K65R with and without S68: a new resistance profile in vivo detected in most patients failing abacavir, didanosine and stavudine

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Antiretroviral treatment with three nucleoside reverse transcriptase inhibitors (NRTIs) is widely used, but the combination of abacavir, didanosine and stavudine has never been investigated.

We describe the surprising and consistent genotypic and phenotypic outcome in patients failing this combination. As part of a Danish multicentre study, 60 antiretroviral-naive patients were randomized to treatment with abacavir, didanosine and stavudine. Failure was defined as one HIV-1 RNA >400 copies/ml. Genotyping was performed using TrueGene® HIV-1 assay (Visible Genetics, London, UK). Phenotypic susceptibilities were determined with the Virco Antivirogram assay.

Eight patients failed treatment with a median viral load of 2,980 copies/ml (range 478–5,950).

At baseline, five patients were wild-type. Three patients harboured nucleoside excision mutations (NEMs), but phenotypic susceptibilities were within normal range. All five patients with wild-type virus developed K65R and four of these patients also acquired the S68G mutation.

Phenotypic susceptibility decreased towards abacavir (median 8.9-fold) and didanosine (median 3.2-fold), while susceptibility towards stavudine was unchanged (median 0.8-fold). Susceptibility towards lamivudine and tenofovir decreased median 14.2- and 4.0-fold, respectively. In two patients with baseline resistance mutations, further accumulation of NEMs and V75T or L74V was observed. One patient developed Q151M.

Failure of a triple NRTI regimen is possible and frequent with only the K65R mutation. Under adequate selection pressure K65R can easily emerge in vivo and may compromise several future treatment options including newer NRTIs. The unexpected high incidence of S68G suggests a functional role of this mutation in viruses harbouring K65R.

Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) are included in most current antiretroviral regimen, along with protease inhibitors (PIs) or a non-nucleoside reverse transcriptase inhibitor (NNRTI). The approval of new NRTIs has extended the options for designing new regimens with possible greater antiviral efficacy and different resistance profiles. Abacavir is a guanosine nucleoside analogue with antiretroviral activity of similar potency as PIs, as judged from monotherapy trials [1,2]. Therefore, abacavir provided the opportunity to design an effective and simplified treatment regimen consisting of NRTI combinations, with the further advantage of preserving other drug classes for future therapy.

Triple NRTI regimens have been evaluated in a number of studies [3–5] and the combination most widely evaluated and used is abacavir, zidovudine and lamivudine. The combination is, however, not considered as potent as classical HAART and is not routinely recommended as initial treatment for patients with high viral loads [6]. One of the main purposes of combination therapy is to broaden the genetic barrier to resistance. Abacavir shares a number of resistance mutations with lamivudine (M184V) [7–9] and zidovudine (M41L, D67N, K70R, L210W, T215Y/F, K219Q) [7,10], and salvage therapy in patients harbouring these mutations has in general been disappointing [11,12]. In theory, it could be possible to widen the genetic barrier and thereby improve the efficacy of triple NRTI regimens, if drugs not sharing resistance mutations were combined.

We analysed the triple NRTI combination of abacavir, didanosine and stavudine in a randomized controlled study. The combination of abacavir with didanosine and stavudine was selected in order to minimize the risk of cross-resistance between the study...
drugs, and thus potentially increase the efficacy compared to other triple NRTI regimens. In vitro selection studies have identified four mutations within the RT-coding region (L74V, M184V, K65R and Y115F) associated with decreased susceptibility to abacavir [9], and results from abacavir monotherapy trials confirmed their appearance in vivo [7,8]. Among these, L74V and K65R could potentially cause cross resistance to didanosine, but they were not considered as major threats because K65R only very rarely is observed during combination therapies [13–16] and because L74V only seldom appears when prescribed in combination with stavudine [17–19].

This report describes the surprising and consistent genotypic and phenotypic outcome in patients failing the above-mentioned combination, an outcome that might have implications for a number of other NRTI regimens.

Materials and methods

The DAPIS (Danish Protease-Inhibitor Study) was a randomized, open-label, multicentre study, designed to compare the virological efficacy of different PI-containing regimens. In January 1999 a new study arm consisting of treatment with abacavir, didanosine and stavudine was opened. Sixty patients were included in this study arm in the time period from January 1999 to December 2000. Only antiretroviral-naïve patients could enrol.

