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REVIEW

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Sorafenib: complexities of Raf-dependent and Raf-independent signaling are now unveiled

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Abstract Hepatocellular carcinoma (HCC) is the most common primary cancer worldwide. The only current drug available for clinical treatment of HCC is sorafenib, which inhibits multiple signaling kinases including Raf family members, platelet-derived growth factor receptor, vascular endothelial growth factor receptors 1 and 2, c-Kit, and Fms-like tyrosine kinase 3. Many studies have revealed that the mechanism underlying the antitumor effect of sorafenib is complex. Because sorafenib inhibits C-Raf more potently than B-Raf, the therapeutic efficacy of sorafenib is strongly influenced by the relative expression and activity of B-Raf and C-Raf and the complex interactions between these factors. Moreover, Raf-independent signaling mechanisms have recently emerged as important pathways of sorafenib-induced cell death. Basic research studies have suggested that using sorafenib as part of a combination therapy may improve its effect, although this has yet to be confirmed by clinical evidence. Further studies of the functional mechanism of sorafenib are required to advance the development of targeted therapy for HCC. To aid future work on sorafenib, we here review the current literature pertaining to sorafenib signaling and its clinical efficacy in both monotherapy and combination therapy.

Key words Hepatocellular carcinoma · Sorafenib · Raf · Myeloid cell leukemia-1 · Endoplasmic reticulum stress

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, with increased rates of incidence in both Asia-Pacific and Western countries. The prognosis of advanced HCC is poor because it frequently recurs or metastasizes and is refractory to standard systemic chemotherapy.[1–4] Furthermore, the majority of HCC cases are ineligible for curative radical surgery because of the extensive liver damage coexisting with HCC and caused by factors such as hepatitis B and C viruses, alcohol abuse, nonalcoholic steatohepatitis, and radioactive agents such as thorotrast.[5–7] Progress in molecular biology has enabled us to identify the oncogenes involved in carcinogenesis, leading to the development of molecular targeting drugs that are clinically useful.

Angiogenesis and cell proliferation play central roles in the development and progression of cancer. The vascular endothelial growth factor (VEGF)-A/VEGF receptor (VEGFR)-2 pathway is a potent inducer of angiogenesis in tumor-associated vascular cells, and its main downstream effector pathway, the Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) axis, has been identified to play a significant role in the tumor cell proliferation.[8] Ras-Raf-ERK signaling is frequently deregulated in many types of cancer cells including hepatoma cells,[9,10] suggesting that drugs specifically targeting VEGF and/or Raf could have favorable effects in the treatment of patients with unresectable tumors. Sorafenib (Nexavar, BAY 43-9006; Bayer HealthCare Pharmaceuticals), an orally bioavailable inhibitor of multiple kinases such as Raf, VEGF receptor, platelet-derived growth factor (PDGF) receptor, and the KIT and Fms-like tyrosine kinase 3 (FLT-3) oncogenes, appears to serve this purpose. Previous studies have reported that sorafenib inhibits tumor cell proliferation by the blockade of Raf signaling, reduces angiogenesis by inhibiting VEGF and PDGF signaling in tumor-associated endothelial cells, and finally increases the apoptotic ratio of cancer cells both in vitro and in vivo.[11,12] Clinical studies involving large numbers of patients demonstrated clear improvements in the overall survival of patients with unresectable HCC following treatment with sorafenib.[13,14] These data suggest that sorafenib could offer significant hope for patients with unresectable HCC.

Unfortunately, accumulating evidence reveals that sorafenib efficacy is somewhat limited clinically. It rarely

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decreases tumor volume in patients, and significant therapeutic effects are only observed in patients retaining adequate liver function.[15,16] In this regard, it should be noted that basic research studies have reported that the blockade of Raf signaling results in unexpected molecular events in cancer cells.[17–19] More importantly, several reports have indicated the possibility that the antitumor effect of sorafenib might be mediated by non-Raf signaling pathways. Thus, to improve the limited efficacy of molecular targeting approaches to HCC therapy, precise and holistic knowledge of sorafenib-mediated signaling networks is an indispensable tool.

Herein, we review key information revealed by the findings of basic research studies investigating sorafenib-mediated Raf- and non-Raf-mediated signaling pathways. We also discuss the possibility of using sorafenib with other drugs in combination therapy, which may be the most promising approach in the treatment of advanced HCC.

Raf signaling and sorafenib

In many types of cancer cells, activated Ras recruits members of the Raf serine/threonine kinase family (A-Raf, B-Raf, and C-Raf) to the plasma membrane, whereupon they are activated by phosphorylation.[20] When activated, Raf members form homologous and heterologous complexes, all of which regulate the MEK/ERK pathway, the downstream effector system for Raf. The functional relationship between B-Raf and C-Raf is nonreciprocal: B-Raf can activate C-Raf, but not vice versa.[21] Accordingly, the kinase activities of Raf complexes vary depending on their constituent components. For example, the kinase activity of B-Raf-C-Raf heterodimers toward MEK is significantly higher than that of homodimers of B-Raf or C-Raf.[22] Mutations in key residues within B-Raf (G466V, G466E, or G596R) dramatically reduce its kinase activity but do not affect its ability to form heterodimers with and activate C-Raf or its ability to activate MEK/ERK. In contrast, when B-Raf is mutated at V600E (a naturally occurring mutation observed in some types of cancer), the kinase activity becomes hyperactivated and B-Raf^{V600E} directly stimulates the MEK/ERK pathway.[21,23] Interestingly, however, a heterologous complex containing C-Raf and B-Raf^{V600E} has reduced kinase activity, and MAPK activation by the C-Raf-B-Raf^{V600E} complex is diminished in comparison with that provoked by unmutated C-Raf-B-Raf dimers.[21] C-Raf thus seems to apply a brake to the hyperactivated B-Raf^{V600E} mutant to preclude overactivation of MAPK signaling, which may explain why oncogenic Ras and B-Raf^{V600E} mutations are generally not found together in human cancers.[21]

It should be noted that sorafenib was originally designed to inhibit C-Raf and was only later shown to also block B-Raf at higher doses. Wilhelm et al.[24] reported that sorafenib inhibits B-Raf^{V600E} at 40 nM, C-Raf at 13 nM, and several other kinases in the low nanomolar range. These different inhibitory concentrations may affect its antitumor efficacy.[25,26] Given the complexity of Raf family interactions, the actions of sorafenib are similarly variable depending on sorafenib concentration and which Raf isotype(s) is expressed. Garnett et al.[21] reported that high doses of sorafenib inhibit MEK/ERK signaling by blocking the activities of C-Raf and B-Raf (either wild type or V600E). In contrast, at lower doses, sorafenib only inhibits C-Raf, resulting in disinhibition of B-Raf and B-Raf^{V600E}. Moreover, low doses of sorafenib induce the formation of heterologous complexes between B-Raf^{V600E} and wild-type B-Raf, which augments B-Raf^{V600E} activity.[21] Heidorn et al.[27] reported that, in cells expressing oncogenic Ras, selective inhibition of B-Raf induces the formation of B-Raf-C-Raf heterodimers, leading to activation of Raf/MEK/ERK signaling. Thus, although sorafenib monotherapy shows tolerable toxicity in HCC patients with hepatitis B virus or C virus infection,[28,29] these findings indicate that sorafenib dosage should be very carefully planned to avoid unintended adverse effects.

To date, few studies have investigated the status of B-Raf in HCC. Tannapfel et al.[30] reported that neither B-Raf nor K-Ras mutations were detected in 25 HCC samples as assessed by direct DNA sequencing. In contrast, B-Raf mutations were frequently found in cholangiocarcinoma. Newell et al.[31] reported that B-Raf was overexpressed in advanced tumors and that its expression was associated with gene amplification, although the gene mutation is unknown. However, given the potential for adverse effects of low-dose sorafenib in patients expressing the B-Raf^{V600E} mutant, further investigation of the mutation status of B-Raf in human HCC cases is required.

Non-Raf signaling (1): Mcl-1 and sorafenib

For clinicians treating advanced malignancies, cancer drug resistance remains one of the most important problems to be solved. Most cancer treatments (standard chemotherapeutic drugs, radiation, molecular targeting agents, etc.) kill tumor cells by apoptosis. Apoptosis is mainly regulated by two major pathways: (1) the death receptor pathway, in which caspase-8 is activated by the actions of tumor necrosis factor-α receptors (TNFRs) or Fas, and (2) the intrinsic apoptosis pathway, in which mitochondria and Bcl-2 family members drive the activation of caspase-9.[32,33] The Bcl-2 family consists of an antiapoptotic subgroup [Bcl-2, Bcl-XL, myeloid cell leukemia-1 (Mcl-1), Bcl-w, A1, Bax, and Bak] and a proapoptotic subgroup (Bax, Bak, Bad, Bid, Bim, Bik, NOXA, and PUMA). Among these, Mcl-1 has been identified as a key target for cancer therapy[34,35] because it is overexpressed in many types of malignancies. Moreover, Mcl-1 is distinctive for having a very short half-life compared with other Bcl-2 family members. Therefore, it is reasonable to hypothesize that manipulation of Mcl-1 expression levels may help to improve the antitumor efficacy of some drugs.

Interestingly, several studies have suggested that the antitumor effect of sorafenib is highly dependent on Mcl-1 but not on Raf signaling. Yu et al.[11] reported that sorafenib decreases Mcl-1 in various types of cancer cells by protea-

some-mediated degradation, suggesting that the degree of downregulation of Mcl-1 may determine the apoptotic effect of this agent. Rahmani et al.[12] showed that sorafenib downregulates Mcl-1 with marked cytochrome *c* release into the cytosol in human leukemia cells, and this effect was found to be independent of the MEK/ERK pathway. They observed no enhanced Mcl-1 ubiquitination in sorafenib-treated cells, but a marked association existed between attenuated Mcl-1 translation and dephosphorylation of Elf4E translation initiation factor. Ulivi et al.[36] reported that both protein and mRNA levels of Mcl-1 were decreased in sorafenib-sensitive pancreatic cancer cells but were upregulated in sorafenib-resistant cells. Furthermore, they showed that U0126 (a MEK inhibitor) did not induce apoptosis in any cell lines, demonstrating independence from MEK/ERK signaling. Silencing of Mcl-1 by siRNA resulted in the same level of apoptosis in both sorafenib-sensitive and sorafenib-resistant cells. Thus, although the mechanism of sorafenib-mediated Mcl-1 downregulation should be further investigated, the apoptotic effect of sorafenib appears to be independent of Raf signaling but dependent upon the decrease in Mcl-1. Alternatively, Mcl-1 may be a useful biomarker in the search for efficient combination therapies incorporating sorafenib.

