HIV

Emtricitabine-Tenofovir Concentrations and Pre-Exposure Prophylaxis Efficacy in Men Who Have Sex with Men

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Drug concentrations associated with protection from HIV-1 acquisition have not been determined. We evaluated drug concentrations among men who have sex with men in a substudy of the iPrEx trial (1). In this randomized placebo-controlled trial, daily oral doses of emtricitabine/tenofovir disoproxil fumarate were used as pre-exposure prophylaxis (PrEP) in men who have sex with men. Drug was detected less frequently in blood plasma and in viable cryopreserved peripheral blood mononuclear cells (PBMCs) in HIV-infected cases at the visit when HIV was first discovered compared with controls at the matched time point of the study (8% versus 44%; P < 0.001) and in the 90 days before that visit (11% versus 51%; P < 0.001). An intracellular concentration of the active form of tenofovir, tenofovir-diphosphate (TFV-DP), of 16 fmol per million PBMCs was associated with a 90% reduction in HIV acquisition relative to the placebo arm. Directly observed dosing in a separate study, the STRAND trial, yielded TFV-DP concentrations that, when analyzed according to the iPrEx model, corresponded to an HIV-1 risk reduction of 76% for two doses per week, 96% for four doses per week, and 99% for seven doses per week. Prophylactic benefits were observed over a range of doses and drug concentrations, suggesting ways to optimize PrEP regimens for this population.

INTRODUCTION

The Joint United Nations Programme on HIV/AIDS (UNAIDS) estimates that there are 2.6 million new HIV-1 infections per year, despite widespread awareness of the modes of transmission and the protective benefits of condom use (2). Men who have sex with men carry a disproportionate burden of infection on all continents (3). Pre-exposure prophylaxis (PrEP) using the antiretroviral drugs emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) is a recently proven strategy for preventing HIV acquisition in men who have sex with men. TDF is a prodrug for tenofovir, which is an analog of deoxyadenosine-monophosphate. These drugs require phosphorylation in mononuclear cells to the pharmacologically active triphosphate anabolites, called emtricitabine-triphosphate (FTC-TP) and tenofovir-diphosphate (TFV-DP).

In the iPrEx trial, HIV-negative men at risk for HIV infection were randomized to daily oral dosing with emtricitabine/tenofovir (TFV) disoproxil fumarate (FTC/TDF) or placebo for PrEP. Randomization

to active drug decreased HIV-1 acquisition by 44% compared with placebo, with greater reductions in HIV-1 acquisition risk associated with higher reported adherence and detectable drug in the blood (1).

In a predefined pharmacology substudy of the iPrEx trial, drug in plasma or peripheral blood mononuclear cells (PBMCs) was detected in 22 of 43 seronegative subjects (51%) versus 3 of 34 HIV-infected subjects from the active arm (9%) (P < 0.001) (1). Predicted efficacy in the iPrEx trial increased from 44% to more than 90% when detectable drug was accounted for. Thus, adherence to daily doses of FTC-TDF was critical for PrEP efficacy in the iPrEx trial, and the same has been found in other PrEP studies (4–7). However, the quantitative relationship between drug concentration and level of adherence with PrEP efficacy has not been determined. The current study expands on the predefined pharmacology substudy of the iPrEx trial and quantifies the concentrations of drugs associated with protection from HIV-1 acquisition, as well as the frequency of PrEP use required to achieve those concentrations.

RESULTS

Intracellular TFV-DP, the active form of tenofovir, was analyzed in PBMCs arising from two separate studies: the iPrEx trial and the STRAND study. Data from these separate studies were combined to understand the relationship between TFV-DP concentration and PrEP efficacy in men who have sex with men. TFV-DP concentrations in PBMCs from the iPrEx trial were used to estimate drug concentrations associated with decreased HIV acquisition. TFV-DP concentrations in PBMCs from the STRAND study were used to establish expected concentrations from two, four, or seven doses per week of directly observed

TDF therapy. PrEP efficacy was then quantified for two, four, and seven doses per week by analyzing TFV-DP concentrations from the STRAND trial with the iPrEx HIV risk reduction model. PBMC collection, processing, and storage for the iPrEx and STRAND trials used identical laboratory protocols, and drug concentration testing was performed in the same laboratory using identical laboratory methods.
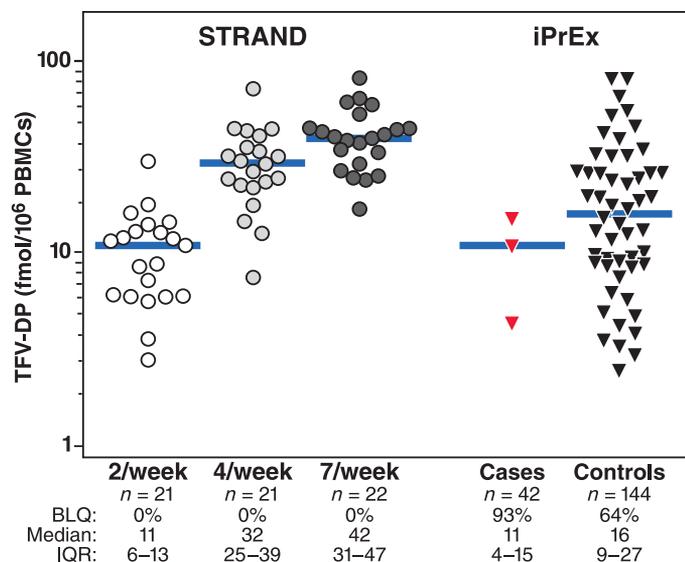
Fig. 1. TFV-DP concentrations in the STRAND and iPrEx trials. The values observed in the STRAND study are shown on the left for two doses per week (open circles), four doses per week (light gray circles), and seven doses per week (dark gray circles). iPrEx values on the right included those from the visit with first evidence of HIV infection in cases (red triangles) and the matched study visit in HIV-negative controls (black triangles). The blue bars represent the medians. The numbers of participants tested (the proportion of PBMCs tested with detectable TFV-DP concentrations), the median concentrations among values in the detectable range, and the IQRs are listed below the x axis. BLQ, below the limit of quantification of the assay.