For the purpose of this analysis, failure was defined as one measurement of plasma viral load >400 copies/ml after initially obtaining undetectable levels, or more than 24 weeks after initiating the treatment regimen. Only one viral load >400 copies/ml was required to meet the definition of failure. From the study database patients with the above-mentioned definition of failure were excluded. The patient files were thoroughly looked through and only patients on randomized treatment were investigated. Patients who had stopped antiretroviral therapy or had been switched to other regimens were excluded.

In patients experiencing failure we performed genotyping on plasma samples from baseline and at time of failure. In patients with a prolonged period with viraemia before switch to second-line therapy, genotyping was also performed on samples from a later time point (late failure) in addition to the time point of early failure. The decision to switch therapy or continue randomized treatment was left at the discretion of the treating physician, who did not have the results of the resistance tests in real-time.

Complete sequencing of the major part of the RT gene (codon 38–248) was done on plasma HIV-1 RNA. HIV-1 RNA was extracted from plasma using the QIAAmp viral RNA minikit (QIAGEN, Erkrath, Germany). In samples with viral loads in the range of 400–1000 copies/ml an ultra centrifugation step (1 ml plasma centrifuged at 50,000 G for 60 min) was added before extraction. The viral RNA was reverse transcribed to cDNA and subsequently amplified by a single-tube RT-PCR with TruGene™ HIV-1 Assay (Visible Genetics, London, UK). Bidirectional sequencing of the amplification products was performed with the CLIP sequencing method (Visible Genetics, London, UK). Each sequencing reaction was loaded on an automated DNA sequencer (MicroGene Clipper, Visible Genetics, London, UK). The assays were base called with GeneObjects (Visible Genetics, London, UK), and aligned and assembled with GeneLibrarian (Visible Genetics, London, UK). The resulting sequences were compared with a standard reference strain (HIV-B-LAV1, Gene Bank accession number: K02013) and any mutations in the patient virus were reported.

Sample mix-up and lab-contamination were excluded by sequencing baseline samples from different time-points and by drawing phylogenetic trees.

All sequences reported in the present study were submitted to GenBank (accession numbers: AY22050–AY222069).

Phenotypic analyses were performed on plasma HIV-1 RNA at VIRCO, Mechelen, Belgium, using the VIRCO Antivirogram assay. This technique encompasses total RNA extraction, cDNA synthesis of the protease and reverse transcriptase (PR-RT) gene, and nested PCR amplification. By recombinant technology the amplified patient PR-RT DNA are introduced into an HIV clone without protease and RT regions. CD4 cells are infected with the recombinant clone. Different concentrations of the NRTIs (zidovudine, lamivudine, didanosine, abacavir, zalcitabine, stavudine, tenofovir) as well as all available NNRTIs and PIs are placed in micro well plates, and dilutions of recombinant virus and CD4 cells with reporter gene systems are placed to each well. After incubation, the amount of HIV replication at each concentration of drug is measured by a computerized system and the susceptibility of the patient virus to each drug is calculated as the concentration of drug required to inhibit virus growth by 50% (IC_{50}). The IC_{50} of the patient virus is compared to the IC_{50} of fully susceptible, non-mutated wild-type virus, and the fold change in IC_{50} compared to wild-type HIV-1 is reported [20].

In the present study phenotypic analyses were done at baseline and failure, and the fold change in susceptibility of zidovudine, lamivudine, didanosine, abacavir, stavudine and tenofovir between baseline and failure were calculated for the individual patient. In patients
with prolonged viraemia, plasma samples from time point of late failure were selected for phenotyping.

Results

Study subjects
Twenty-one patients experienced at least one viral load >400 copies/ml. Hereof 13 patients had either been switched to another treatment regimen before time of failure or had stopped taking the study medication. These 13 patients were excluded from further analyses in the present study.

Eight patients experienced at least one viral load >400 copies/ml while on randomized treatment and were included in the following analyses.

Viral load and CD4 cell count
Median viral load and CD4 cell count at baseline was 244 500 copies/ml (range 58 825–1 060 000) and 100 cells/mm³ (range 10–190). At failure the corresponding values were 2980 copies/ml (478–5950) and 260 cells/mm³ (range 96–650). Median time to failure was 38 weeks (range 25–52).