Non-Raf signaling (2): endoplasmic reticulum stress and sorafenib

Very recently, endoplasmic reticulum (ER) stress has emerged as an alternative mechanism of Raf-independent, sorafenib-induced cell death.[37] The ER is a eukaryotic organelle that serves many general functions, including protein folding, transportation of synthesized proteins, and activation of chaperone proteins via the storage of intracellular calcium. ER stress is caused by accumulation of misfolded proteins in the cells exposed to various types of stimuli (hypoxia, oxidative stress, hypoglycemia, viral infection, etc.), and a network of signaling pathways comprising the "unfolded protein response" (UPR) is evoked for ameliorating the effects of the ER-located unfolded proteins.[38,39] At present, several of the main signaling molecules in the UPR have been identified. PKR-like kinase (PERK) inactivates eukaryotic initiation factor 2 (Elf2α) by phosphorylation and plays an important role in attenuating the translation of newly synthesized proteins. Inositol requiring-1 (IRE1) and activating transcription factor-6 (ATF6) induce calcium-dependent protein chaperones such as GRP78/BiP to maintain proper protein folding.[39,40] Interestingly, although the ER stress-related response is often linked to cell survival or chemoresistance, it also causes autophagy-related cell death when the degree of ER stress is excessive.[41]

Although the detailed mechanism(s) of sorafenib-induced ER stress remains unclear, Rahmani et al.[37] first showed that treatment with sorafenib strongly induces ER stress in human leukemia cells, leading to PERK and Elf2α phosphorylation, immediate mobilization of stored calcium, and the reduction of protein synthesis. Importantly, introduction of constitutively active MEK1 was unable to rescue the cell from sorafenib-mediated lethality, demonstrating that ER stress may be an independent mediator of sorafenib-induced cell death and acts without the involvement of the Raf/MEK/ERK pathway.[37] However, the same group reported that overexpression of PDGFRβ and FLT3 was essential for sorafenib-induced autophagy,[42] suggesting that receptor tyrosine kinases may be involved in ER stress. Very recently, Niessner et al.[43] reported that sorafenib-induced apoptosis was associated with the upregulation of ER stress-related transcription factors p8 and CAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) and the downregulation of Mcl-1. Thus, although there have been only a few reports of the role of ER in the effect of sorafenib, ER stress-mediated cell death may be deeply involved in the antitumor efficacy of sorafenib.

Angiogenesis and sorafenib

It has been widely known that angiogenesis is a critical process to support the growth of hepatoma cells, and the activated RAF/MEK/ERK cascade plays important roles in angiogenesis in endothelial cells.[24] Intriguingly, sorafenib can exert antiangiogenic effects against tumors in both in vitro and in vivo. Sorafenib reduces the microvessel area in tumor xenografts, possibly through the blockade of VEGFR and PDGFR signaling in the endothelial cells.[44] Moreover, angiogenic properties of the supernatant of cultured osteosarcoma were found to be reduced by sorafenib because it inhibits the production of VEGF and matrix metalloproteinase-2 by tumor cells.[45] More recently, it has been reported that sorafenib significantly inhibited the VEGF secretion by myeloma cells.[46] These lines of evidence may suggest that the clinical significance of sorafenib would become more apparent in the near future.

Perspectives of sorafenib-based combination therapy

As just described, the concept that Raf is the therapeutic target of sorafenib remains controversial. Newer theories describing Raf-independent signaling mechanisms such as Mcl-1 and ER stress are now regarded as more plausible targets of sorafenib. Several lines of evidence indicate that modulating multiple pathways may be necessary to improve the therapeutic effect of sorafenib. Recent studies have suggested that combination therapy using sorafenib with other non-Raf inhibitors may potentiate its efficacy.[47–49] Improved sorafenib efficacy has been reported when it is used in conjunction with agents targeting other signaling components, such as TNFα-related apoptosis-inducing ligand (TRAIL)[49–52] and inhibitors of histone deacetylase[53] and Bcl-2.[54]

Some studies have described new combination regimens that might have potential for therapeutic usage if their effects can be translated to the clinic (Table 1). Rausch et al.[55] reported that cotreatment with sorafenib and

Table 1. Combination studies of sorafenib with other agents

Combined agents		Objective	Reference
Experimental studies			
Rapamycin	mTOR inhibitor	Hepatoma cells	Wang et al.[64]
CI-1040	MEK inhibitor	Hepatoma cells	Ou et al.[65]
AZD6244	MEK/ERK inhibitor	Hepatoma cells	Huynh et al.[66]
Sulforaphane	NF-κB inhibitor	Pancreatic cancer cells	Rausch et al.[55]
Vitamin K	Fat-soluble vitamins	Hepatoma cells	Wei et al.[57]
Zoledronic acid	Bisphosphonate	Hepatoma cells	Zhang et al.[58]
Bortezomib	Proteasome inhibitor	Hepatoma cells	Chen et al.[67]
Erlotinib/cetuximab	EGFR inhibitors	Colorectal cancer cells	Martinelli et al.[68]
ABT-737	Bcl-xL inhibitor	Hepatoma cells	Hikita et al.[69]
PI-103	PI3K/mTOR inhibitor	Hepatoma cells	Gedaly et al.[70]
Clinical trial			
Octreotide	Somatostatin	HCC patients (phase II)	Prete et al.[71]
Radiation	Conformal radiation	HCC patients (phase I/II)	Zhao et al.[72]
Doxorubicin	Anticancer drug	HCC patients (phase II)	Abou-Alfa et al.[73]

mTOR, mammalian target of rapamycin; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; EGFR, epidermal growth factor receptor; PI3K, phosphoinositide 3-kinase

sulforaphane (SF), a broccoli isothiocyanate capable of inhibiting NF-κB activity without toxicity, completely abrogated the clonogenicity and spheroid formation of pancreatic cancer stem cells. Wei et al.[56,57] found that the combination of vitamin K_1, K_2, and K_5 with sorafenib enhanced sorafenib-induced apoptosis in pancreatic cancer cells and in hepatoma cells. In this combination protocol, phospho-ERK and Bcl-2 levels were decreased but levels of other Bcl-2 family members were unchanged, indicating that vitamin K may help to suppress the Raf/MEK/ERK pathway. Moreover, Zhang et al.[58] reported that zoledronic acid, clinically available as an antitumor drug for bone metastasis, prevented macrophage recruitment to the tumor and significantly reduced tumorigenesis when used with sorafenib. Finally, a randomized clinical trial of the combination of sorafenib with an inhibitor of mammalian target of rapamycin (mTOR) is now enrolling patients with HCC.[59] Because mTOR inhibitors have been proven to inhibit the recurrence rate of posttransplant HCC patients,[60] this trial holds great promise for patients with advanced HCC.

Conclusion

HCC is historically unique among many types of malignancies from the aspect of its etiology. Until recently, HCC was thought to occur in individuals with chronic hepatitis or cirrhosis with unknown etiology. In 1965, Baruch Blumberg discovered an antigen identical to hepatitis B surface antigen in the blood of Australian aboriginals, which was later found to be one of the major risk factors for hepatocarcinogenesis.[61] In 1989, hepatitis C virus was identified using molecular biology techniques.[62] These two breakthrough discoveries greatly advanced our knowledge of HCC etiology, but the findings have yet to translate to effective treatments for HCC. Because hepatoma cells are refractory to standard chemotherapy,[1-4] the prognosis for HCC patients remains dismal.

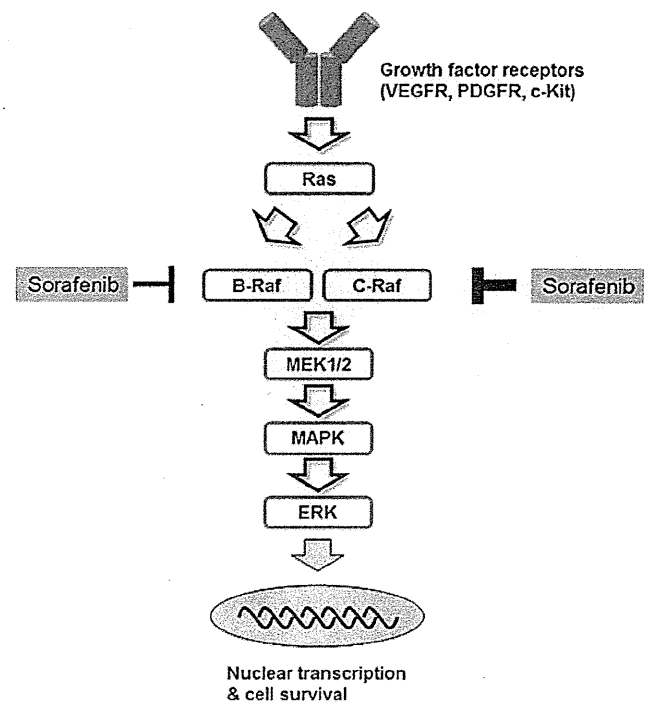

Fig. 1. Sorafenib modulates Raf signaling pathway. Sorafenib inhibits C-Raf more potently than B-Raf, leading to the inhibition of MAPK/ERK activation. *MEK*, mitogen-activated protein kinase; *MAPK*, mitogen-activated protein kinase; *ERK*, extracellular signal-regulated kinase

Sorafenib is currently the only treatment option for late-stage HCC. Initially considered to be a Raf-1 inhibitor (Fig. 1), recent studies have revealed a complex regulatory mechanism of B-Raf and C-Raf, and several Raf-independent signaling pathways are now regarded as important molecular targets of sorafenib (Fig. 2). Interestingly, sorafenib was recently found to efficiently block hepatitis C virus (HCV)