Predicted TFV-DP concentrations in the STRAND trial

STRAND was an open-label crossover study of oral TDF in 24 HIV-negative adults (FTC was not included), each of whom received two, four, and seven doses per week for 6 weeks. Dosing was directly observed Monday to Friday, including all of the two and four doses per week (Tuesday/Wednesday and Monday/Tuesday/Thursday/Friday, respectively), and participants provided confirmation of the date and time of doses taken on Saturday and Sunday by text message or telephone contact on the day of use. TFV-DP concentrations in PBMCs were measured at the end of the 6 weeks of each dosing regimen. The median [interquartile range (IQR)] values in STRAND were 11 fmol/10^6 PBMCs (6 to 13) for two doses per week, 32 fmol/10^6 PBMCs (25 to 39) for four doses per week, and 42 fmol/10^6 PBMCs (31 to 47) for seven doses per week (left portion of Fig. 1). The median (IQR) times from the last dose to PBMC collection were 24 (20 to 141) hours, 25 (22 to 62) hours, and 24 (21 to 25) hours, respectively. TFV-DP concentrations were quantifiable in all STRAND participants at all visits.
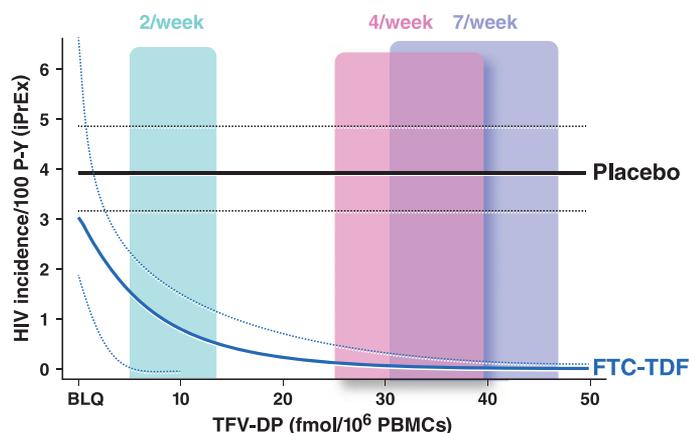
Drug detection at the visit with first evidence of HIV infection in the iPrEx trial

The iPrEx study was used to identify drug concentrations associated with different levels of protection from HIV-1 acquisition. Plasma and/or PBMCs were tested for FTC-TDF concentrations in all 48 seroconverters at the visit when HIV-1 infection was first detected, using either plasma viral RNA, serum antibodies, or both. The median time between the last HIV-negative test and the first evidence of HIV was 33 days (IQR, 28 to 48 days). Each HIV-infected case was matched to three seronegative controls in the FTC-TDF arm by study site and duration of the study. One of the three controls was selected on the basis of having reported high-risk sexual practices, to ensure comparable HIV-1 exposure with cases, and the other two were selected randomly. The cases and controls were comparable with respect to HIV risk factors, level of schooling, and alcohol use (Table 1). Both plasma and PBMCs were tested in 42 of 48 (88%) of cases at the visit when HIV-1 infection was first detected and in 144 of 144 (100%) of controls at the matched time point of the study. Plasma and/or PBMCs were also tested from other longitudinal study visits in the

Table 1. Demographic and HIV risk characteristics at baseline among analysis groups in the iPrEx study.

	Cases (n = 48)	Controls (n = 144)	P
Baseline			
Age in years, mean (SD)	24 (6)	27 (8)	0.01
Calculated CrCl ml/min, mean (SD)	125 (23)	121 (22)	0.30
Male partners in past 6 months, mean (SD)	12 (32)	13 (46)	0.78
Secondary school, n (%)	31 (66)	109 (76)	0.19
Alcohol use (≥5 drinks per day on days when drinking), n (%)	24 (52)	74 (54)	0.87
Unprotected receptive anal intercourse, n (%)	34 (71)	109 (76)	0.57
History of sexually transmitted infection in past 6 months, n (%)	15 (31)	52 (36)	0.60
Herpes seropositive, n (%)	24 (50)	69 (48)	0.87
At time of HIV detection in cases			
Weeks on study, mean (SD)	63 (35)	65 (36)	0.73
Unprotected receptive anal Intercourse, n (%)	19 (40)	74 (51)	0.18

cases and controls. Drug detection was defined as any quantifiable moiety among plasma TFV, FTC, and intracellular TFV-DP and FTC-TP. Drug detection in plasma and PBMCs in the iPrEx trial was concordant in >95% of all pairwise comparisons, indicating that plasma and PBMC analysis yielded similar estimates of the proportion of groups having detectable drug.

Any drug moiety was detected 5.5-fold less frequently (8% versus 44%; $P < 0.001$) in HIV-positive cases at the visit when HIV infection was first detected compared with the matched time point in HIV-negative controls (Fig. 2). Lower drug detection among cases versus controls (11% versus 51%; $P < 0.001$) was also observed within the 90 days before the HIV infection visit or matched time point in controls. At time points more than 90 days before HIV was first detected, the proportion of cases and controls with any detectable drug was comparable (36% versus 41%; $P = 0.77$), indicating that HIV infection occurred during periods of low drug exposure in the active arm of the iPrEx trial. These drug detection rates suggest that a substantial fraction of iPrEx participants were dosing with fewer than two doses per week, given the 100% TFV-DP detection rate for two doses per week in the STRAND trial (right portion of Fig. 1).

