Thymidine-sparing triple-nucleoside regimens have exhibited poor virologic response despite apparent phenotypic susceptibility to 2 of 3 regimen components at early time points. Phenotypic resistance masking by wild-type virus may explain this discrepancy. Consistent with this notion were (1) the presence of low-level nucleoside reverse-transcriptase inhibitor–resistant human immunodeficiency virus in subjects receiving failing first-line regimens consisting of tenofovir (TDF), abacavir (ABC), and lamivudine (3TC); (2) lower fold resistance associated with mixtures versus mutants in a clinical-isolate database; and (3) dose-dependent changes in susceptibility to ABC, 3TC, TDF, and didanosine on titration of K65R and/or M184V with wild-type virus. These findings underscore the limitations of stand-alone phenotypic susceptibility measures and emphasize the importance of complementary and/or more sensitive techniques. (ClinicalTrials.gov identifier: NCT00053638.)

Several clinical studies of thymidine-sparing (zidovudine or stavudine) triple-nucleoside-analogue therapy in treatment-naive HIV-1–infected subjects have been prematurely halted when an unacceptably high proportion of subjects (33%-63%) experienced early virologic failure. The regimens included the combination of tenofovir (TDF for studies in vivo, and TFV for in vitro assays), lamivudine (3TC), and didanosine (ddI) or abacavir (ABC) [1–5]. In these studies, high rates of selection for the reverse-transcriptase (RT) mutations K65R and M184V were observed by population sequencing. Phenotypic analyses indicated that, although the majority of samples had reduced susceptibility to 3TC, very few had apparent reduced susceptibility to other study drugs.

To better understand why virologic failure occurred despite apparent susceptibility to 2 of the 3 drugs in the regimen, we evaluated minor variants via longitudinal clonal analysis. For this assessment, HIV genotypes and phenotypic susceptibilities were determined for 2 subjects with early virologic nonresponse who were receiving a TDF/ABC/3TC regimen in the ESS30009 study. To further understand the phenotypes of mutant plus wild-type mixtures, we determined the susceptibility of clinical-isolate samples with mixtures at positions 65 and 184 in RT across a large clinical database. Finally, we assessed the ability of a phenotypic assay to detect reduced nucleoside RT inhibitor (NRTI) susceptibility in samples containing defined mixtures of wild-type and K65R and/or M184V.

Methods. The ESS30009 study was approved by the institutional review boards of each site, and all participants provided written informed consent. A limited number of the 41 subjects in the ESS30009 study receiving the TDF/ABC/3TC regimen who experienced virologic nonresponse had baseline and week 12 HIV genotypic and phenotypic data (PhenoSense GT; Monogram Biosciences) as well as plasma stored from weeks 2, 4, and 8. Additional genotypic analysis was done with week 2, 4, and 8 samples for 2 subjects by means of the TruGene HIV-1 genotyping assay (Bayer HealthCare).

For clonal genotypic analysis, individual RT sequences from patient isolates were cloned from the TruGene RT PCR product by the ZERO Blunt Topo PCR cloning method (Invitrogen). The RT coding region from each individual bacterial colony was amplified by PCR and sequenced by standard methods.

Phenotypic susceptibility analysis of NL4-3–based and HXB2–based plasmid mixtures with K65R and/or M184V mutations was performed at Monogram Biosciences, using the cell-based portion of the PhenoSense assay. Clinical cutoffs for reduced drug susceptibility (fold change in IC50 vs. reference [FC]) using the phenotypic assay are as follows: for TDF, 1.4; for ddI, 1.3; for ABC, 4.5; and for 3TC, 3.5.
The sensitivity of the PhenoSenseGT assay for the detection of minority species on the basis of genotype is dependent on the relative chromatogram peak heights of each particular mutation. Detection of low levels of viral variants is optimized by combining computerized analysis with intensive manual review by 2 operators. All mixtures are confirmed with chromatogram sequence data generated from both DNA strands. In DNA and virus mixture reconstruction experiments, the sensitivity for the detection of minor populations of resistance-associated mutations ranged from 10% to 30% (Monogram GeneSeqHIV assay validation; data on file). Overall, detection of viruses with reduced phenotypic susceptibility was dependent on the drug and the mutant virus in question.

The Monogram Biosciences database of phenotype and genotype results from clinical samples was queried for samples with the K65R and/or M184I/V mutations, either apparently unmixed or as mixtures with the wild-type amino acid. Note that use of parentheses at position 184 of M184M(I/V) indicates M184M plus I and/or V (a slash always indicates “and/or”). Other NRTI-associated resistance mutations (M41L, D67N, K70R, L74x, V75x, T69x, Y115F, Q151M, L210W, T215F, T215Y, or K219x, where x represents any change from wild type) were excluded. Samples used in this analysis were collected between January 2002 and April 2004.

