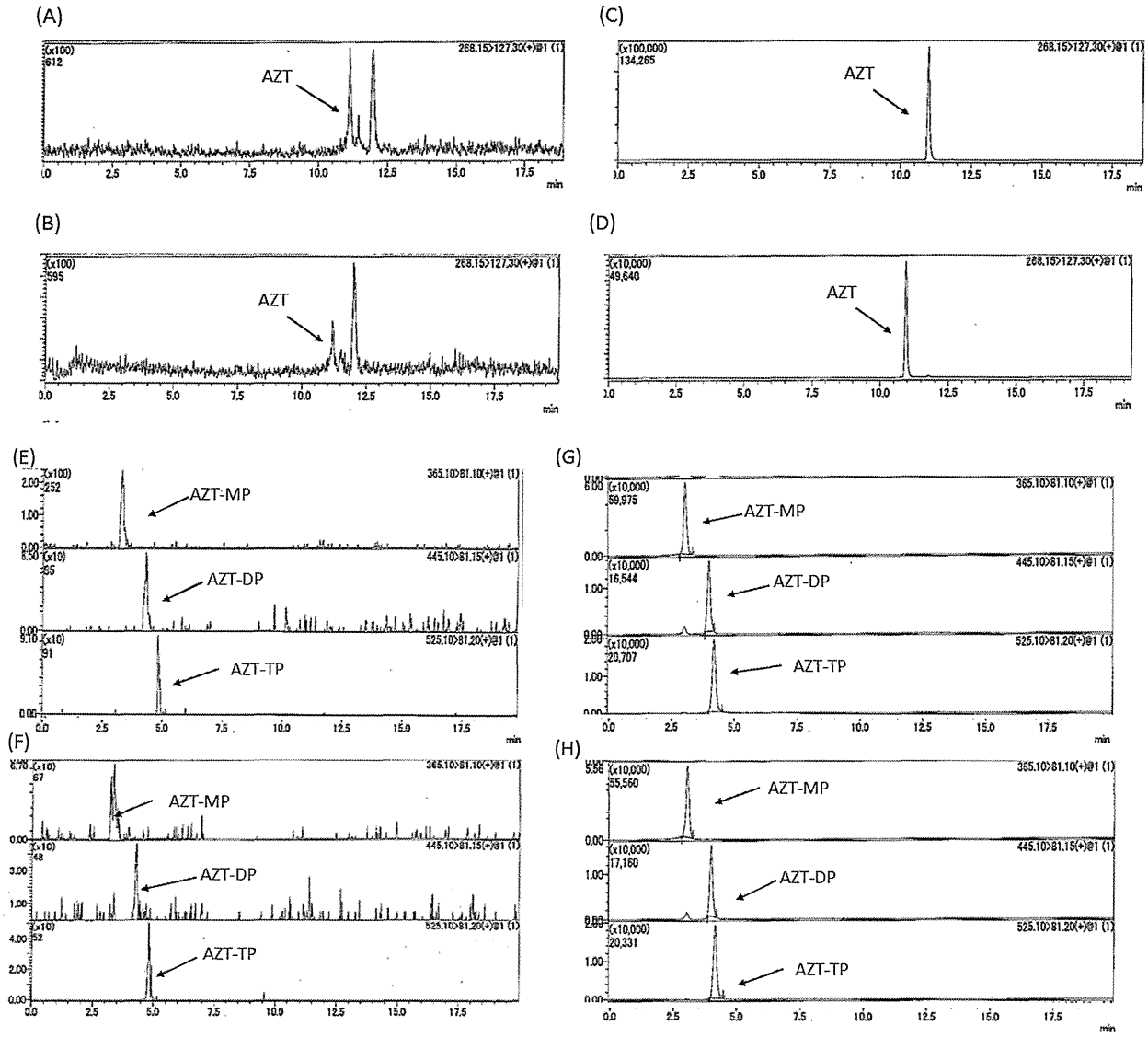

Figure 4. Multiple reaction monitoring ion chromatograms of (A) lower limit of quantification (LLOQ:6 fmol per sample) of AZT in standard solution; (B) LLOQ of AZT spiked in plasma; (C) high calibration point (6000 fmol per sample) of AZT in standard solution; (D) high calibration point of AZT in spiked plasma; (E) LLOQ (6, 10 and 10 fmol per sample) of AZT-MP, -DP and -TP in standard solution; (F) LLOQ of AZT-MP, -DP and -TP in spiked peripheral blood mononuclear cells (PBMCs); (G) high calibration point of AZT-MP (6000 fmol per sample), -DP (10 000 fmol per sample) and -TP (10 000 fmol per sample) in standard solution; (H) high calibration point of AZT-MP, -DP and-TP in spiked PBMCs.

(from 103 to 9640 fmol/10^6 cells) in accordance with the 10 000-fold increase in exposure to AZT (from 1 to 10 000 nmol/L). Especially, AZT-TP increased only 2.6-fold (from 3703 to 9640 fmol/10^6 cells) when exposure to AZT increased by 100-fold from 100 to 10 000 nmol/L. Consequently, AZT-MP levels were lower than those of AZT-DP and -TP at low AZT exposure (less than 100 nmol/L), which are comparable to the range in AZT-treated HIV-infected adult patients. Although AZT-MP can be underestimated because of the wash-out of AZT-MP through the extraction procedures, it is suggested that AZT-MP can rapidly be metabolized into AZT-DP and -TP upon exposure to low levels of AZT. Our highly sensitive method first highlighted the pharmacokinetics of AZT metabolites at extremely low concentrations of AZT (1 and 10 nmol/L), which has not been determined even by conventional methods.