Genotypic and phenotypic results
RT-genotypes were obtained for all patients at baseline and failure.

In patient number 1, 2, 3, 4, 5, 7 and 8 genotyping was performed on plasma samples from the very first time point of failure. In patient number 6 genotyping was performed 11 weeks after the first time point of failure, due to lack of plasma samples from earlier time points.

Phenotyping was successfully performed at baseline and failure in six patients, while in two patients no plasma was available for phenotypic analyses neither at baseline, nor failure (patient 4 and 7, Figure 1).

Baseline
In three patients mutations at sites known to be associated with resistance were present at baseline. One patient (patient 6, Figure 1) had D67N and K219Q, one patient (patient 7, Figure 1) had M41L, L210W and T215N, while the third patient (patient 8, Figure 1) harboured K70T at baseline.

The phenotypic susceptibilities were, however, within the normal range in patient 6 and 8 (Table 1). No phenotype was obtained for patient 7.

The remaining five patients had wild-type virus at baseline and phenotypic susceptibilities within normal ranges (Table 1).

Failure
Three different patterns of resistance mutations were detected, namely K65R, NEMs or Q151M.

K65R – The five patients with wild-type virus at baseline all developed the K65R mutation (patient 1–5, Figure 1 and 2). Four of these patients also acquired a new polymorphism at a codon not known to confer resistance, namely S68G. One patient with the K65R and S68G mutation also acquired K219R (patient 1, Figure 1).

Phenotypic analyses were done in four of the five patients, three harbouring K65R and S68G (patient 1–3, Figure 1), and one harbouring K65R only (patient 5). In patient 4, no phenotype was achieved. In patients with K65R (±S68G) phenotypic susceptibility towards abacavir decreased median 8.9-fold (range 5.0–13.0), towards didanosine 3.2-fold (range 1.9–11.0), while susceptibility towards stavudine was largely unchanged (median 0.8-fold, range 0.5–2.1). Susceptibility towards drugs that the patients had never been exposed to decreased 14.2-fold (range 8.9–40.6) for lamivudine and 4.0-fold (range 3.1–9.8) for tenofovir. Susceptibility towards zidovudine was only marginally affected (median decrease 1.8-fold, range 0.8–4.0) (Table 1).

NEMs – In two patients we detected NEMs at baseline (patient 6 and 7, Figure 1). These two patients showed a completely different resistance pattern at time of failure, consisting of the classical NEMs (M41L, D67N, K70R, L210W, T215V/Y and K219Q/R). Patient 6, who harboured D67N and K219Q at baseline further accumulated K70R and T215V in addition to the stavudine-linked mutation V75T. Patient 7, who harboured M41L, L210W and T215N at baseline, acquired D67N and K219R while T215N shifted into T215Y. In addition, the didanosine-linked mutation L74V was detected (Figure 1 and 3).

In patient 7, no phenotypic analysis was achieved. In patient 6 (harbouring D67N, K70R, V75T, T215V, K219Q), phenotypic susceptibility towards abacavir decreased 2.2-fold, towards stavudine 2.0-fold, while susceptibility towards didanosine remained the same (0.9-fold). Also, decreased susceptibility towards drugs not part of the failing regimen, was observed (zidovudine: 2.0-fold, lamivudine: 4.1-fold and tenofovir: 2.0-fold) (Table 1).

Q151M – In one patient (patient 8) who harboured the rare K70T mutation at baseline, the multiple dideoxynucleoside resistance mutation Q151M was detected at failure.

However, the usual concurrent occurrence of A62V, V75I, F77L and F116Y was absent, though the patients virus was sequenced during time of failure at two time points with an interval of 26 weeks. At time of early failure only Q151M and K70E was found,
**Figure 1. Mutations in the reverse transcriptase genome at time of failure**