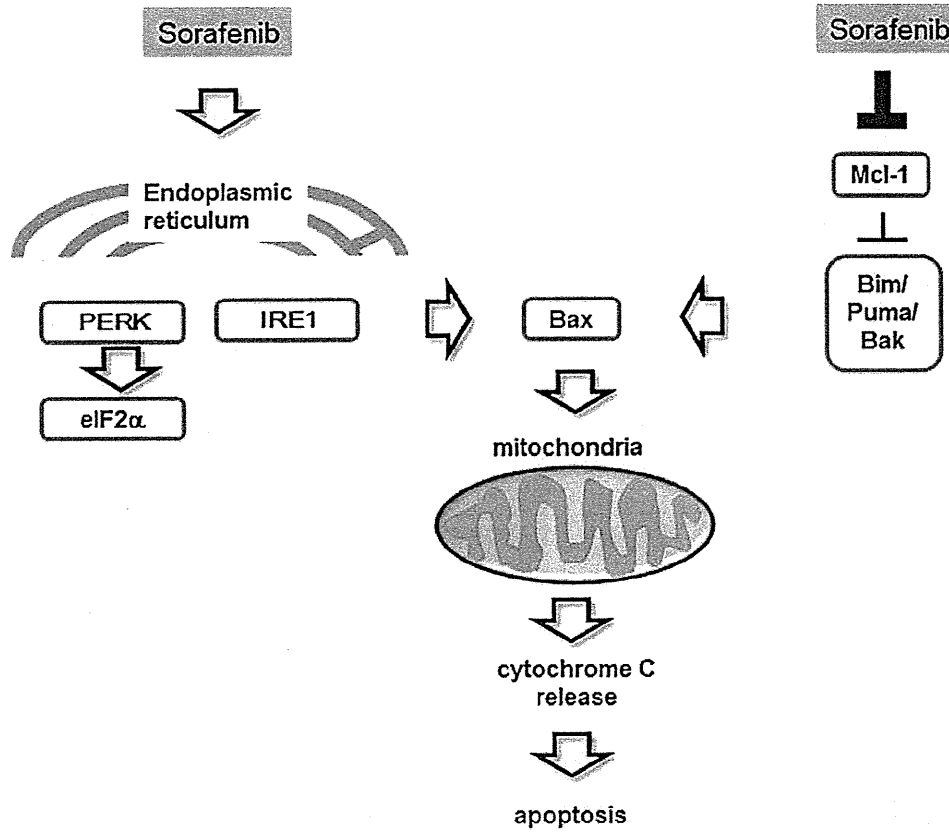

Fig. 2. Sorafenib induces apoptosis via non-Raf signaling. Besides inhibiting Raf, sorafenib induces cell apoptosis via stimulating endoplasmic reticulum stress as well as inhibiting Mcl-1. *PERK*, PKR-like kinase; *eIF2α*, eukaryotic initiation factor 2α; *IRE1*, inositol requiring-1; *Mcl-1*, myeloid cell leukemia-1

replication via inhibiting the recruitment of the viral non-structural 5A protein to c-Raf,[63] suggesting that it should be further explored as an antiviral strategy for patients with chronic HCV infection. Although the efficacy of sorafenib alone is still limited, extensive basic research may enable us to unravel the complexity of sorafenib-mediated cell signaling and drive the development of novel molecular targeting therapies.

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K70Q Adds High-Level Tenofovir Resistance to "Q151M Complex" HIV Reverse Transcriptase through the Enhanced Discrimination Mechanism

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Abstract

HIV-1 carrying the "Q151M complex" reverse transcriptase (RT) mutations (A62V/V75I/F77L/F116Y/Q151M, or Q151Mc) is resistant to many FDA-approved nucleoside RT inhibitors (NRTIs), but has been considered susceptible to tenofovir disoproxil fumarate (TFV-DF or TDF). We have isolated from a TFV-DF-treated HIV patient a Q151Mc-containing clinical isolate with high phenotypic resistance to TFV-DF. Analysis of the genotypic and phenotypic testing over the course of this patient's therapy lead us to hypothesize that TFV-DF resistance emerged upon appearance of the previously unreported K70Q mutation in the Q151Mc background. Virological analysis showed that HIV with only K70Q was not significantly resistant to TFV-DF. However, addition of K70Q to the Q151Mc background significantly enhanced resistance to several approved NRTIs, and also resulted in high-level (10-fold) resistance to TFV-DF. Biochemical experiments established that the increased resistance to tenofovir is not the result of enhanced excision, as K70Q/Q151Mc RT exhibited diminished, rather than enhanced ATP-based primer unblocking activity. Pre-steady state kinetic analysis of the recombinant enzymes demonstrated that addition of the K70Q mutation selectively decreases the binding of tenofovir-diphosphate (TFV-DP), resulting in reduced incorporation of TFV into the nascent DNA chain. Molecular dynamics simulations suggest that changes in the hydrogen bonding pattern in the polymerase active site of K70Q/Q151Mc RT may contribute to the observed changes in binding and incorporation of TFV-DP. The novel pattern of TFV-resistance may help adjust therapeutic strategies for NRTI-experienced patients with multi-drug resistant (MDR) mutations.

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Introduction

Nucleos(t)ide reverse transcriptase inhibitors (NRTIs) are used in combination with other classes of drugs for the treatment of patients infected with human immunodeficiency virus type-1 (HIV-1). This approach is known as highly active anti-retroviral therapy (HAART) and has been remarkably successful in reducing the viral loads and increasing the number of CD4+ cells in patients' plasma. However, prolonged therapies inevitably result in resistance to all of the available drugs. Several mutations in the reverse transcriptase (RT) are known to cause resistance to NRTIs through two basic mechanisms:

1) The excision mechanism, which is based on an enhanced capacity of RT to use adenosine triphosphate (ATP) as a nucleophile for the removal of the chain-terminating nucleotide from the DNA terminus. The excision reaction products are a 5′, 5′-dinucleoside tetraphosphate and an unblocked primer with a free 3′-OH, allowing DNA synthesis to resume [1,2,3]. Increased excision of NRTIs is imparted by Excision Enhancement Mutations, typically M41L, D67N, K70R, T215Y/F, L210W, and K219E/Q (also known as Thymidine Associated Mutations, or TAMs). Other mutations have also been reported to enhance excision, including insertions or deletions at the tip of the β3- β4 loop of the fingers subdomain in the background of other excision enhancement mutations [4,5,6,7,8,9,10,11].

2) The other mechanism of NRTI resistance is the exclusion mechanism, which is caused when NRTI-resistance mutations in RT enhance discrimination and reduce incorporation of the NRTI-triphosphate (NRTI-TP). This mechanism is exemplified by the resistance of the M184V RT mutant to lamivudine (3TC) and emtricitabine (FTC) due to steric clash between the β-branched Val or Ile at position 184 and the oxathiolane ring of the inhibitors [12,13]. Another example of the exclusion mechanism is the multi-drug resistant (MDR) HIV-1 RT known as Q151M complex (Q151Mc). This RT contains the Q151M mutation together with a cluster of four

additional mutations (A62V/V75I/F77L/F116Y) [14,15]. Q151M by itself causes intermediate- to high-level resistance to zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), and low level resistance to abacavir (ABC) [15,16,17] without reducing viral fitness [18,19]. Addition of the four associated mutations increases replication capacity of RT and results in high-level resistance to AZT, ddI, ddC, and d4T, 5-fold resistance to ABC and low-level resistance to lamivudine (3TC) and emtricitabine (FTC) [17,18,19,20,21]. Miller *et al.* and Smith *et al.* reported a 1.8-fold and 3.6-fold increase in resistance to tenofovir (TFV), respectively [22,23].

Biochemical studies on the mechanism of Q151Mc resistance to multiple NRTIs have revealed that the mutations of this complex decrease the maximum rate of NRTI-TP incorporation without significantly affecting the incorporation of the natural nucleotides [21,24,25]. Structurally, the Q151 residue interacts with the 3′-OH of a normal deoxynucleoside triphosphate (dNTP) substrate [26]. It appears that the Q151Mc mutations cause resistance to multiple NRTIs by affecting the hydrogen bond network involving protein side chains in the vicinity of the dNTP-binding site and the NRTI triphosphate lacking a 3′-OH [25,26,27]. The Q151Mc set of mutations was also reported to decrease pyrophosphate PPi- and ATP-mediated excision [25].

K65R is another mutation near the polymerase active site that confers NRTI resistance through the exclusion mechanism. Specifically, K65R RT has reduced susceptibility to the acyclic nucleotide analog, TFV and other NRTIs, including ddI, ddC, ABC, FTC and 3TC [28,29,30,31]. Biochemical studies with K65R RT have demonstrated that this enzyme decreases the incorporation rate of these NRTIs [32,33,34]. The crystal structure of K65R RT in complex with DNA and TFV diphosphate (TFV-DP) revealed that R65 forms a molecular platform with the conserved residue R72, and the platform enhances the ability of K65R RT to discriminate NRTIs from dNTPs [35]. HIV carrying the Q151Mc mutations has been reported to be susceptible to TFV disoproxil fumarate (TFV-DF), the oral prodrug of TFV that enhances its oral bioavailability and anti-HIV activity [22,36]. While the K65R mutation appeared in several patients treated for more than 18 months with TFV-DF, no patient developed multi-NRTI resistance through appearance of Q151Mc [37].

Here we report the identification of unique HIV clinical isolates that have acquired the K70Q mutation in the background of Q151Mc during TFV-DF-containing therapy. We have used a combination of virological, biochemical, and molecular modeling methods to derive the mechanism by which this mutation confers resistance to TFV.

Materials and Methods

Clinical samples

HIV was isolated from fresh plasma immediately after collection of clinical samples from study participants at the outpatient clinic of the AIDS Clinical Center (ACC), International Medical Center of Japan. The Institutional Review Board approved this study (IMCJ-H13-80) and a written consent was obtained from all participants.

Construction of recombinant clones of HIV-1

Recombinant infectious clones of HIV-1 carrying various mutations were prepared using standard site-directed mutagenesis protocols as described previously [38]. The NL4-3-based molecular clone was constructed by replacing the *pol*-coding region with the HIV-1 BH10 strain. Restriction enzyme sites *Xma I* and *Nhe I* were introduced by silent mutations into the molecular clone at positions corresponding to HIV-1 RT codons 15 and 267, respectively [39]. Each molecular clone was transfected into COS-7 cells. Cells were grown for 48 h, and culture supernatants were harvested and stored at −80°C until use.

Single-cycle drug susceptibility assay

Susceptibilities to various RT inhibitors were determined using the MAGIC-5 cells which are HeLa cells stably transfected with a β-galactosidase gene under the control of an HIV long terminal repeat promoter, and with vectors that express the CD4 receptor and the CCR5 co-receptor under the control of the CMV promoter as described previously [40]. Briefly, MAGIC-5 cells were infected with diluted virus stock (100 blue forming units) in the presence of increasing concentrations of RT inhibitors, cultured for 48 h, fixed, and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside). The stained cells were counted under a light microscope. Drug concentrations reducing the number of infected cells to 50% of the drug-free control (EC_{50}) were determined from dose response curves.