TFV-DP concentrations and risk of HIV acquisition

In the iPrEx seronegative controls having quantifiable TFV-DP concentrations (36% of the total), the median concentration was 16 fmol/10^6 PBMCs (IQR, 9 to 27), which was between the median concentrations observed for two and four doses per week in the STRAND trial (Fig. 1). Only 18% of seronegative controls had TFV-DP concentrations in the range associated with daily dosing in the STRAND study. This suggests that TFV-DP concentrations below those associated with daily dosing were exerting antiviral effects to give the 44% overall efficacy in the group randomized to FTC/TDF versus placebo. Only three HIV-infected cases had detectable TFV-DP concentrations at the time HIV was first detected. These concentrations were in the range of those associated with

two doses per week in the STRAND trial and none were in the range of daily dosing.

The TFV-DP concentration in PBMCs associated with HIV-1 acquisition was estimated using a Cox proportional hazards model that assessed the risk of HIV infection in the iPrEx trial as a function of TFV-DP (or FTC-TP) concentrations in active-arm participants. Exponential regression was also used in a second analysis. Multiple imputation (8, 9) was used to construct a data set with drug concentrations at each visit when an HIV-1 test was performed. If a drug concentration was not available, imputation was used using information about the participant [age, site, time on study, numbers of partners, creatinine clearance, alcohol use, secondary education, infection with herpes simplex virus (HSV), sexually transmitted disease, pharmacy drug dispensation records, and unprotected receptive anal intercourse (URAI) at baseline and follow-up] to estimate the missing concentration. This approach allowed HIV-1 risk to be estimated for the entire cohort and to estimate how drug exposure as a time-dependent variable was associated with HIV risk compared with the placebo arm. Drug concentrations below the limit of quantitation (BLQ) were set to 0 fmol/10^6 PBMCs.

The relationship between drug concentrations and HIV infection risk was significant for both TFV-DP ($P = 0.016$) and FTC-TP ($P = 0.004$) among those assigned to receive oral FTC/TDF. Compared with HIV incidence for the placebo arm, the TFV-DP concentrations associated with 50, 90, and 99% reduced HIV-1 acquisition were 3 [95% confidence interval (CI), <1 to 7], 16 (95% CI, 3 to 28), and 33 (6 to 60) fmol/10^6 PBMCs, respectively. For FTC-TP, the corresponding values were 0.82 (0.1 to 1.6), 3.7 (1.2 to 6.1), and 7.7 (2.6 to 12.9) pmol/10^6 PBMCs, respectively. However, neither TFV-DP nor FTC-TP concentrations were independent of the other in a model that included both; therefore, further analyses focused on TFV-DP concentrations so that TFV-DP concentrations from the STRAND trial could be used.

Dosing and risk of HIV acquisition

The exponential regression shown in Fig. 3 demonstrated that the risk of HIV infection was comparable among participants in the active arm with undetectable TFV-DP concentrations compared to the placebo arm (relative hazard, 0.78; 95% CI, 0.49 to 1.06; $P = 0.19$). Similar to the Cox analysis above, a TFV-DP concentration of 16 fmol/10^6 PBMCs was associated with 90% HIV risk reduction (EC_{90}) relative to placebo. For perspective, the concentrations from the STRAND study are shown in colored panels in Fig. 3. The EC_{90} (16 fmol/10^6 PBMCs) was 38% of the median TFV-DP concentration observed in those taking seven doses per week in STRAND (42 fmol/10^6 PBMCs).

Sensitivity analyses were conducted to evaluate the robustness of the estimated EC_{90} TFV-DP concentration in PBMCs. An analysis that adjusted for URAI (the main risk factor for HIV acquisition in this population) and other factors also used for multiple imputation, yielded an estimate of 15 (95% CI, 3 to 27) fmol/10^6 PBMCs. Allowing drug concentrations below the limit of quantitation to vary uniformly between 0 and 5 yielded an estimate of 20 (95% CI, 7 to 33) fmol/10^6 PBMCs. Using the averaged drug concentrations from the visit closest to HIV infection with values in the previous 90 days yielded an estimate of 23 (5 to 41) fmol/10^6 PBMCs. An analysis that brought drug detection at subsequent seroconversion time points to the time of the first detection of HIV-1 RNA yielded an estimate of 19 (95% CI, 4 to 33) fmol/10^6 PBMCs. Adjustment of TFV-DP concentration (from 4.19 to 13.4 fmol/10^6 PBMCs) in one person whose blood specimen was

Fig. 2. Longitudinal drug detection relative to the time of first HIV-1 detection in the iPrEx trial. Shown are the percent of case and control time points with any drug detection in plasma or PBMCs, including TFV, FTC, TFV-DP, or FTC-TP, at the time of first laboratory evidence of HIV in cases or the matched time point in controls (dashed vertical line), and at pre- and post-HIV infection time points. The pre- and post-HIV infection time points were divided into those within 3 months (90 days) before or after HIV infection, and those within 6-month intervals distal to these time points. The number of time points with either plasma or PBMCs available for testing is listed for each time period. *$P < 0.05$, cases versus controls.

Fig. 3. Estimated HIV incidence from exponential regression model of TFV-DP concentrations (fmol/10^6 PBMCs) in the iPrEx trial. The HIV infection rate in the placebo arm is shown as a horizontal black line at 3.9 infections per 100 person-years (P-Y), with the relative rate in the FTC-TDF arm (blue solid line) according to TFV-DP concentrations (x axis). Dashed lines represent the 95% CIs. The IQRs of TFV-DP concentrations associated with directly observed dosing in the STRAND study are provided as colored panels overlying the curves for HIV incidence versus TFV-DP concentration in the iPrEx trial. BLQ, below the limit of quantification of the assay.

Fig. 4. Percent of case and control time points with TFV-DP concentrations ≥16 fmol/10^6 PBMCs (estimated EC_{90}) in the iPrEx trial. Black circles represent HIV-negative controls, and red circles represent HIV-infected cases. The x axis represents time relative to the visit with first evidence of HIV infection in the case and the matching time point in controls. Pre- and post-HIV infection time points were divided into those within 3 months (90 days) before or after HIV infection, and those within 6-month intervals distal to these time points. The number of time points with PBMCs tested for TFV-DP concentrations is listed for each time period. *$P < 0.05$, cases versus controls.

available 7 days after stopping oral FTC/TDF at seroconversion yielded an estimate of 20 (95% CI, 4 to 36) fmol/10^6 PBMCs. Estimating a TFV-DP concentration associated with a 90% HIV risk reduction in cases versus controls was also possible using conditional logistic regression without multiple imputation. This analysis yielded a similar estimate for the EC_{90} for PBMCs of 16 fmol/10^6 PBMCs (95% CI, 8 to 44). All estimates were comparable with the initial EC_{90} estimate of 16 fmol/10^6 PBMCs and well within the range of concentrations achieved with four to seven tablets per week in the STRAND trial.