The site-directed mutations K65R and M184V were made in a NL4-3 plasmid at Monogram Biosciences. The K65R/M184V double mutant was created in a plasmid containing the HIV-1 HXB-2-RT at GlaxoSmithKline. Mixtures were prepared using plasmid DNA quantitated by UV spectroscopy or by comparison with known standards after resolution by gel electrophoresis.

Results. At the time of the unplanned ESS30009 interim analysis, 102 subjects had been randomized to receive TDF/ABC/3TC, and had ≥8 weeks of HIV RNA data available [2]. Of these 102 subjects, 50 (49%) met the unplanned analysis criteria for virologic nonresponse, and 41 (82%) of these 50 subjects had paired baseline and week 12 samples for which HIV genotype and phenotype was determined. Plasma-derived viruses from these 41 virologic nonresponders were susceptible to all study drugs at baseline, with a median baseline HIV RNA level of 4.8 log_{10} copies/mL. At week 12, the median HIV RNA level was 4.1 log_{10} copies/mL. By population sequencing, 40 (98%) of the 41 subjects had virus with M184M/I/V, and 22 (54%) had K65R or K65K/R mixtures. At week 12, susceptibility to ABC and TDF was observed in 33 (80%) and 39 (95%) of the 41 nonresponders, respectively. Examples of the virologic response and the resistance profile from 2 representative subjects from the ESS30009 study are shown in figure 1.

Clonal sequence analysis for subject 1 (figure 1A) revealed wild-type virus (1/22 [5%]) as well as genomes with the single mutations K65R (4/22 [19%]), M184I (11/22 [50%]), and M184V (6/22 [27%]) at week 2. Dual K65R/M184V mutants were detected at week 4 (1/43 [2%]) and week 8 (5/24 [21%]).

Subject 2 (figure 1B) experienced a brief period of virologic suppression to <1000 copies/mL, followed by viral load rebound to baseline levels at week 12 and a >1 log decrease by week 20. Viral mixtures containing M184M/I/V were detected at week 8. By week 12, population sequencing revealed the presence of K65KR and M184M/I/V, and phenotypic analysis indicated high-level 3TC resistance but minimal or no reduced susceptibility to ABC (FC, 2.3) or TDF (FC, 0.4). Clonal analysis indicated wild-type virus was still present at week 12 in 1 of 36 clones; however, the majority of viruses (35/36 [97%]) contained single mutations in RT at codon 184 and/or codon 65. Viruses with K65R/M184V were minority species (4/36 [11%]) at week 12; however, by week 20 all clones (28/28) harbored K65R/M184V.
To determine the phenotypic susceptibilities of clinical samples with K65R and/or M184I/V, we used the Monogram Biosciences database of 22,494 clinical samples with paired genotypic and phenotypic data collected from 2002 through 2004 to identify 2999 samples containing K65R and/or M184I/V as the only NRTI mutations. Of these, 25% appeared to be mixed mutant/wild-type isolates. There were clear differences in phenotypic susceptibilities; more apparently pure mutant samples had reduced susceptibility to nonthymidine NRTIs than did matching mixture samples. More specifically, when K65R was detected with no wild-type or other apparent NRTI mutants (n = 139 samples), 98% exhibited reduced susceptibility to

Figure 2. Possible overestimation of abacavir, lamivudine, tenofovir, and didanosine susceptibility when K65R and/or M184V are present as mixtures. In panels A–D, the fold changes (FCs) in drug susceptibility (IC50 vs. reference) of syngenic virus construct mixtures containing either the single mutation K65R or M184V, combined in differing percentages with wild-type HIV, are shown for abacavir, lamivudine, tenofovir, and didanosine. In panels E–H, the FCs in susceptibilities are shown for the K65R/M184V double-mutant mixtures.
3TC, 86% to TFV, and 0.7% to ABC. However, if both wild type and mutant (K65R/R; n = 33) were observed in the isolate, only 15% of the samples exhibited reduced susceptibility to 3TC and 12% to TFV, and all were fully susceptible to ABC. Similarly, 100% of isolates that appeared to harbor pure M184I or M184V (n = 2165) exhibited reduced susceptibility to 3TC, whereas only 49% of those containing M184M/I/V mixtures (n = 414) appeared to be resistant to 3TC.

For dual-mutation samples, when K65R was present in combination with M184I/V (n = 105) with no wild type detected at position 65 by population sequencing, the number of samples with reduced susceptibility to ABC or TFV (95% or 20%, respectively) was higher than what was observed for the K65R/R mixtures with M184I/V (n = 85; 24% and 0% for ABC and TFV, respectively). Similarly, when K65R was present in combination with the M184M/I/V mixture (n = 19) in the absence of apparent wild type, the number of samples with reduced susceptibility to ABC or TFV (21% and 42%, respectively) was higher than what was observed for the K65R/R mixture with the M184M/I/V mixture (n = 39; 0% and 2.6% for ABC and TFV, respectively).