Enzymes

RT sequences coding for the p66 and p51 subunits of BH10 were cloned in the pRT dual vector, which is derived from pCDF-2 with LIC duet minimal adaptor (Novagen), using restriction sites *PpuMI* and *SacI* for the p51 subunit, and *SacII* and *AvrII* for the p66 subunit. RT was expressed in the *Escherichia coli* strain BL21 (Invitrogen) and purified by nickel affinity chromatography and MonoQ anion exchange chromatography [41]. RT concentrations were determined spectrophotometrically based on absorption at 260 nm using a calculated extinction coefficient (261,610 M^{-1} cm^{-1}). The active site concentration of the various RT preparations was calculated as described below.

Nucleic acid substrates

DNA oligomers were synthesized by Integrated DNA Technologies (Coralville, IA). An 18-nucleotide DNA primer fluorescently labeled with Cy3 at the 5′ end (P_{18}; 5′-Cy3 GTC CCT GTT CGG GCG CCA-3′) and a 100-nucleotide DNA template (T_{100}; 5′-TAG TGT GTG CCC GTC TGT TGT GTG ACT CTG GTA ACT AGA GAT CCC TCA GAC CCT TTT AGT CAG TGT GGA AAA TCT CTA GCA GTG GCG CCC GAA CAG GGA C-3′) were used in primer extension assays. An 18-nucleotide DNA primer 5′-labeled with Cy3 (P_{18}; 5′-Cy3 GTC ACT GTT CGA GCA CCA-3′) and a 31-nucleotide DNA template (T_{31}; 5′- CCA TAG CTA GCA TTG GTG CTC GAA CAG TGA C-3′) were used in the ATP rescue assay and pre-steady state kinetic experiments.

Active site titration and determination of the dissociation constant for DNA binding ($K_{D\text{-}DNA}$)

Determination of active site concentrations in the different preparations of WT and mutant RTs were performed using pre-steady state burst experiments. A fixed concentration of RT (80 nM, determined by absorbance measurements) was pre-incubated with increasing concentrations of DNA/DNA template/primer (T_{31}/P_{18}), followed by rapidly mixing with a reaction mixture containing $MgCl_2$ and dATP, at final concentrations of 5 mM and 50 μM, respectively. The reactions were quenched at various times (10 ms to 5 s) by adding EDTA to a final concentration of 50 mM. The amounts of product (P_{18}-dAMP) were quantitated and fit to the following burst equation:

$$P = A(1 - e^{-kobst}) + k_{ss}t \quad (1)$$

where A is the amplitude of the burst phase that represents the RT-DNA complex at the start of the reaction, k_{obs} is the observed burst rate constant for dNTP incorporation, k_{ss} is the steady state rate constant, and t is the reaction time. The rate constant of the linear phase (k_{cat}) can be estimated by dividing the slope of the linear phase by the enzyme concentration. The active site concentration and template/primer binding affinity ($K_{D\text{-}DNA}$) were determined by plotting the amplitude (A) against the concentration of template/primer. The data were fit using non-linear regression to a quadratic equation:

$$A = 0.5(K_D + [RT] + [DNA]) - \sqrt{0.25(K_D + [RT] + [DNA])^2 - ([RT][DNA])} \quad (2)$$

where K_D is the dissociation constant for the RT-DNA complex, and [RT] is the concentration of active polymerase molecules. Subsequent biochemical experiments were performed using corrected active site concentrations [42,43].

Primer extension assay

To examine the DNA polymerase activity of WT and mutant RTs and the inhibition of DNA synthesis by TFV, the primer extension assays were carried out on the T_{100}/P_{18} template/primer (P_{18} was 5′-Cy3 labeled) in the presence or absence of 3.5 mM ATP [41]. The enzyme (20 nM active sites) was incubated with 20 nM template/primer at 37°C in a buffer containing 50 mM Tris-HCl, pH 7.8 and 50 mM NaCl. The DNA synthesis was initiated by the addition of 1 μM dNTP and 10 mM $MgCl_2$. The primer extension assays were carried out in the presence or absence of varying concentrations of TFV-DP. The reactions were terminated after 15 min by adding equal volume of 100% formamide containing traces of bromophenol blue. The extension products were resolved on a 7 M urea-15% polyacrylamide gel, and visualized by phosphor-imaging (FLA 5100, Fujifilm, Tokyo). We followed standard protocols that utilize the Multi Gauge software (Fujifilm) to quantitate primer extension [41,44]. The results from dose response experiments were plotted using Prism 4 (GraphPad Software Inc., CA) and IC_{50} values for TFV-DP were obtained at midpoint concentrations.

ATP-dependent rescue assay

Template/primer (T_{31}/P_{18}) terminated with TFV (T/P_{TFV}) was prepared as described in Michailidis et al [41]. 20 nM of T/P_{TFV} was incubated at 37°C with HIV-1 RT (60 nM), either at various concentrations of ATP (0–7 mM) for 30 minutes, or for various times (0–120 minutes) with 3.5 mM ATP, in RT buffer containing 50 mM Tris-HCl, pH 7.8, and 50 mM NaCl, and 10 mM $MgCl_2$. The assay was performed in the presence of excess competing dATP (100 μM) that prevented reincorporation of the excised TFV, 0.5 μM dTTP and 10 μM ddGTP. Reactions were quenched with 100% formamide containing traces of bromophenol blue and analyzed as described above. The dissociation constants (K_d) of the various enzymes for ATP used in the rescue reactions were determined by fitting the rescue data at various ATP concentrations, using non-linear regression fitting to hyperbola.

Kinetics of dNTP incorporation by WT and mutant enzymes

To determine the binding affinity of WT and mutant enzymes to the dNTP substrate ($K_{D\text{-}dNTP}$) and to estimate the maximum rate of dNTP incorporation by these enzymes (k_{pol}), we carried out transient-state experiments using a rapid quench instrument (RQF-3, Kintek Corporation, Clarence, PA) at 37°C in RT buffer (50 mM Tris-HCl, pH 7.8 and 50 mM NaCl). HIV-1 RT (50 nM active sites) was pre-incubated with 50 nM T_{31}/P_{18} in one syringe (Syringe A), whereas varying concentrations of dNTP and 10 mM $MgCl_2$ were kept in another syringe (Syringe B). The solutions were rapidly mixed to initiate reactions, which were subsequently quenched at various times (5 ms to 10 s) by adding EDTA to a final concentration of 50 mM. The products from each quenched reaction were resolved, quantitated, and plotted as described above. The data were fit by non-linear regression to the burst equation (Eq 1).

To obtain the dissociation constant $K_{D\text{-}dNTP}$ for dNTP binding to the RT-DNA complex, the observed burst rates (k_{obs}) were fit to the hyperbolic equation (Eq. 3) using nonlinear regression:

$$k_{obs} = (k_{pol}[dNTP])/(K_{D-dNTP} + [dNTP]) \quad (3)$$

where k_{pol} is the optimal rate of dNTP incorporation.

The kinetics of TFV incorporation by the WT and mutant enzymes were carried out in a manner similar to that employed for natural dNTP substrate except the time of reactions. It was noted that the mutant enzymes required longer time to incorporate TFV compared to the WT HIV-1 RT (detailed in the Results section).

Molecular Modeling

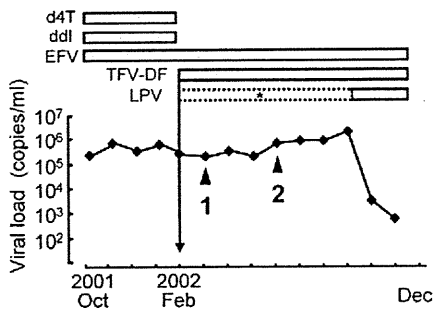
Molecular models of mutant enzymes were generated using SYBYL (Tripos Associates, St. Louis, MO). The starting protein coordinates were from the crystal structure of HIV-1 RT in complex with DNA template/primer and TFV-DP (PDB file 1T05) [45]. They were initially modified by the Protein Preparation tool (Schrodinger Molecular Modeling Suit, NY), which deletes unwanted water molecules, sets charges and atom type of metal ions, corrects misoriented Gln and Asn residues, and optimizes H-atom orientations. Amino acid side chains were substituted in by Maestro (Schrodinger, Molecular Modeling Suite, NY). Molecular dynamics simulations of the WT and mutant RT models were carried out to obtain the most stable structures by Impact, interfaced with Maestro at constant temperature, and OPLS_2005 force field. The molecular dynamics simulations were performed for 1000 steps with 0.001 ps intervals. The temperature relaxation time was 0.01 ps. The Verlet integration algorithm was used in simulations. The structures were imported into Pymol (http://www.pymol.org) for visualization and comparison.

Results

Phenotypic resistance to TFV-DF in the absence of any known TFV resistance mutations

During phenotypic and genotypic evaluation of the clinical isolates we identified a unique virus that exhibited an apparent discordance between the phenotypic and genotypic results. The clinical history of the patient and the corresponding genotypic and phenotypic changes during the course of the therapy are summarized in Fig. 1. (Also see Table S1). The patient's treatment before Feb 2002 included d4T, ddI, and EFV and did not decrease significantly the viral loads (Fig 1A). Hence, the therapeutic regimen was switched to TFV-DF, EFV, and the protease inhibitor lopinavir (LPV). However, the patient's immunological and virological responses still did not improve due to poor adherence, especially to LPV. Genotypic and phenotypic analyses on March 2002 (point 1) and June 2002 (point 2) revealed

A



B Phenotypic profile

Isolate	Fold increase in EC_{50}								
	AZT	ddI	d4T	3TC	ABC	**TFV·DF**	FTC	NVP	EFV
1	61	12	9	13	10	**2**	9	>200	>500
2	57	>40	>50	31	15	**6**	5	>200	>500

C Genotypic profile

Isolate	Resistance-associated mutation		Other newly acquired mutations
	NRTI	NNRTI	
1	Q151M complex,	V106M, V179D	-
2	Q151M complex,	V106M, V179D, V108I	**K70Q**, K102R

Figure 1. Clinical course of patient and drug resistance profile. (A) The two clinical isolates were collected from the patient at the time points indicated by triangles. Both isolates had no known resistant mutations in the protease region. During the period indicated by asterisk, LPV was administrated but the patient demonstrated poor adherence due to undesirable side effects. After instruction on the use of antiretroviral drugs, the viral loads successfully decreased below the detection limit (<50 copies/ml). (B) Phenotypic drug susceptibility assays of clinical isolates in at least three independent experiments are shown as a relative increase in EC_{50} compared to HIV-1 NL4-3 strain which served as WT (see also Table S1). (C) Mutations observed in the isolates that are defined as the NRTI and NNRTI resistance associated mutations deposited in the HIV Drug Resistance Database maintained by International AIDS Society 2009 [58] and the Stanford University (http://hivdb.stanford.edu/) were shown. Abbreviations of drugs used: d4T, stavudine; ddI, didenosine; EFV, efavirenz; TFV-DF, tenofovir disoproxil fumarate; LPV, lopinovir; AZT, zidovudine; 3TC, lamivudine; ABC, abacavir; FTC, emtricitabine; NVP, nevirapine.
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resistance to multiple RT inhibitors, including NNRTIs (Fig 1B). Resistance to all NRTIs, except AZT and FTC, was enhanced in the point 2 isolate (Fig. 1B). Notably, this isolate showed an increase in resistance to TFV-DF in the absence of the canonical TFV resistance mutation (K65R) and in the presence of Q151Mc mutations (Fig. 1C). Previously, it has been shown that Q151Mc remains susceptible to TFV [22] although Smith *et al.* reported that Q151Mc had a 3.6-fold increase in TFV resistance [23]. Suppression of the viral load was finally achieved by improvement in drug adherence to LPV and by the addition of FTC in the therapeutic regimen, since no protease resistance mutations were found within the protease coding region.