The fraction of case and control time points that exceeded the EC_{90} over time is shown in Fig. 4. At the visit when HIV was first detected, no cases (0 of 42) had TFV-DP concentrations ≥16 fmol/10^6 PBMCs, compared with 18% (26 of 144) of seronegative controls at the matched time point ($P < 0.001$; Fig. 4). The proportion of iPrEx participant time points with TFV-DP concentrations at or above the EC_{90} decreased among cases ($P = 0.02$) and controls ($P < 0.001$) over time. Reported risk behavior associated with exposure to HIV-1 also decreased over time among iPrEx participants (1).

TFV-DP concentrations arising from two, four, and seven directly observed doses per week in the STRAND study were analyzed with the exponential regression model from the iPrEx trial described above. The estimated PrEP efficacy was 76% (95% CI, 56 to 96%) for two doses per week, 96% (95% CI, 90 to >99%) for four doses per week, and 99% (95% CI, 96 to >99%) for seven doses per week. The proportions of people who attained the TFV-DP EC_{90} were 14% for two tablets per week, 90% for four tablets per week, and 100% for seven tablets per week.

DISCUSSION

This study identified that a TFV-DP concentration of 16 fmol/10^6 PBMCs was associated with 90% reduced risk of HIV-1 acquisition

in the iPrEx trial. The number of tablets per week to consistently reach that concentration was estimated to be four or more using information from a directly observed dosing study, STRAND. These findings together suggest that oral FTC-TDF PrEP is a robust intervention for men having sex with men, with a relatively wide prophylactic window.

Drug exposure in HIV-infected cases in the iPrEx trial was critically low at the time of first laboratory evidence of HIV infection, providing a likely explanation for HIV acquisition in these participants. Other evidence indicated negligible drug exposure near the time of HIV infection in cases: Plasma HIV-1 RNA levels were comparable in the placebo and active arms, and there was no evidence of TDF or FTC resistance among emergent infections in the active arm (1). Drug detection in controls was higher than in cases but was not commensurate with daily dosing in the majority. Only 44% of controls had any detectable drug moiety at the matching time point of the case. Only 18% of seronegative controls had TFV-DP concentrations above 16 fmol/10^6 PBMCs, a level achieved by 90% of STRAND trial participants taking four or more doses per week, and a level associated with a 90% HIV infection risk reduction.

The minimum protective drug concentrations in the blood and the number of tablets per week required to achieve those concentrations may differ depending on the route and frequency of exposure to HIV (10). For men who have sex with men, the tissue of greatest relevance to the acquisition of HIV-1 infection is the rectal mucosa. Oral dosing has been shown to deliver 20- to 100-fold higher TFV-DP in rectal tissue compared with blood or vaginal/cervical tissue (11, 12). TFV-DP delivery to penile tissue, relevant for male insertive exposures, has not been determined to our knowledge. The high delivery of TFV-DP to rectal mucosa suggests that pharmacological findings relevant for men who have sex with men, such as in this study, may not directly extrapolate to parenteral, vaginal, or penile exposures.

This study has demonstrated that the EC_{90} in PBMCs (16 fmol/10^6 PBMCs) was 38% of the median for daily dosing in the STRAND trial

(42 fmol/10^6 PBMCs). With this information, a rectal mononuclear cell EC_{90} can be estimated. The rectal mononuclear cell concentration observed with daily oral dosing was reported to be 1846 fmol/10^6 cells (95% CI, 931 to 3659) (11). Assuming that the kinetics of TFV-DP in rectal mononuclear cells are similar to those in PBMCs, the EC_{90} in rectal mononuclear cells would be 38% of this value, or about 700 fmol/10^6 rectal cells (95% CI, 350 to 1400). This estimate makes several assumptions that require further validation but nevertheless provides a starting point for a target cell concentration in tissue to translate into animal or ex vivo systems for validation (13, 14). The threshold identified here is analogous to the TFV concentration threshold of 1000 ng/ml in vaginal fluid identified as protective in another PrEP trial, the CAPRISA 004 study, and in ex vivo assays (5, 14).

Drug concentrations were measured as close to HIV infection as possible (at the time HIV infection was first discovered and within 90 days of this time point). Through use of multiple imputations, drug concentrations were assigned to all active-arm participants at the time of HIV infection in placebo cases. However, the model could not account for variations in dosing patterns and relationship to timing of HIV exposure and transmission risk.

Confounding is also possible in this analysis, because there may be factors that link higher adherence with lower exposure to HIV. The finding that HIV acquisition among active-arm participants with undetectable drug concentrations was not higher than the placebo rate argues against confounding (Fig. 3). In addition, the statistical analysis adjusted for several markers of HIV incidence, including numbers of sexual partners, unprotected anal intercourse, sexually transmitted diseases, age, level of schooling, and substance use.

Viable cryopreserved PBMCs were available for drug analysis, whereas freshly processed and lysed PBMCs are traditionally used for cell pharmacology studies (15). Measurements of TFV-DP concentrations in PBMCs from the STRAND trial were a median of 48% (IQR, 38 to 67%) that of freshly lysed PBMCs also collected in that study. Processing PBMCs also introduced additional variability in TFV-DP/FTC-TP measurements. Despite this added variation, drug concentrations in blood were found to be strongly associated with reduced HIV risk in the active arm compared with placebo in the iPrEx trial. This finding suggests that drug concentration monitoring could inform HIV acquisition risk in persons taking FTC/TDF for PrEP. Other specimens, such as hair or dried blood spots, may afford more convenient long-term measures of drug exposure that will be particularly useful if they can be correlated with protective drug concentrations in PBMCs (16, 17).