NL4-3–based plasmids with K65R or M184V site-directed mutations were mixed in defined proportions with wild-type parent plasmid and tested for phenotypic susceptibilities to an NRTI panel. The effects of increasing percentage of M184V and/or K65R on drug susceptibility to ABC, TFV, 3TC, and ddI is shown in figure 2A–2D. For ABC (figure 2A), the FC increase for both single mutants without wild type was below the clinical cutoff (FC, 4.5). For 3TC (figure 2B), mixtures containing >50% M184V were required to increase the FC to above the clinical cutoff of 3.5, consistent with previously reported assay validation data [6]. Mixtures containing >80% K65R were necessary to increase the FC to above the 3TC clinical cutoff. For TFV (figure 2C), >80% K65R was necessary to increase the FC to above the clinical cutoff of 1.4, and 100% K65R resulted in a FC of 2.1 for TFV. As the percentage of M184V relative to wild type increased, TFV susceptibility increased. For ddI (figure 2D), a mixture containing >70% K65R was required to increase the FC to over the clinical cutoff of 1.3, whereas no amount of M184V was sufficient to increase the FC to above the clinical cutoff. HXB2–based plasmids containing the K65R/M184V double mutant were used for mixing experiments in combination with wild-type HIV (figure 2E–2H). For the double mutant, >75%, >50%, and >50% of the mutant virus was needed to reach the clinical cutoffs for ABC, 3TC, and ddI, respectively (figure 2E, 2F, and 2H). The clinical cutoff for TFV was not reached at any point in the K65R/M184V dilution scheme (figure 2G).

Discussion. Phenotypic testing is advantageous for identifying nonalgorithmic resistance and mutational interactions, especially in complex mixtures of mutations with divergent phenotypic effects. However, for subjects experiencing virologic nonresponse in the ESS30009 study, resistance was typically not detected for ABC or TDF until months after failure. Possible explanations for this failure and profile include antagonistic pharmacokinetics with these drug combinations and low-abundance HIV variants resistant to the drugs that were not detected by the assay employed. The first possibility seems unlikely to be a major factor in light of recent data [7–9], and the second is supported by clonal analysis of early virologic failure.

Also consistent with the latter hypothesis, samples from subjects with relevant mutant mixtures identified in the Monogram Biosciences clinical database were more likely to be susceptible to ABC, TFV, or 3TC than samples that were characterized as fully mutant by population sequencing.

The data from the mixing experiments confirms that the sensitivity of genotypic assays for the detection of minority virus populations with reduced drug susceptibility varies by mutation and drug, as shown in prior studies [6, 10]. In the present study, >75% of the K65R/M184V double-mutant virus was needed to reach the clinical cutoff (FC, 4.5) for ABC and >80% of K65R was needed to reach the clinical cutoff (FC, 1.4) for TDF, whereas >50% of M184V sufficed to reach the clinical cutoff (FC, 3.5) for 3TC.

These data may represent an extreme example of a consistent phenomenon: failure of first-line therapy despite susceptibility to 1 or more components of triple-antiretroviral therapy. Boosted protease inhibitor regimens typically fail with minimal resistance, although failure is often attributed to patient nonadherence. Failure of dual NRTI/nonnucleoside RT inhibitor (NNRTI) regimens is often associated with early presentation of primary NNRTI mutations and M184V. These mutations are accompanied by high-level resistance to the NNRTI used and 3TC or FTC, whereas resistance to the third agent (e.g., zidovudine [ZDV], ABC, or TDF) is less frequent and slower to emerge. For other triple-NRTI regimens with substantially better response rates (e.g., ABC/3TC/ZDV or TDF/3TC/ZDV), M184V is frequently the first mutation to emerge, though failure is often attributed to patient nonadherence. Failure of dual-NNRTI regimens is often associated with early presentation of primary NNRTI mutations and M184V. These mutations are accompanied by high-level resistance to the NNRTI used and 3TC or FTC, whereas resistance to the third agent (e.g., zidovudine [ZDV], ABC, or TDF) is less frequent and slower to emerge. For other triple-NRTI regimens with substantially better response rates (e.g., ABC/3TC/ZDV or TDF/3TC/ZDV), M184V is frequently the first mutation to emerge, and susceptibility to the noncytidine components is retained. In these cases, the virologic rebound is generally much less, and >2 log reduction in HIV-1 RNA level are often maintained for many months [11].

These data suggest that the level of ABC, ddI, 3TC, and TFV resistance may be underestimated when K65R and/or M184V are present as mixtures and that virologic failure may be associated with apparent susceptibility when mixtures are present. These data also emphasize the value of performing combined genotypic and phenotypic analyses.

References