|     | M41 | K65 | D67 | K70 | L74 | V75 | Q151 | L210 | T215 | K219 | N54 | N57 | V60 | K64 | S68 | R72 | T107 | D113 | L120 | E138 | R143 | I178 | I195 | D218 | I244 |
|-----|-----|-----|-----|-----|-----|-----|------|------|------|------|-----|-----|-----|-----|-----|-----|------|------|------|------|------|------|------|------|------|------|
| **1** | *   | R   | *   | *   | *   | *   | *    | R    | *    | *    | G   | *   | A   | *   | *   | A   | *    | *    | *    | *    | *    | *    | *    | *    | *    | *    |
| **2** | *   | R   | *   | *   | *   | *   | *    | *    | *    | *    | G   | *   | E   | *   | *   | *   | *    | *    | *    | *    | *    | *    | *    | *    | *    | *    |
| **3** | *   | R   | *   | *   | *   | *   | *    | *    | *    | *    | G   | *   | E   | *   | *   | *   | *    | *    | *    | *    | *    | *    | *    | *    | *    | *    |
| **4** | *   | R   | *   | *   | *   | T   | *    | *    | *    | *    | G   | *   | I   | *   | L   | *   | *    | *    | *    | *    | *    | *    | *    | *    | *    |
| **5** | *   | R   | *   | *   | *   | *   | *    | *    | *    | *    | *   | *   | *   | *   | *   | *    | *    | *    | *    | *    | *    | *    | *    | *    | *    |
| **6** | *   | N   | R   | T   | *   | V   | Q    | *    | *    | *    | *   | N   | *   | *   | *   | *    | M   | *    | *    | *    | *    | *    | *    | *    | *    |
| **7** | L   | N   | V   | *   | W   | Y   | R    | *    | *    | *    | I   | N   | *   | K   | *   | *    | I   | *    | *    | *    | *    | *    | *    | *    |
| **8** | *   | *   | E   | *   | *   | M   | *    | *    | *    | *    | K   | Y   | *   | G   | *   | E   | D   | *    | *    | *    | *    | *    | K    | *    | *    | *    |

- **Acquired mutations at resistance sites**
- **Mutations present at baseline**
- **Acquired polymorphisms**
while at late failure the S68G polymorphism was acquired in addition to Q151M and K70E (Figure 1 and 3).

Phenotypic analysis revealed a reduced susceptibility towards abacavir of 9.2-fold, towards didanosine of 14.4-fold and towards stavudine of 11.0-fold. Susceptibility towards drugs not part of the failing regimen was reduced 2.9-fold for zidovudine, 2.5-fold for lamivudine and 2.4-fold for tenofovir (Table 1).

No evidence of genotypic resistance towards NNRTIs or PIs was observed at any time point. Marginal phenotypic resistance towards indinavir was observed in patient 1 (4.9-fold) in whom the secondary protease mutations K20M and M36I were detected, and towards ritonavir in patient number 3 (3.8-fold), who had the secondary protease mutation L10I.

Follow-up after failure
The median period of follow-up after failure was 81 weeks (range 41–138).
One patient (patient 2) discontinued treatment and subsequently rebounded to viral loads >200 000 copies/ml. In a plasma sample obtained during this period off treatment, the concurrent disappearance of K65R and S68G was detected (Figure 2).

Patients 4 and 5 (harbouring K65R only) continued randomized treatment and experienced low-level viraemia (<50–300 copies/ml). After approximately 60 weeks patient 4 rebounded with viral loads at maximal 1,460 copies/ml and accumulation of additional mutations was detected (V75T and S68G) (Figure 2).

Response to salvage therapy
Five patients were switched to second-line regimens (patient 1, 3, 6, 7 and 8).

Patient 3 (harbouring K65R and S68G) switched to stavudine, lamivudine and efavirenz, but experienced persistent viraemia (2000–3000 copies/ml), though no compromised adherence was suspected (Figure 2).

Four patients achieved sustained virological suppression (<50 copies/ml) on second-line regimens within a follow up period of median 68 weeks (range 12–118 weeks): patient 1 (harbouring K65R, S68G and K219R) who switched to efavirenz, ritonavir and saquinavir (Figure 2), patient 8 (harbouring Q151M, S68G and K70E) who switched to didanosine, lamivudine and efavirenz (Figure 3), patient 6 (harbouring D67N, K70R, V75T, T215V, K219Q) who switched to didanosine, abacavir, indinavir and ritonavir (Figure 3) and patient 7 (harbouring M41L, D67N, L74V, L210W, T215Y and K219R) who switched to zidovudine, lamivudine and efavirenz (Figure 3).