This study identified a relationship between systemic drug exposure and reduction of HIV acquisition risk in one important population. Protective TFV-DP concentrations were readily achieved with four or more doses per week. This study focused on TFV-DP because independent relationships for FTC-TP could not be identified in the iPrEx study, and the STRAND trial did not include FTC-TP; thus, expected concentrations for nondaily dosing are not known. FTC coadministration is not expected to affect intracellular concentrations of TFV-DP in PBMCs (18). The TFV-DP EC_{90} in the iPrEx trial was calculated with the presence of FTC-TP, indicating that reaching these protective TFV-DP concentrations with FTC-TDF dosing is relevant for PrEP efficacy in men who have sex with men. Nevertheless, future studies should aspire to evaluate the contribution of FTC-TP, as well as TFV-DP, to PrEP efficacy.

Alternative dosing regimens, such as pre- and post-intercourse dosing, warrant controlled clinical trials to evaluate the acceptability, behav-ioral feasibility, and pharmacokinetics of nondaily regimens. The 95% CI for the estimate of the TFV-DP EC_{90} was 3 to 28 fmol/10^6 PBMCs. The lower bound (3 fmol/10^6 PBMCs) would be achievable after a single dose (19), and 28 fmol/10^6 PBMCs was just below the median achieved with four doses per week in STRAND. Whereas animal studies indicate that both pre- and post-exposure dosing are important (20), more information is needed to define the timing and duration of drug exposure that is required to prevent infection. Dose optimization in the absence of a surrogate marker of protection would require prohibitively large trials, so better definition of pharmacological parameters associated with differing dosing strategies (for example, dose and dosing interval) and protection will be essential to move the PrEP field forward. Ultimately, recommendations for daily PrEP use may be more robust because daily regimens encourage a routine and afford drug concentrations that are expected to persist in the protective range even if some doses are missed. The low fraction of iPrEx participants with TFV-DP concentrations in the range of daily dosing suggests that demonstration projects should optimize ways to promote daily dosing.

In conclusion, this study identified a target TFV-DP concentration for protecting against HIV-1 acquisition in men who have sex with men. This threshold should enable further studies to be conducted in men who have sex with men to evaluate new ways of promoting the consistent use of PrEP, thus maximizing the probability of PrEP efficacy.

MATERIALS AND METHODS

iPrEx trial
The design, conduct, and outcomes of the iPrEx trial have been published previously (1). Briefly, the iPrEx initiative was a randomized, double-blinded, controlled trial of daily FTC-TDF versus placebo in HIV-negative men and transgender women 18 years or older who have sex with men, meeting behavioral criteria that put them at risk for sexual acquisition of HIV. Two thousand four hundred ninety-nine participants were randomized and followed monthly through a median of 87 weeks of therapy (IQR, 61 to 125). In the final analysis, 83 infections were observed in the placebo arm versus 48 infections in the FTC-TDF arm (efficacy, 42%; 95% CI, 18 to 60%) (21).

Plasma specimens were stored every 12 weeks, and viable cryopreserved PBMC specimens were stored every 24 weeks and at the time of study discontinuation or seroconversion. Each participant from the active arm who contracted HIV during the study was included in this pharmacology substudy (cases). For each visit week when HIV was detected in cases, samples were selected from three HIV-negative controls from the active arm at the same site: two randomly and one from among those reporting URAI in a recall period before the specimen collection. The latter control was chosen to enrich the control sample for people exposed to HIV, to better match the HIV-infected cases. Specimens were tested from the time of first evidence of HIV infection in the cases, the nearest visit week in controls, as well as longitudinally at other available time points during the treatment period.

STRAND study
The STRAND study was an open-label, randomized, six-sequence, three-period, single-site, crossover trial in 24 HIV-negative adults (12 men and 12 women) that tested the effects of three different oral TDF dosing regimens on TFV concentrations in hair, plasma, and PBMCs. The six sequences were different orders of the three dosing

strategies: seven doses per week, four doses per week, and two doses per week. The two doses per week were taken on Tuesday and Wednesday, and the four doses per week on Monday, Tuesday, Thursday, and Friday. Each dosing period lasted 6 weeks. All doses scheduled Monday to Friday were directly observed by study staff, and the doses on the weekend were confirmed by telephone or text message. Stored viably cryopreserved PBMCs (using the same method as in iPrEx) were collected at the end of each 6-week dosing period. Freshly processed/lysed PBMCs were available from a subset of participants. The study was funded by the U.S. National Institutes of Health and was approved by the University of California, San Francisco, Committee on Human Research.

Analytical pharmacology

A liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay was validated for the determination of TFV and FTC in human plasma (22). The method includes protein precipitation and stable isotopic internal standards. The linearity of the concentration curves was in the range of 10 to 1500 ng/ml for both analytes (250 μl of plasma extracted). The lower limit of quantification was 10 ng/ml for both analytes.

The viable PBMC processing procedure involved a quick thaw in a 37°C water bath and mixing by inversion (1 min). Cells were immediately transferred to a 15-ml centrifuge tube that already contained 10 ml of prewarmed (37°C) phosphate-buffered saline followed by gentle mixing by inversion. Cells were then pelleted and the supernatant was discarded. If red blood cell (RBC) contamination was visible, the cells were treated with an RBC lysing buffer. Cells were then counted with an automated hemocytometer. Viability and total cell count were recorded; cells were kept on crushed ice through the process. Cells were washed and lysed. Median (IQR) viability at the time HIV was discovered was 66% (56 to 76%) overall, 71% (56 to 79%) in cases, and 64% (56 to 73%) in controls.