To identify the mutation(s) responsible for the unexpected resistance to TFV-DF we sequenced the entire RT coding region at time-points 1 and 2 (Figure S1, GenBank Accession Number AB506802 and AB506803). Of the three substituted residues (70, 102, and 108) amino acids 102 and 108 are part of the structurally distinct NNRTI binding pocket [46], which can mutate during EFV-based therapeutic regimens. However, residue 70 is located in the β3-β4 hairpin loop of the p66 "fingers" subdomain of HIV-1 RT, which interacts with the incoming dNTP substrate [10,27]. Different mutations at this site have been previously implicated in NRTI resistance [47], suggesting that the observed K70Q mutation may be involved in the increased resistance to TFV-DF.

NRTI resistance enhancement by mutation at residue 70

Several mutations at position 70 of HIV-1 RT (R, G, E, T, N and Q) have been reported to the Stanford HIV-1 Drug Resistance Database (http://hivdb.stanford.edu/, accessed on Feb. 27th 2010). K70Q is rarely observed in treatment-naïve patients (0.04%), but appears more often in clinical samples from NRTI-treated patients (0.1%, $p<0.0001$ compared with the frequency of K70Q in treatment-naïve patients) but not NNRTI-treated patients. Furthermore, K70Q is observed in 0.5% of the clinical samples from patients infected with HIV-1 Q151M. There have been no previous reports on a possible role of K70Q in NRTI resistance.

To examine the effect of K70Q on drug susceptibility we generated a series of HIV variants with mutations at RT codon 70 (Figure 2A and also Table S2). The HIV-1_{K70Q} variant exhibited marginal resistance to ddI and 3TC (5- and 3.3-fold, respectively), but no significant resistance to other NRTIs. We further examined whether the mutations at residue 70 affect susceptibility to NRTIs in the Q151Mc background (Figure 2B and also Table S3). HIV-$1_{K70G/Q151Mc}$ had enhanced resistance to d4T (4.6-fold) as compared to HIV-1_{Q151Mc}. Notably, HIV-$1_{K70Q/Q151Mc}$ also showed enhanced resistance to ddI and d4T (2.4- and 4.4-fold, respectively, compared to HIV-1_{Q151Mc}). In addition, HIV-$1_{K70Q/Q151Mc}$ displayed 5-fold increased resistance to TFV-DF compared to HIV-1_{Q151Mc}. Other K70 mutations exhibited little or no resistance to TFV-DF.

Primer Extension and ATP-based Rescue Assays

As mentioned earlier, a key mechanism of NRTI resistance is the excision mechanism, which is based on the enhanced ability of NRTI-resistant enzymes to use ATP for unblocking chain-terminated primers and allow for further DNA synthesis to continue [2,3,48]. To determine whether the K70Q mutation causes TFV resistance through the excision mechanism we measured the susceptibility of WT and mutant RTs to inhibition by TFV in the presence or absence of ATP. In gel-based assays, an enhancement in excision would manifest as an increase in the production of fully extended DNA when 3.5 mM ATP is included in the extension reaction [49,50]. Our extension assays in the absence of ATP (no-excision conditions) showed that addition of the K70Q mutation to Q151Mc HIV-1 RT enhances resistance to TFV-DP. However, this enhancement is not influenced by the presence of ATP (Table 1, Fig. 3A and Figure S2A). In fact, excision enhancement due to the presence of ATP measured as [IC_{50} with ATP]/[IC_{50} without ATP] was similar for all enzymes, including the WT RT (from 2.7-fold to 2.9-fold for WT, K70Q, Q151Mc, and K70Q/Q151Mc RTs) (Table 1). Using a related type of assay, the ATP-mediated rescue assay, we compared the rates by which the WT and mutant RTs unblock TFV-terminated primers and extend products past the point of chain-termination. We find that the ATP-based rescue activity of WT RT is not slower, but 1.5-, 2.5-, and 3-fold faster than that of K70Q, Q151Mc, and K70Q/Q151Mc RTs, respectively (Fig. 3B and Figure S2B). In addition, the ATP-based rescue activity of WT RT was saturated at lower concentrations of ATP than K70Q,

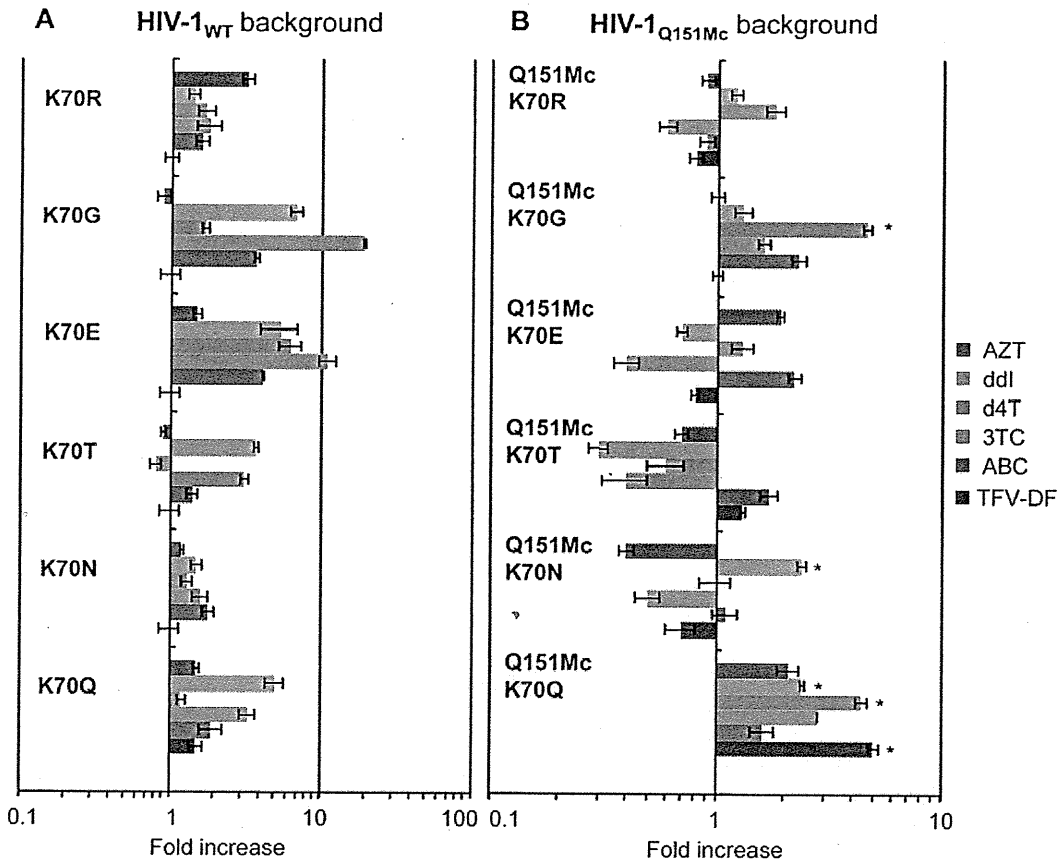

Figure 2. NRTI resistance of HIVs with mutations at RT residue 70 in the background of WT or Q151Mc. Antiviral activities of HIV-1s carrying mutations at residue 70 (K70R, K70G, K70E, K70T, K70N, or K70Q) in the WT (A) or Q151Mc (B) background were determined by the MAGIC5 assay. The data for each clone were compared to WT (A) and Q151Mc (B) HIV-1 and are shown as fold increase; AZT (red), ddI (green), d4T (cyan), 3TC (orange), ABC (blue), and TFV-DF (purple). Error bars represent standard deviations from at least three independent experiments (see also Table S2 and S3). The asterisk indicates statistically significant in EC_{50} values ($P<0.0001$ by t-test).
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Table 1. Primer extension assay in the presence or absence of ATP.