### Discussion

The present study investigated the mutational profile selected by triple nucleoside therapy with abacavir, didanosine and stavudine. All patients without NEMs at baseline developed the K65R mutation. On the basis of in vitro results this mutation has been expected to emerge on a number of occasions, but has with few

### Table 1. Phenotypic susceptibility

<table>
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Phenotypic susceptibilities (fold change in IC₅₀ compared to wild-type HIV-1) at baseline and failure. For the individual patients, fold change in susceptibility between baseline and time of failure are listed in column 5. The median value of fold change in susceptibility for patients with the K65R mutation (patient 1–5) is listed in column 6.
exceptions failed to show up in combination therapies [3–5,15].

The present study shows that K65R can be consistently selected for in vivo if the selection pressure is adequate. When didanosine and stavudine are given in combination, the failing patients usually develop NEMs [17–19] instead of the L74V, K65R and V75T mutations reported in vitro and after monotherapy [21,22]. In the present study it could be expected that most failing patients would develop NEMs and perhaps M184V, which is the same profile as is usually detected in regimen with abacavir, lamivudine and zidovudine [13,15,23].

Therefore, the frequency of patients in the present study displaying a resistance pattern of K65R without NEMs, is surprisingly high. The significance of this finding is further supported by the very low overall prevalence of K65R in virus isolates (<2%) [14,16] and is in contrast to a common assumption that K65R is difficult to select during combination therapies.

Position 65 is part of the highly flexible β3–β4 loop in the fingers domain of the reverse transcriptase enzyme. The lysine side chain makes important contacts with the γ-phosphate of the incoming dNTP, which helps positioning and binding of the dNTP for subsequent catalytic incorporation into the growing DNA chain during polymerization [24]. K65R is thought to exert its effect mainly by an increased ability to discriminate between the natural dNTPs and the nucleoside analogues [25], though recent research points towards ATP-mediated removal of chain terminators as a possible contributing mechanism [26]. The discriminatory ability is in particular pronounced for binding of ddCTP (mimicked by lamivudine) and ddATP (mimicked by didanosine and tenofovir) [27,28]. In agreement with this, we found the thymidine analogues to be unaffected by the K65R mutation in the phenotypic analysis (Table 1). Also, a slightly decreased affinity for the natural dCTP and dATP, and sometimes a decreased processivity has been found, which could explain the
reduced replication capacity of viruses containing K65R [26].

Another characteristic of virus harbouring K65R is the apparent increased fidelity of the RT enzyme, with a significantly reduced error rate [29]. In theory, the reduced number of mutations will on one hand reduce the amount of non-viable viruses, but on the other hand diminish the capacity for resistance development of the virus. To what extent this property influences disease course or resistance patterns remains to be determined, but the further accumulation of mutations in K65R isolates from the present study, was limited (Figure 1 and 2).

Surprisingly, K65R appeared in conjunction with S68G in four of five patients, and S68G was detected in 63% of the study population at failure. S68G is commonly present in approximately 3% of wild-type viruses [30]. Due to the unexpected high incidence of S68G in combination with K65R in the present study, we performed a search on the Stanford Database for sequences containing these two mutations (http://hivdb.stanford.edu/cgi-bin/RTMut.cgi).

Thirty-eight isolates from 31 patients were retrieved, of which 29 (76%) also encompassed the Q15M mutation. Only nine sequences harboured K65R, S68G and wild-type Q151.

A fitness-increasing role for S68G was suggested by Gerardo García-Lerma et al. [30], who investigated the possible mutational pathways to the Q151M mutation, which requires a two-base transversion to occur. He demonstrated that the two possible intermediates were not viable in a laboratory-adapted viral background, but that introduction of the S68G mutation restored replicative capacity of the Q151L intermediate. Since S68G has a crucial role in viruses developing the Q151M complex, the presence of S68G in patient 8 is not unexpected. On the contrary, the high frequency of S68G in combination with K65R and without Q151M is significant, and indicates a role for S68G in the development of K65R. The absence of S68G at baseline, the disappearance of both S68G and K65R during treatment interruption (patient 2, Figure 2), and the accumulation of S68G at a second viral breakthrough (patients 4, Figure 2) suggest that S68G...
is not just a polymorphism, but might increase the replicative capacity of the mutant virus.