A validated LC-MS/MS assay was used for the determination of TFV-DP and FTC-TP from lysed intracellular matrix (15). The method used a strong anion exchange isolation of mono-, di-, and triphosphates from intracellular matrix. The triphosphate fraction was then dephosphorylated to the parent moiety, yielding a molar equivalent to the original nucleotide analog intracellular concentration. The analytical portion included desalting/concentration by solid-phase extraction and detection by LC-MS/MS. The quantifiable linear range for TFV-DP was 2.5 to 2000 fmol per sample and that for FTC was 0.1 to 200 pmol per sample. Stable labeled isotopic internal standards facilitated accuracy and precision in various cell matrices (15). Two million total cells were typically extracted, constituting the "sample," and results were corrected for cell viability and normalized to femtomole or picomole per million viable cells.

Stored viable PBMC samples such as in this study have been used for the measurement of intracellular TFV-DP in previous studies [see Appendix in (1)]. These studies demonstrated that values in viably cryopreserved PBMCs (median, about 40 fmol/10^6 viable PBMCs) were lower than those measured in pharmacology studies among HIV-infected subjects, where PBMC samples were processed and lysed immediately (median, about 90 fmol/10^6 PBMCs) (23, 24).

To evaluate viable cells in more detail, an internal study called iOptimum enrolled 10 HIV-infected participants for a single blood draw to assess the TFV-DP and FTC-TP in viable cryopreserved cells. Self-reported adherence in the previous 30 days was 63 to 100%, and

viral loads were largely suppressed (range, <40 to 240 copies/ml), suggesting good adherence to treatment. Aliquots of viable cells were stored in liquid nitrogen for 4, 12, and 24 weeks. Freshly lysed cells were also collected. Both TFV-DP and FTC-TP were quantifiable in all viable PBMC samples and above the lower limit of quantitation by several fold. The median (IQR) ratio of viable PBMCs/PBMCs at weeks 4 and 12 in liquid nitrogen storage was 0.3 (0.22 to 0.38). The median (IQR) value for 24 weeks in liquid nitrogen was 0.56 (0.38 to 0.83), which was similar to STRAND, 0.48 (0.38 to 0.67). Median (IQR) time in liquid nitrogen for iPrEx and STRAND samples was 70 (46 to 108) weeks and 57 (52 to 65) weeks, respectively. We have observed no loss in viable cell concentrations for storage times in liquid nitrogen through 119 weeks (2.3 years) (1).

Statistical methods

All analyses were performed with Stata 12.1 (25).

Model for the effect of intracellular drug concentration

This analysis fits a (stratified by site) Cox proportional hazards model to the entire cohort with the following model:

$$h_k(t; Z(t), \text{rx}) = h_{0k}(t)\exp(\beta_1 \text{rx} + \beta_2 Z(t)) \qquad (1)$$

$h_k(t)$ is the hazard of HIV infection at the kth site and rx = 1 if the participant is assigned to FTC/TDF and 0 if the participant is assigned to placebo. The variable $Z(t)$ is a time-dependent covariate for a quantitative drug concentration (for example, TFV-DP), where $Z(t)$ is set to be a drug concentration of 0 if the participant is on the placebo group. We examined quadratic and logarithmic transformations of the concentration $Z(t)$, but neither improved the fit.

The primary analyses have used models stratified by clinic site to avoid confounding the association between drug concentrations and HIV acquisition. Stratification was incorporated into the design of the nested case-control study by matching controls by study site. Stratification can be highly efficient relative to an unstratified model even if confounding by site is weak (26).

Model 1 uses the placebo group as the baseline hazard function permitting comparison of the hazard of HIV acquisition at a given drug concentration to the placebo group. For instance, the concentration $Z(t)$, which is associated with a d% reduction in risk relative to placebo, is as follows:

$$\{\log(1 - d/100) - \beta_1\}/\beta_2$$

The concentration associated with a d% reduction in risk relative to a 0/BLQ concentration on the FTC/TDF arm is as follows:

$$\log(1 - d/100)/\beta_2$$

Here, β_1 is the log hazard ratio of a 0/BLQ level on the FTC/TDF arm (relative to placebo), β_2 quantifies the change in risk with the concentrations of drug, and $h_{0k}(t)$ is the baseline hazard of HIV seroconversion on the placebo arm.

We also fit

$$\alpha(t; Z, \text{rx}) = \alpha \exp\{\alpha_1 \text{rx} + \alpha_2 Z(t)\} \qquad (2)$$

where this exponential model is parameterized to yield the annualized incidence of HIV. The fit of this model to the data yielded similar results to the fit of model 1 and was used as the basis of the annualized HIV incidence graphed in Fig. 3.

Note that models 1 and 2 require a complete set of drug concentrations for participants in the active arm. We must confront two types of missing data. First, only a randomly selected subset of time points had drug concentrations tested. Second, intracellular drug (TFV-DP, FTC-TP) was only tested every 6 months and at the time of HIV seroconversion. Hence, even if a control has drug concentration tested, the information is sparse and may not correspond to an ideal time match to their HIV case.

We used two strategies for fitting model 1 with missing data in the drug concentrations: multiple imputation and conditional logistic regression. Conditional logistic regression functions only as a sensitivity analysis because it is unable to use the placebo group as a reference and does not handle the uncertainty in drug concentrations in the controls because of the long periods of time between measurements of drug concentrations.

Multiple imputation

The concentration of drug for the HIV-infected cases at the time of HIV infection was taken as the intracellular concentration observed at the first laboratory evidence of HIV infection. Six participants lacked an intracellular specimen at the first laboratory evidence of HIV infection—all had a plasma specimen at the first evidence of infection and all were BLQ for TFV and FTC. In the case-control study of 355 plasma specimens that are BLQ for TFV and FTC, 96% were BLQ for TFV-DP and 95% were BLQ for FTC-TP. Hence, the TFV-DP and FTC-TP for these six seroconverters were set as BLQ.

For controls, we attempted to infer the drug concentration at the identical day of follow-up as seroconverters from the same site. We used the closest intracellular drug concentration within 45 days as the drug concentration if one was available and considered that as a measured drug concentration. If there was no tested drug concentration within 45 days, then drug concentrations were imputed. Varying the 45-day window had little effect on the results.