Figure 5(B) shows intracellular concentrations of AZT metabolites using different incubation times with 10 μmol/L of AZT. The levels of AZT metabolites increased rapidly after the start of incubation, then reached plateaus within 2 h of incubation. Interestingly, AZT metabolites did not increase after longer exposure to AZT or high levels of AZT.

Quantification of AZT metabolites in AZT-treated adult patients

The demographic characteristics of the patients, sampling time, AZT plasma concentrations and intracellular concentrations of AZT-MP, -DP and -TP are listed in Table 3. Low levels of intracellular AZT-TP (<10 fmol/10^6 cells) were noted in eight samples from five patients. These results were consistent with those determined by the conventional but highly sensitive method of radioimmunoassay (10–40 fmol/10^6 cell).[8,17] In contrast, these values were lower than those reported previously in a study using LC–MS/MS assay (50–200 fmol/10^6 cells).[9,10,14]

Table 2. Matrix effect of human PBMC number on quantitation of AZT, AZT-MP, -DP and -TP

	Theoretical concentration (fmol/sample)			Theoretical concentration (fmol/sample)					
	Low	High		Low			High		
	18	6000		18	30	30	6000	10 000	10 000
	AZT	AZT		AZT-MP	AZT-DP	AZT-TP	AZT-MP	AZT-DP	AZT-TP
Plasma %			Cells ($\times 10^6$)						
25	0.085	17.12	3	0.0069	0.0027	0.0014	14.69	7.055	4.582
50	0.099	16.76	6	0.0077	0.0025	0.0016	15.30	6.793	4.484
100	0.092	17.60	9	0.0079	0.0026	0.0015	16.10	7.179	4.711
			12	0.0071	0.0026	0.0013	14.92	6.525	4.367
			15	0.0075	0.0023	0.0014	15.80	6.806	4.446
Mean	0.092	17.16		0.0074	0.0026	0.0014	15.36	6.872	4.518
SD	0.007	0.422		0.0005	0.0001	0.0001	1.058	0.272	0.171
CV%	7.9	2.5		6.9	9.8	11.2	6.9	4.0	3.8

Figures represent mean ratio ($n = 6$) of chromatographic peak area of analytes to internal standard.