Enzyme[a]	IC_{50} (nM) of TFV-DP[b] (fold increase[c])		Excision enhancement due to ATP[d]
	Without ATP	With ATP	
WT	641±83	1854±197	2.9
	(1)[b]	(1)[b]	
K70Q	802±99	2306±270	2.9
	(1.3)	(1.2)	
Q151Mc	1503±90	3996±341	2.7
	(2.3)	(2.1)	
K70Q/Q151Mc	2392±353	7001±226	2.9
	(3.7)	**(3.8)**	

[a]The sequence of HIV RT WT and mutant derived from BH10.
[b]Data are means ± standard deviations from at least three independent experiments.
[c]The relative increase in IC_{50} value compared with each HIV-1 RT WT without, or with ATP is given in parentheses. Bold indicates an increase in fold increase value greater than 3-fold.
[d]Excision enhancement due to ATP is calculated as IC_{50} with ATP/IC_{50} without ATP.
doi:10.1371/journal.pone.0016242.t001

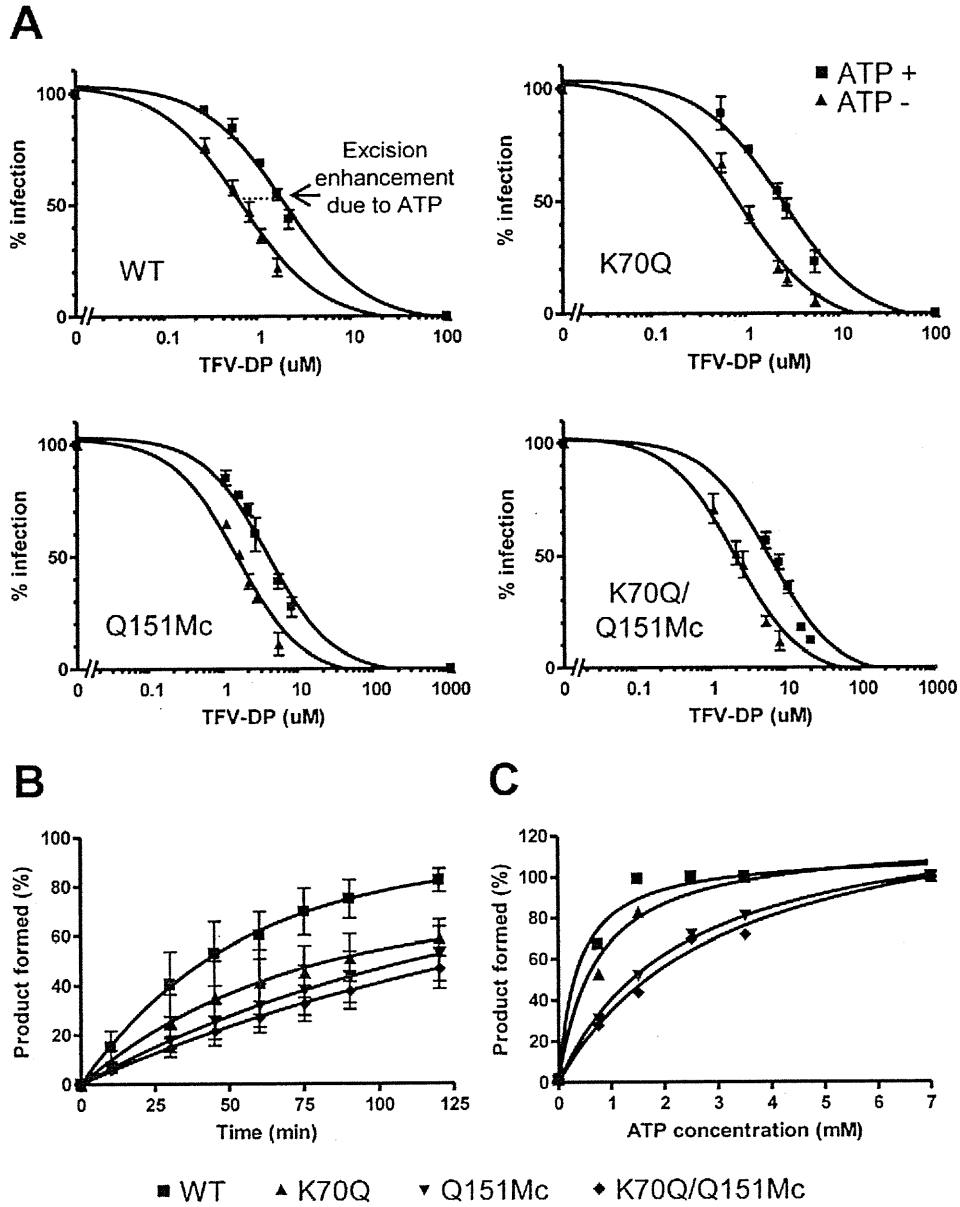

Figure 3. Effects of RT mutations K70Q, Q151Mc, or K70Q/Q151Mc on DNA primer extension activity and on ATP-based excision activities. (A) Effect of varying concentrations of TFV-DP on the primer extension activities of HIV-1 WT and mutant RTs. The experiments were carried out in the presence (■) or absence (▲) of 3.5 mM ATP (B) Time dependence of ATP-based rescue of TFV-terminated primers. TFV-terminated T_{31}/P_{18} oligos (20 nM) were incubated with 60 nM RT and 3.5 mM ATP. The reaction mixture also included excess of competing dATP (100 μM) that prevented reincorporation of TFV-DP and 0.5 μM dTTP, and 10 μM ddGTP that allowed extension of the rescued primer by two nucleotides and chain termination. Rescue products (WT [■], K70Q [▲], Q151Mc [▼] and K70Q/Q151Mc [♦]) were analyzed at indicated time points. (C) ATP-based rescue was dependent on concentration of ATP. Reactions were as in (B), but for 30 minutes and at varying concentrations of ATP. Rescue products at 7 mM ATP are defined as 100% product formed.
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Q151Mc, and K70Q/Q151Mc RTs (the apparent $K_{D\text{-}ATP}$ for WT, K70Q, Q151Mc and K70Q/Q151Mc were 0.4, 0.7, 2.3, and 3.1 mM, respectively), suggesting that a better binding of ATP may contribute to the slightly enhanced excision activity of WT RT (Fig. 3C and Figure S2C). Collectively, these results rule out the possibility that K70Q/Q151Mc becomes resistant to TFV through the excision mechanism.

Pre-Steady Kinetic Constants for Binding and Incorporation of dATP and TFV-DP

To determine whether the resistance by K70Q/Q151Mc is caused by an increased preference of physiological dATP substrate over TFV-DP, we carried out pre-steady state transient kinetic analyses of WT, K70Q, Q151Mc, and K70Q/Q151Mc enzymes. The kinetic constants $k_{pol\text{-}dATP}$ and $K_{D\text{-}dATP}$ for WT and mutant

enzymes are presented in Table 2 and Fig. 4 (and also in Figure S3). The results reveal that K70Q, Q151Mc, and K70Q/Q151Mc RTs have increased $k_{pol\text{-}dATP}$ as well as $K_{D\text{-}dATP}$. Both Q151Mc and K70Q/Q151Mc enzymes incorporate dATP faster than WT (17.9 and 14.6 s^{-1}, respectively *vs.* 6.3 s^{-1}) but have a weaker binding affinity for dATP than WT RT (5.4 and 5.0 μM, respectively *vs.* 2.6 μM). Hence, the catalytic efficiency ratio of dATP incorporation remains similar for all enzymes ($k_{pol\text{-}dATP}/K_{D\text{-}dATP}$ ratios for WT, K70Q, Q151Mc, and K70Q/Q151Mc were 2.4, 2.2, 3.3, and 2.9 $\mu M^{-1}\cdot s^{-1}$, respectively). On the contrary, a significant change in the incorporation efficiency of TFV was observed. The K70Q and K70Q/Q151Mc enzymes had more than 4.5-fold reduced affinity for TFV than the WT enzyme ($K_{D\text{-}TFV}$ values were 8.6 and 8.9 μM compared to 1.9 μM). In addition, the turnover rates of TFV incorporation by the WT and K70Q enzymes were comparable ($k_{pol\text{-}TFV}$ were 2.8 and 3.1 s^{-1}, respectively). The addition of the K70Q mutation to Q151Mc also reduced the k_{pol} for TFV-DP. The net effect of these changes was a significant reduction in the TFV-DP incorporation efficiencies of the mutant enzymes compared to the WT enzyme ($k_{pol\text{-}TFV}/K_{D\text{-}TFV}$ ratios for WT, K70Q, Q151Mc, and K70Q/Q151Mc were 1.47, 0.36, 0.3, and 0.11 $\mu M^{-1}\cdot s^{-1}$, respectively; Table 2). WT RT incorporated TFV-DP most efficiently, followed by K70Q>Q151Mc>K70Q/Q151Mc enzymes. As a direct measure of the enzyme's ability to discriminate between the natural dATP substrate and the TFV, we determined the "selectivity", defined as the ratio of efficiency of the enzyme to incorporate dATP over TFV-DP ($k_{pol\text{-}dATP}/K_{D\text{-}dATP}/k_{pol\text{-}TFV}/K_{D\text{-}TFV}$). The selectivity values demonstrate that the K70Q/Q151Mc enzyme favors incorporation of dNTP over TFV-DP 26.3 times compared to 1.6 times by the WT enzyme, leading to a 16.4-fold resistance to TFV (defined as $selectivity_{mutant}/selectivity_{WT}$; Table 2). This resistance is more than twice the TFV resistance of Q151Mc and 4 times the TFV resistance of K70Q.

Molecular modeling

Molecular dynamics simulations on the control structural coordinates of the WT RT/DNA/TFV-DP crystal structure [45] did not cause any significant structural changes, suggesting that the modeling protocols do not alter the structures in ways that are not related to the K70Q or Q151Mc mutations. The root mean square deviation (rmsd) between the Cα atoms of the WT structures before and after simulation was 0.1 Å. Similarly, the rmsd between the Cα atoms of WT and mutant RT molecular models were also very low (~0.1 Å). Comparison of these models showed a significant repositioning of residue 65 in Q151Mc/K70Q (Fig. 5), and to a lesser extent in K70Q or Q151Mc RTs (not shown). Additional smaller changes in the side chains of residues 151, 70, and 72 were also observed (Fig. 5). The structure of TFV-DP was also slightly adjusted, possibly as a result of the changes in the surrounding residues (Fig. 5). While residue 70 is located proximal to residue 65, and to the phosphates of the incoming TFV-DP, it does not appear to interact directly with these structural elements.

Discussion

We have discovered a novel HIV mutation that causes high-level resistance to TFV-DF. We have also determined the biochemical mechanism of this resistance. TFV-DF is a valuable NRTI therapeutic option for patients infected with multi-drug resistant Q151Mc HIV-1 [22]. We demonstrate here that Q151Mc can acquire an additional mutation, K70Q, which expands the multi-drug resistance to include high-level resistance to TFV-DF. We identified this mutant during genotypic analysis of clinical isolates from an HIV-infected patient who was not responding to TFV-DF. The K70Q/Q151Mc set of mutations is currently rare among HIV-infected patients. However, we believe that similar to K65R, its prevalence will increase, as tenofovir use continues to rise. Our virological studies with recombinant viruses confirmed that the observed enhancement and expansion of multi-drug resistance is the consequence of the addition of K70Q to Q151Mc HIV. Recently, the concept of clinical cut-offs (CCOs) has been introduced to improve the prediction of drug resistance during antiretroviral therapies. CCOs are better correlated with virologic response than biological cut-offs [51,52]. Importantly, K70Q/Q151Mc is 10 times less susceptible to TFV-DF than WT HIV-1, whereas the CCOs for TFV-DF is defined as a 2.1-fold reduction in virologic response to this inhibitor. Moreover, K70Q/Q151Mc is at least twice as resistant to TFV as the well-known TFV-resistant K65R in the background of Q151Mc (as reported in the Stanford HIV Drug Resistance Database).