If the visit had plasma but not intracellular concentrations (PBMCs), we used whether the plasma had detectable or not in our imputation model. We performed multiple imputations (9) of TFV-DP, FTC-TP, and any detection of drug in plasma or PBMCs using medication possession ratio (defined as the number of tablets dispensed at the previous visit divided by days since the last visit at which medication was dispensed), study week, URAI at baseline, URAI at follow-up (most recent report on or after the time point), baseline HSV status, number of male sexual partners at screening, participant report of a sexually transmitted infection in the 6 months before enrollment, secondary education, age at enrollment (in years), and baseline (estimated) creatinine clearance (by Cockcroft-Gault equation). Plasma and PBMC concentrations have monotone missingness; hence, chained imputation was used to create joint imputations. The imputation model for detection of drug in plasma was based on logistic regression, and imputations for TFV-DP and FTC-TP were based on predictive means matching (8). Predictive means matching used a regression model to identify observed values in the data, which form the most plausible value for the imputations, yielding nonparametric imputations that must follow the observed distribution of the drug concentrations. This method seamlessly imputed concentrations of TFV-DP and FTC-TP that were BLQ. This permitted us to form imputations in a first stage of analysis and to vary the strategy for quantifying BLQ values in a second stage. We performed 20 imputations per observation.

Conditional logistic regression

Analyses with conditional logistic regression mimic a (stratified) Cox proportional hazards model among participants on the active arm of the form

$$h_k(t; Z) = h_{0k}(t)\exp\{\beta_3 Z(t)\} \qquad (3)$$

where k is the clinic site and $Z(t)$ is a time-dependent covariate for drug concentration. Prentice and Breslow demonstrated that by measuring covariates in cases and a sample of (time matched) controls, it was possible to fit model 3 using a conditional logistic regression model (27). Hence, the coefficient in the conditional logistic regression is a valid estimator of the log hazard ratio β_3, which is the risk reduction compared to a drug concentration of 0 on the active arm. As a sensitivity analysis, we compared the results of our estimated protective concentrations by calculating the intracellular drug concentrations associated with a d% reduction in risk relative to a 0/BLQ concentration on the FTC/TDF arm as follows:

$$\log(1 - d/100)/\beta_3$$

Inferred protection

On the basis of estimates for model 1 fit using multiple imputation, we estimated the protective effect of dosing regimens used in STRAND. For this, we combined the parameter estimates from model 1 with the observed TFV-DP from the two, four, and seven tablets per week regimens in STRAND. The estimated relative risk reduction was approximated by the following equation:

$$100\left(1 - n_D^{-1}\sum_{i=1}^{n_D} \exp\{\hat{\beta}_1 + \hat{\beta}_2 Z_{iD}\}\right)$$

where $(Z_{1D}, \ldots, Z_{n_D D})$ were the n_D observed TFV-DP concentrations for D tablet per week regimen, and the parameter estimates were taken from fitting model 1 using multiple imputation with associated 0.95 level Wald-based CIs.

REFERENCES AND NOTES

1. R. M. Grant, J. R. Lama, P. L. Anderson, V. McMahan, A. Y. Liu, L. Vargas, P. Goicochea, M. Casapía, J. V. Guanira-Carranza, M. E. Ramirez-Cardich, O. Montoya-Herrera, T. Fernández, V. G. Veloso, S. P. Buchbinder, S. Chariyalertsak, M. Schechter, L. G. Bekker, K. H. Mayer, E. G. Kallás, K. R. Amico, K. Mulligan, L. R. Bushman, R. J. Hance, C. Ganoza, P. Defechereux, B. Postle, F. Wang, J. J. McConnell, J. H. Zheng, J. Lee, J. F. Rooney, H. S. Jaffe, A. I. Martinez, D. N. Burns, D. V. Glidden; iPrEx Study Team, Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. N. Engl. J. Med. 363, 2587–2599 (2010).
2. UNAIDS, 2010.
3. S. Baral, F. Sifakis, F. Cleghorn, C. Beyrer, Elevated risk for HIV infection among men who have sex with men in low- and middle-income countries 2000–2006: A systematic review. PLoS Med. 4, e339 (2007).
4. D. Donnell et al., paper presented at the 19th Conference on Retroviruses and Opportunistic Infections, Seattle, WA, 5 to 8 March 2012.
5. S. S. Karim, A. D. Kashuba, L. Werner, Q. A. Karim, Drug concentrations after topical and oral antiretroviral pre-exposure prophylaxis: Implications for HIV prevention in women. Lancet 378, 279–281 (2011).
6. M. C. Thigpen et al., paper presented at the 6th IAS Conference on HIV Pathogenesis, Treatment and Prevention, Rome, Italy, 2011.
7. L. Van Damme et al., paper presented at the 19th Conference on Retroviruses and Opportunistic Infections, Seattle, WA, 5 to 8 March 2012.
8. R. J. A. Little, Missing-data adjustments in large surveys. J. Bus. Econ. Stat. 6, 287–296 (1988).
9. J. L. Schafer, Multiple imputation: A primer. Stat. Methods Med. Res. 8, 3–15 (1999).

10. A. D. Kashuba, K. B. Patterson, J. B. Dumond, M. S. Cohen, Pre-exposure prophylaxis for HIV prevention: How to predict success. *Lancet* **379**, 2409–2411 (2012).

11. P. L. Anderson *et al.*, paper presented at the 19th Conference on Retroviruses and Opportunistic Infection, Seattle, WA, 2012.

12. K. B. Patterson, H. A. Prince, E. Kraft, A. J. Jenkins, N. J. Shaheen, J. F. Rooney, M. S. Cohen, A. D. Kashuba, Penetration of tenofovir and emtricitabine in mucosal tissues: Implications for prevention of HIV-1 transmission. *Sci. Transl. Med.* **3**, 112re4 (2011).