AZT, zidovudine; MP, monophosphate; DP, diphosphate; TP, triphosphate; PBMC, peripheral blood mononuclear cells; LLOQ. lower limit of quantification; CV, intraday precision; SD, standard deviation.

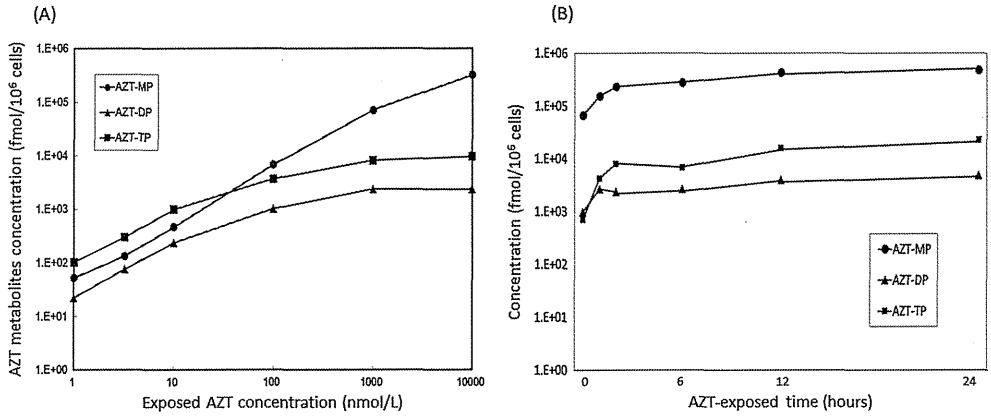

Figure 5. *In vitro* assay using phytohemagglutinin-activated peripheral blood mononuclear cells. Intracellular AZT-MP, -DP and -TP concentrations by (A) 24 h-incubation of different concentrations of AZT and by (B) different incubation time period using 10 μmol/L of AZT.

Interestingly, no marked increases in intracellular AZT phosphate levels were noted from 0 to 4 h in the two patients, despite the rapid increases in plasma AZT levels. Several studies have also reported that AZT phosphorylation is quite poor,[18–20] and recent clinical data also confirmed that the efficiency of AZT phosphorylation is far lower than tenofovir DF.[17] AZT is phosphorylated sequentially by thymidine kinase, thymidylate kinase and nucleoside diphosphate kinase, with thymidylate kinase acting as a rate-limiting enzyme in this process.[21] Our study also showed that AZT-DP levels are comparable to those of AZT-TP, which added support to the aforementioned hypothesis.

Our study identified relatively low levels of intracellular AZT-MP concentrations compared with those reported in previous studies.[9,10,14] The different findings can be explained as follows: first, low plasma AZT concentrations (<100 nmol/L), which are observed in the majority of AZT-treated adult patients, are associated with low intracellular AZT-MP levels. Second, AZT-MP concentration decreases rapidly due to the short half-life.[10] Third, low levels of AZT-MP can be easily washed out during the PBMC extraction procedures, because AZT-MP rapidly diffuses out of the cell membrane. Furthermore, in Japan, AZT is commonly administered in a small dose of 200 mg twice daily based on the high incidence of severe nausea in adult patients treated with the standard dose. Thus, a smaller dose of AZT is associated with low plasma AZT concentrations and consequently results in low intracellular AZT-MP levels.

AZT is exclusively and widely used in infants to prevent mother-to-infant HIV-1 transmission. The pharmacokinetics of AZT phosphates in infants have not been fully evaluated except for one study that employed the LC–MS/MS assay.[4] However, 4 mL of blood sample was required for analysis in the earlier study, which can be a heavy burden in most infants. In contrast, our assay determined AZT phosphates level using smaller volume of blood sample due to the improved sensitivity. Therefore, our sensitive assay is potentially useful for the evaluation of the pharmacokinetics of intracellular phosphates in infants and children using only a small amount of blood samples.