Previous studies have offered insights into the drug resistance mechanism of similar mutations (K70E, K70G, K70R, and K70T). Specifically, K70E was selected in patients with virological failure after TFV-DF-based antiviral therapy [53,54,55]. K70T emerged in the background of Q151Mc during *in vitro* selection by TFV-DF [56]. K70R is a key mutation involved in resistance to AZT and appears in the background of other excision enhancement mutations [2,3,57]. In our case, a new mutation (K70Q) was

Table 2. Pre-steady state kinetic constants for binding and incorporation of dATP and TFV-DP by WT, K70Q, Q151Mc and K70Q/Q151Mc HIV-1 RT.

Pre-steady state kinetic constants[a]								
Enzyme[b]	dATP			TFV-DP			Selectivity[c]	Resistance[d]
	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d ($\mu M^{-1}\cdot s^{-1}$)	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d ($\mu M^{-1}\cdot s^{-1}$)		
WT	6.3±0.5	2.6±0.1	2.4±0.2	2.8±0.08	1.9±0.2	1.47±0.07	1.6	-
K70Q	8.4±0.4	3.8±0.6	2.2±0.4	3.1±0.4	8.6±1.5	0.36±0.08	6.1	3.8
Q151Mc	17.9±0.4	5.4±0.5	3.3±0.3	1.3±0.03	4.3±0.8	0.3±0.06	11	6.9
K70Q/Q151Mc	14.6±1.6	5.0±0.07	2.9±0.3	1.0±0.03	8.9±2.1	0.11±0.03	26.3	16.4

[a]Data are means ± standard deviations from at least three independent experiments.
[b]The sequence of HIV RT WT and mutant derived from BH10.
[c]Selectivity is defined as $(k_{pol}/K_d)_{dATP}/(k_{pol}/K_d)_{TFV\text{-}DP}$.
[d]Resistance (fold) is calculated as $selectivity_{mutant}/selectivity_{WT}$.
doi:10.1371/journal.pone.0016242.t002

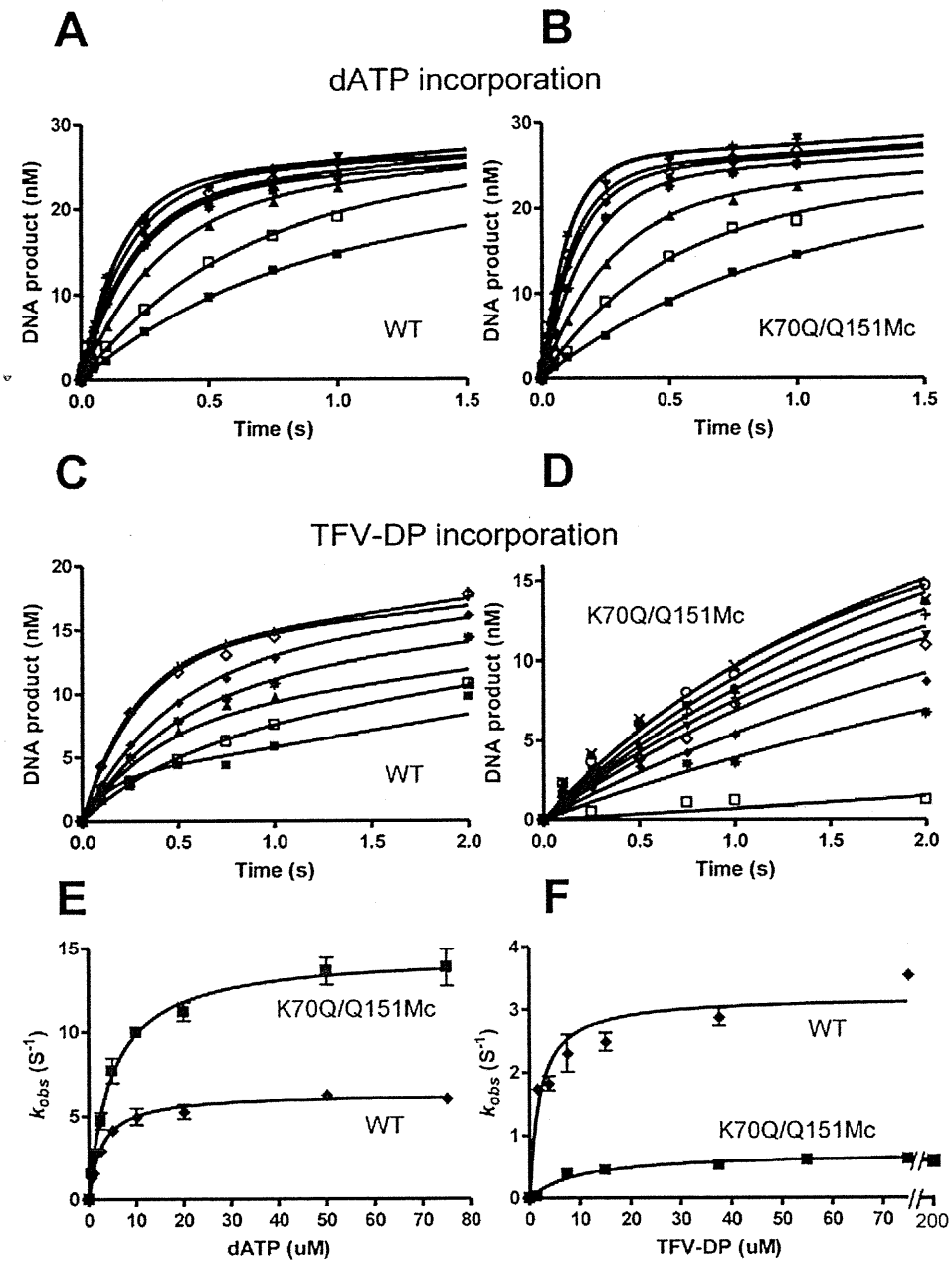

Figure 4. Pre-steady state kinetics of incorporation of dATP or TFV-DP by WT and K70Q/Q151Mc HIV-1 RTs. Single-nucleotide incorporation of dATP (panels A, B, and E) or TFV-DP (panels C, D, and F) by WT (panels A, C, E, and F) and K70Q/Q151Mc (panels B, D, E, and F). Formation of extended primer products in the reactions with WT RT and K70Q/Q151Mc RT were measured at 5 ms to 5 s time points, using the following dATP concentrations: 0.5 (■), 1 (□), 2.5 (▲), 5 (*), 10 (♦), 20 (◇), 50 (▼) and 75 μM (+). Incorporation of TFV was measured at 0.1–10 s reactions and at the following TFV-DP concentrations: 0.75 (■), 1.5 (□), 3.75 (▲), 7.5 (*), 15 (♦), 37.5 (◇) and 75 μM (+) for reactions with WT RT (panel C), and 1.5 (□), 7.5 (*), 15 (♦), 37.5 (◇), 55 (▼), 75 (+), 112.5 (•), 150 (○) and 200 μM (x) for reactions with K70Q/Q151Mc RT (panel D). (E) The amplitudes of the burst phases from the dATP reactions shown in panels A (WT, [♦]) and B (K70Q/Q151Mc, [■]) were plotted as a function of dATP concentrations. (F) The amplitudes of the burst phases from the TFV-DP reactions shown in panels C (WT, [♦]) and D (K70Q/Q151Mc, [■]) were plotted as a function of TFV-DP concentrations. The solid lines in panels A, B, C, and D represent the best fit of data to the burst equation. Each point represents the average values of three experiments.
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identified in a patient infected with Q151Mc HIV-1 during the course of TFV-DF-based antiviral therapy. The International AIDS Society-USA publishes [58] every year a list of HIV-1 drug resistance mutations compiled by a panel of experts charged with the goal of delivering accurate, unbiased, and evidence-based information for use by HIV clinical practitioners. In order for a novel mutation to be accepted in the list it should meet at least *one* of the following criteria: 1) *in vitro* passage experiments or

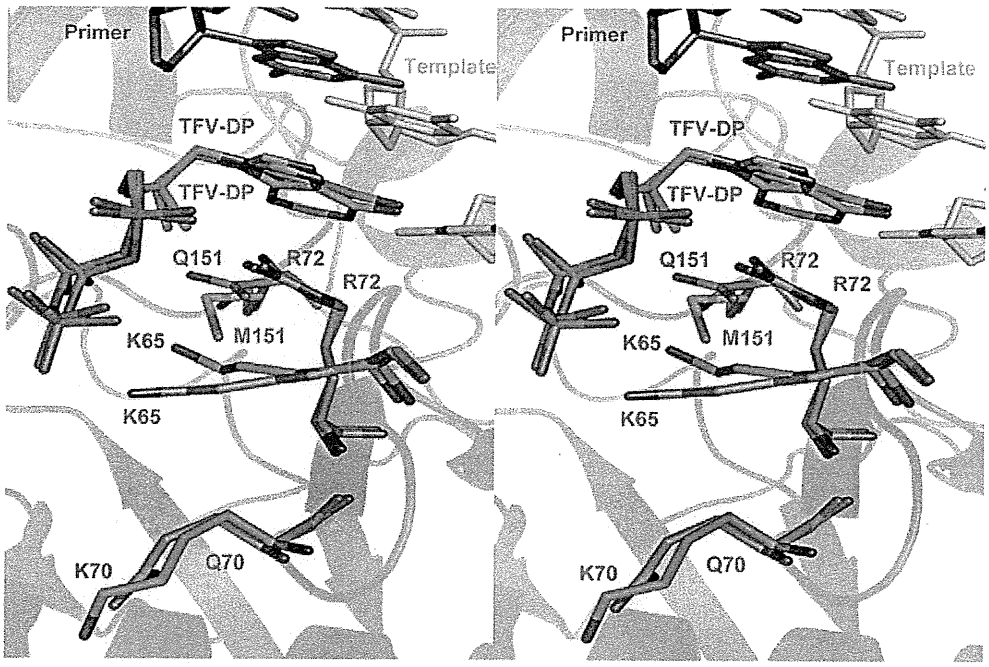

Figure 5. Stereo view of TFV-DP in the polymerase active site of WT RT and K70Q/Q151Mc RT. WT RT residues are shown as cyan sticks, K70Q/Q151Mc RT residues are shown as purple sticks. The primer strand is shown as dark gray sticks, template strand as light gray sticks. The fingers and palm subdomains are shown as blue and red cartoons, respectively.
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validation of contribution to resistance by using site-directed mutagenesis; 2) susceptibility testing of laboratory or clinical isolates; 3) nucleotide sequencing of viruses from patients in whom the drug is failing; 4) correlation studies between genotype at baseline and virologic response in patients exposed to a drug. Our study has unambiguously demonstrated that K70Q meets at least the first three criteria: evidence for criterion #1 is shown in Figure 2; for criterion #2 in Figures 1 and 2; and for criterion #3 in Figure 1 and Figure S1. Therefore, the K70Q mutation meets the criteria of a clinically relevant mutation.