The third resistance pattern in the present study was observed in patient 8, who developed Q151M, S68G and K70E. The multidrug resistance mutation (MDR) Q151M is in general observed in <2% of patient samples [14], but in three studies of naive patients treated with stavudine and didanosine the incidence of Q151M was 3/21 (14%) [19], 4/34 (12%) [17] and 4/39 (10%) [18]. This mutational pattern in the present study is, therefore, not surprising, but it is none the less worrying, because the above-mentioned connection between K65R, S68G and Q151M could indicate that the viruses containing K65R and S68G are set on a mutational pathway towards Q151M. Certainly, the advantage of S68G in providing a genetic background for viable intermediates of Q151M is not reassuring.

To our surprise, three patients had mutations at codons known to confer resistance present at baseline. Though one of the patients (patient 8) only had the K70T mutation, which is probably a polymorphism, these findings were unexpected as the central monitoring of primary resistance in Denmark has detected only very limited transmission. Therefore, all patient files were carefully looked through, several samples from different time points before the initiation of therapy were sequenced and phylogenetic trees were drawn, but no evidence of contamination, sample mix up or undiscovered previous drug exposure were found. Therefore, true transmission of resistant virus must be assumed. A triple NRTI regimen is more sensitive towards transmitted NRTI resistance than a combination of several drug classes. Sequencing of baseline samples from fully suppressed as well as failing patients in the other study arms, which included PIs or NNRTIs plus PIs, could possibly reveal the same extent of primary resistance.

The triple nucleoside regimen of the present study was selected in order to yield a high genetic barrier and since HAART regimens have previously failed due only to the lamivudine-induced M184V mutation, lamivudine was particularly excluded from the regimen. To our surprise, the patients still failed due to one single mutation, even one with broader cross resistance than M184V and, therefore, with possible implications for the sequencing of NRTIs.

Since K65R confers low to intermediate levels of resistance to lamivudine, abacavir, tenofovir and didanosine, the only fully susceptible NRTIs left are zidovudine and stavudine, which are antagonistic. It has been suggested that K65R and the NEMs do not commonly appear together because they exert opposing selective pressures. This is supported by the findings of the present study in which patients with NEMs at baseline did not develop the K65R mutation and is in agreement with the selection against K65R observed in patients treated with zidovudine and abacavir [13,23]. The low frequency of K65R overall could, therefore, be due to the widespread use of zidovudine instead of a relative inability of the virus to develop K65R.

Though the present study is limited by a small sample size, it raises some concern about antiretroviral regimens consisting of drugs prone to select for K65R (tenofovir, abacavir and didanosine) [7–9,22,31], without the concurrent use of zidovudine.

The issue is addressed in a larger scale in the Gilead Study, in which 299 antiretroviral-naive patients were randomized to treatment with tenofovir, lamivudine and efavirenz. Twenty-nine patients failed the regimen and in 24% (seven patients) the K63R mutation was detected [32], thus confirming the relative ease with which K63R emerges.

In the present study the outcomes of second-line treatment were encouraging, which was also the case in the Gilead Study. This could be due to introduction of new drug classes (NNRTI and boosted PIs) and antiviral effect of thymidine-analouges. However, the two patients who continued study medication (patient 4 and 5, Figure 2) had only low-grade viraemia, suggesting that a reduced replicative capacity and remaining pressure from the NRTIs to which the K63R mutation confers resistance is likely to play a role.

The fact that all the failing patients harboured resistance mutations conferring varying degrees of cross resistance, questions the virological safety of this specific triple nucleoside regimen. Triple NRTI treatment is not routinely recommended for patients with high viral loads [6] and in the present study seven out of eight failing patients had baseline viral loads exceeding 100.000 copies/ml. Especially in a patient population with high viral loads, and with even a small risk of harbouring transmitted resistance, this specific combination is not advisable. Furthermore, accumulating evidence that mitochondrial toxicity is associated with the use of didanosine and stavudine [33–35], renders the present study arm even less attractive.

In conclusion, virological failure of this triple NRTI combination consisting of abacavir, didanosine and stavudine is possible and frequent with only the K63R mutation. K63R can easily emerge in vivo under the right selection pressure. The mutation confers varying degrees of cross resistance and may compromise several future treatment options including newer NRTIs.

Support

The present work was supported by an unrestricted grant form the Danish AIDS Foundation.
Resistance development on abacavir, didanosine and stavudine

References