13. P. Anton *et al.*, paper presented at the 18th Conference on Retroviruses and Opportunistic Infections, Boston, MA, 2011.

14. M. J. Keller, R. P. Madan, N. M. Torres, M. J. Fazzari, S. Cho, S. Kalyoussef, G. Shust, P. M. Mesquita, N. Louissaint, J. Chen, H. W. Cohen, E. C. Diament, A. C. Lee, L. Soto-Torres, C. W. Hendrix, B. C. Herold, A randomized trial to assess anti-HIV activity in female genital tract secretions and soluble mucosal immunity following application of 1% tenofovir gel. *PLoS One* **6**, e16475 (2011).

15. L. R. Bushman, J. J. Kiser, J. E. Rower, B. Klein, J. H. Zheng, M. L. Ray, P. L. Anderson, Determination of nucleoside analog mono-, di-, and tri-phosphates in cellular matrix by solid phase extraction and ultra-sensitive LC-MS/MS detection. *J. Pharm. Biomed. Anal.* **56**, 390–401 (2011).

16. J. Castillo-Mancilla *et al.*, paper presented at the 19th Conference on Retroviruses and Opportunistic Infections, Seattle, WA, 5 to 8 March 2012.

17. A. Liu *et al.*, paper presented at the 17th Conference on Retroviruses and Opportunistic Infections, San Francisco, CA, 2010.

18. K. Borroto-Esoda, J. E. Vela, F. Myrick, A. S. Ray, M. D. Miller, In vitro evaluation of the anti-HIV activity and metabolic interactions of tenofovir and emtricitabine. *Antivir. Ther.* **11**, 377–384 (2006).

19. P. L. Anderson *et al.*, paper presented at the 18th Conference on Retroviruses and Opportunistic Infections, Boston, MA, 27 February to 2 March 2011.

20. J. G. García-Lerma, M. E. Cong, J. Mitchell, A. S. Youngpairoj, Q. Zheng, S. Masciotra, A. Martin, Z. Kuklenyik, A. Holder, J. Lipscomb, C. P. Pau, J. R. Barr, D. L. Hanson, R. Otten, L. Paxton, T. M. Folks, W. Heneine, Intermittent prophylaxis with oral Truvada protects macaques from rectal SHIV infection. *Sci. Transl. Med.* **2**, 14ra4 (2010).

21. R. Grant *et al.*, paper presented at the 6th IAS Conference on HIV Pathogenesis, Treatment and Prevention, Rome, Italy, 2011.

22. T. Delahunty, L. Bushman, B. Robbins, C. V. Fletcher, The simultaneous assay of tenofovir and emtricitabine in plasma using LC/MS/MS and isotopically labeled internal standards. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **877**, 1907–1914 (2009).

23. G. Baheti, J. J. Kiser, P. L. Havens, C. V. Fletcher, Plasma and intracellular population pharmacokinetic analysis of tenofovir in HIV-1–infected patients. *Antimicrob. Agents Chemother.* **55**, 5294–5299 (2011).

24. J. J. Kiser, C. V. Fletcher, P. M. Flynn, C. K. Cunningham, C. M. Wilson, B. G. Kapogiannis, H. Major-Wilson, R. M. Viani, N. X. Liu, L. R. Muenz, D. R. Harris, P. L. Havens; Adolescent Trials Network for HIV/AIDS Interventions, Pharmacokinetics of antiretroviral regimens containing tenofovir disoproxil fumarate and atazanavir-ritonavir in adolescents and young adults with human immunodeficiency virus infection. *Antimicrob. Agents Chemother.* **52**, 631–637 (2008).

25. StataCorp, *Stata 12.1* (StataCorp LP, College Station, TX, 2011).

26. D. V. Glidden, E. Vittinghoff, Modelling clustered survival data from multicentre clinical trials. *Stat. Med.* **23**, 369–388 (2004).

27. R. L. Prentice, N. E. Breslow, Retrospective studies and failure time models. *Biometrika* **65**, 153–158 (1978).

Editor's Summary

PrEParing to Stop HIV Acquisition

Pre-exposure prophylaxis (PrEP) using the antiretroviral drugs emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) is a recently proven strategy for preventing HIV acquisition. These drugs require phosphorylation in mononuclear cells to the pharmacologically active triphosphate moieties, called emtricitabine-triphosphate (FTC-TP) and tenofovir-diphosphate (TFV-DP). The iPrEx study was a randomized placebo-controlled trial of daily oral doses of FTC-TDF as PrEP in HIV-negative men who have sex with men. Participants all received a comprehensive package of HIV prevention services. HIV infections were reduced by 44% overall in the FTC-TDF arm relative to placebo. HIV risk was reduced by more than 90% among those having detectable drug in blood, indicating that adherence was a powerful determinant of drug efficacy at preventing HIV acquisition. A new study by Anderson *et al.* estimates specific drug concentrations and adherence levels associated with protection from HIV-1 acquisition in the iPrEx trial. A regression analysis predicted that a TFV-DP concentration of 16 fmol/10^6 peripheral blood mononuclear cells (PBMCs) (95% confidence interval, 3 to 28) was associated with a 90% reduction in HIV acquisition relative to placebo in the iPrEx study. To determine the number of tablets required to achieve this drug concentration, TFV-DP concentrations from another study called STRAND were used to establish expected TFV-DP concentrations. TFV-DP was detected in the blood at all dosing levels in all participants with a median (interquartile range) TFV-DP concentration of 11 fmol/10^6 PBMCs (6 to 13) after two doses per week, 32 fmol/10^6 PBMCs (25 to 39) after four doses per week, and 42 fmol/10^6 PBMCs (31 to 47) after seven doses per week. When the iPrEx study's regression model was used to analyze the STRAND TFV-DP concentrations, the predicted HIV risk reductions were 76, 96, and 99% for two, four, and seven doses per week, respectively. These findings suggest that PrEP using oral FTC-TDF tablets is a robust intervention for preventing HIV acquisition among men who have sex with men.

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