In addition to the clinical and virological studies, we used biochemical techniques to determine the mechanism of TFV resistance imparted by the K70Q mutation to Q151Mc RTs. We used primer extension assays to show that K70Q/Q151Mc RT is less susceptible to TFV-DP than WT and Q151Mc RTs. We demonstrated that the mechanism of this resistance is not based on excision. On the contrary, we showed that the ATP-based excision of the mutant enzymes was slightly decreased with respect to WT RT, possibly because of decreased affinity of the mutant enzymes for the ATP excision substrate, incurred by changes in the binding environment of ATP, such as the loss of lysine at position 70.

Using transient-state kinetics we unambiguously established that the overall mechanism of K70Q/Q151Mc resistance to TFV is due to enhanced discrimination between the natural dATP substrate and TFV-DP. While all mutant enzymes had comparable efficiency of dATP incorporation, they displayed varying affinity and turnover rates of incorporation. It appears that the stronger effect of the enhanced discrimination overcomes the slight increase in sensitivity due to the small increase in excision. As a result, the mutant enzymes are resistant to the inhibitor.

Mutations at position 70 of RT have been known to confer NRTI resistance by two distinct mechanisms: K70R combined with at least two excision enhancing mutations, D67N and T215Y, enhances ATP-mediated excision of AZT and d4T [1,2,3,48] (*excision–dependent mechanism*). On the other hand, K70E causes resistance to 3TC, TFV, and ABC by lowering the maximum rate of inhibitor incorporation by RT (k_{pol}-*dependent exclusion mechanism*) [55]. Our results establish that in the background of Q151Mc, K70Q causes TFV resistance through a third mechanism: by decreasing the binding affinity of the inhibitor (K_d-*dependent exclusion mechanism*). Taken together, these findings highlight the remarkable ability of RT to use separate mutations at a single position to acquire NRTI resistance through three different mechanisms.

Our cell-based assays with infectious HIV-1 show that Q151Mc remains susceptible to TFV-DF, a finding consistent with previous reports [22]. Similarly, clinical isolates deposited at the Stanford HIV resistance database and carrying the Q151Mc mutation were also susceptible to TFV-DF, unless they also had the K65R mutation. However, pre-steady state characterization of TFV-DP incorporation by Q151Mc in this work (Table 2) and by others [59] showed that Q151Mc is less susceptible to TFV-DP than WT RT. This small discrepancy may be the result of potential differences in DNA-dependent and RNA-dependent DNA synthesis, or the result of the slightly increased excision of Q151Mc RT compared to WT RT (Fig. 3B and C).

To gain insights into the possible structural changes caused by the addition of K70Q to Q151Mc, we compared the molecular model of K70Q/Q151Mc RT/DNA/TFV-DP with the crystal structure of WT RT/DNA/TFV-DP [45] (Fig. 5). The network of hydrogen bonds involving the side-chains of K65, R72, and Q151 in the WT structure [26,27,54], is disrupted in the mutant structure. Also, Q151M and associated mutations A62V, V75I, and F77L are likely to modify the hydrophobic core of the fingers. We and others have previously shown that the side-chains of residues 72 and 65 interact with each other [35] and with Q151

and the α- and γ-phosphates of the incoming dNTP [26] or TFV-DP [45]. The functions of these residues have been established by several biochemical studies [21,25,60,61,62,63]. The reduction in polymerase rate (k_{pol}) and in binding affinity for TFV-DP (increased $K_{d.TFV\text{-}DP}$) may be the consequence of one or more such structural changes. Our molecular dynamics simulation experiments suggested a re-arrangement in the position of the side chain of K65, which is a catalytically important residue. While the precise effect of this change is not clear at this point, such changes could influence the overall binding of the substrate and/or the rate of nucleotide incorporation. Moreover, such movement of K65 in the presence of a mutation at position 70 is consistent with our previously reported crystallographic data, which established that there is an interplay between the positioning of the side chains at positions 70 and 65 [64]. Ongoing crystallographic studies are expected to provide more detailed structural insights into the role of K70Q in drug resistance.

In summary, we report here clinical data showing that addition of the K70Q mutation to the Q151Mc background confers high-level HIV resistance to TFV-DF and enhances resistance to other NRTIs. The biochemical mechanism of the TFV resistance is based on reduced binding affinity and incorporation of TFV-DP. Detection of this novel pattern of TFV-DF resistance may help adjust therapeutic regimens for the treatment of patients infected with multi-drug resistant HIV-1.

Supporting Information

Figure S1 Amino acid sequence alignment of the RT regions (amino acid 32 to 560) of the clinical isolates at time points 1 to 2 (see Figure 1A).
(DOC)

Figure S2 Effects of RT mutations K70Q, Q151Mc, or K70Q/Q151Mc on DNA primer extension activity and on ATP-based excision activities. (A) Effect of varying concentrations of TFV-DP on the primer extension activities of HIV-1 WT and mutant RTs. The experiments were carried out in the presence and absence of 3.5 mM ATP (marked as ATP (+) and ATP (−), respectively). Addition of ATP in the polymerization mixture allows measurement of the net sum of DNA polymerization and ATP-based excision activities. (B) Time dependence of ATP-based rescue of TFV-terminated primers. (C) ATP-based rescue was dependent on concentration of ATP.
(PPTX)

Figure S3 Pre-steady state incorporation of dATP or TFV-DP by K70Q and Q151Mc HIV-1 RTs. Single-nucleotide incorporation of dATP (panels A, B, and E) or TFV-DP (panels C, D, and F) by K70Q (panels A, C, E, and F) and Q151Mc (panels B, D, E, and F). Formation of extended primer products in the reactions with K70Q RT and Q151Mc RT were measured at 5 ms to 5 s time points, using the following dATP concentrations: 0.5 (■), 1 (□), 2.5 (▲), 5 (*), 10 (♦), 20 (◇), 50 (▼) and 75 μM (+). Incorporation of TFV was measured at 0.1–10 s reactions and at the following TFV-DP concentrations: 0.75 (■), 1.5 (□), 3.75 (▲), 7.5 (*), 15 (♦), 37.5 (◇) and 75 μM (▼) for reactions with K70Q RT (panel C), and 3.75 (▲), 7.5 (*), 37.5 (◇), 55 (▼), 75 (+) and 112.5 (•) for reactions with Q151Mc RT (panel D). (E) The amplitudes of the burst phases from the dATP reactions shown in panels A (K70Q, [▲]) and B (Q151Mc, [▼]) were plotted as a function of dATP concentrations. (F) The amplitudes of the burst phases from the TFV-DP reactions shown in panels C (K70Q, [▲]) and D (Q151Mc, [▼]) were plotted as a function of TFV-DP concentrations. The solid lines in panels A, B, C, and D represent the best fit of data to a burst equation. Each point represents average values of three experiments.
(PPTX)

Table S1 Drug susceptibility of clinical isolates.
(DOC)

Table S2 Drug susceptibility of HIV-1 variants carrying mutation at residue 70.
(DOC)

Table S3 Drug susceptibility of HIV-1 variants carrying mutation at residue 70 in the background of Q151M complex.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: AH ENK SO SGS. Performed the experiments: AH MMS KAK EM YS KS. Analyzed the data: AH MMS KAK KS SGS. Contributed reagents/materials/analysis tools: AH ENK SGS OS. Wrote the paper: AH ENK KS SGS.

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Biochemical, inhibition and inhibitor resistance studies of xenotropic murine leukemia virus-related virus reverse transcriptase

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ABSTRACT

We report key mechanistic differences between the reverse transcriptases (RT) of human immunodeficiency virus type-1 (HIV-1) and of xenotropic murine leukemia virus-related virus (XMRV), a gammaretrovirus that can infect human cells. Steady and pre-steady state kinetics demonstrated that XMRV RT is significantly less efficient in DNA synthesis and in unblocking chain-terminated primers. Surface plasmon resonance experiments showed that the gammaretroviral enzyme has a remarkably higher dissociation rate (k_{off}) from DNA, which also results in lower processivity than HIV-1 RT. Transient kinetics of mismatch incorporation revealed that XMRV RT has higher fidelity than HIV-1 RT. We identified RNA aptamers that potently inhibit XMRV, but not HIV-1 RT. XMRV RT is highly susceptible to some nucleoside RT inhibitors, including Translocation Deficient RT inhibitors, but not to non-nucleoside RT inhibitors. We demonstrated that XMRV RT mutants K103R and Q190M, which are equivalent to HIV-1 mutants that are resistant to tenofovir (K65R) and AZT (Q151M), are also resistant to the respective drugs, suggesting that XMRV can acquire resistance to these compounds through the decreased incorporation mechanism reported in HIV-1.

INTRODUCTION

Xenotropic murine leukemia virus-related virus (XMRV) is a gammaretrovirus that was first identified in some prostate cancer tissues (1,2) While some subsequent reports confirmed the presence of XMRV in prostate cancer samples (3–6), several others found little or no evidence of the virus in patient samples (7–9). XMRV DNA was also reported in 67% of patients with chronic fatigue syndrome (CFS) (10), but several subsequent studies in Europe and the USA failed to identify XMRV DNA in CFS patients or healthy controls (11–15). Hence, the relevance of XMRV to human disease remains unclear (16) and have been challenged (17). Most recently, it has been reported that XMRV has been generated through recombination of two separate proviruses suggesting that the association of XMRV with human disease is due to contamination of human samples with virus originating from this recombination event (18). Nonetheless, as a retrovirus that can infect human cells, XMRV can be very helpful in advancing our understanding of the mechanisms of retroviral reverse transcription, inhibition and drug resistance.

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