Rapid Communication

Xenotropic murine leukemia virus-related virus is susceptible to AZT

Ryuta Sakuma [a,1], Toshie Sakuma [a], Seiga Ohmine [a], Robert H. Silverman [b], Yasuhiro Ikeda [a,*]

[a] Department of Molecular Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55906, USA
[b] Department of Cancer Biology, NB40, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA

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ABSTRACT

The xenotropic murine leukemia virus-related virus (XMRV) is a human retrovirus, recently isolated from tissues of prostate cancer patients with impaired RNase L activity. In this study, we evaluated 10 licensed anti-HIV-1 compounds for their activity against XMRV, including protease inhibitors (PI), nucleoside reverse transcriptase (RT) inhibitors (NRTI), non-nucleoside RT inhibitors (NNRTI) and an integrase inhibitor. No PI affected XMRV production; even high concentrations of Ritonavir failed to inhibit the maturation of XMRV Gag polyproteins. Among the NRTI, NNRTI and integrase inhibitors used in this study, only AZT blocked XMRV infection and replication through inhibition of viral reverse transcription. This sensitivity of XMRV to AZT may be explained by the modest homology in the motif D sequences of HIV-1 and XMRV reverse transcriptases. If XMRV becomes established as an etiological agent for prostate cancer or other diseases, AZT may be useful for preventing or treating XMRV infections in humans.

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Prostate cancer is the most frequently diagnosed noncutaneous malignancy among men in industrialized countries (Simard et al., 2002). In the United States, one in eight men will develop prostate cancer during his lifetime. Prostate cancer has a complex etiology influenced by androgens, diet and other environmental and genetic factors (Nelson et al., 2003). A positive family history is among the strongest epidemiological risk factors for prostate cancer, and a number of genetic mutations have been implicated in prostate cancer. An R462Q mutation in the RNase L protein, an important effector of the innate antiviral response, was implicated in up to 13% of unselected prostate cancer cases (Casey et al., 2002). The link between prostate cancer and the RNase L R462Q mutation, which impairs its catalytic activity, suggested the possible involvement of a viral infection in prostate cancer in individuals harboring the R462Q variant. Indeed, Urisman et al. (2006) used a viral detection DNA microarray composed of oligonucleotides corresponding to the conserved sequences of all known viruses, and identified the sequences of a novel human gammaretrovirus in cDNA samples from 7 of 11 R462Q-homozygous cases, and in 1 of 8 heterozygous and

homozygous wild-type cases. This newly identified virus was termed as xenotropic murine leukemia virus (MLV)-related virus (XMRV). Further study found XMRV infection in 8 of 20 R462Q homozygous cases, while only 1 in 66 heterozygous and homozygous wild-type homozygous cases (Urisman et al., 2006).

Gammaretroviruses such as MLV, feline leukemia virus and Gibbon ape leukemia virus are associated with leukemogenesis in their respective host species (Kawakami et al., 1972, 1967; Rask-Nielsen, 1963). The two well-understood processes in gammaretrovirus-mediated leukemogenesis are insertional activation of proto-oncogenes and direct introduction of proto-oncogenes through recombination with a retroviral genome (Fan, 1997). XMRV is a gammaretrovirus, most closely related to xenotropic MLV, and uses the same XPR1 (xenotropic and polytropic retrovirus receptor 1) as their receptor (Battini et al., 1999; Dong et al., 2007). Although the role of XMRV in prostate cancer remains to be determined, XMRV infection has been observed in prostatic stromal cells in vivo (Urisman et al., 2006) and in human 22Rv1 prostate carcinoma cells (Knouf et al., 2009). The latter report suggests that viral integration could contribute to oncogenesis through insertional activation of an adjacent oncogene. Alternately, infection of prostatic stromal cells could promote prostate cancer development by secreting growth factors, cytokines or other factors that stimulate cell proliferation or aid the tumor microenvironment.

Many anti-retroviral drugs are currently available for the highly active anti-retroviral therapy (HAART) for HIV-1 treatment. In this

* Corresponding author. Department of Molecular Medicine, Mayo Clinic College of Medicine, Guggenheim 18-11c, 200 First Street SW, Rochester, MN 55905, USA. Fax: +1 507 266 2122.
E-mail address: ikeda.yasuhiro@mayo.edu (Y. Ikeda).
[1] Current address: Department of Molecular Virology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan.

study, we screened the antiviral activities of 10 major antiviral drugs on XMRV replication, and found that AZT strongly blocked XMRV replication through inhibition of viral reverse transcription. If XMRV is established as a human pathogen AZT could be useful or treating or preventing these infections.

Production of a GFP-carrying XMRV

In order to monitor XMRV production and infectivity, we generated a GFP-expressing XMRV (XMRV-GFP) by cross-packaging a GFP-encoding MLV vector genome with a full-length XMRV clone VP62. Semi-confluent 293T cells in a 6-well plate were co-transfected with a GFP-carrying MLV-based gene transfer vector construct (Noser et al., 2006) and an infectious XMRV proviral plasmid, VP62/pcDNA3.1(-) (Dong et al., 2007) using 6 μl of FuGene6 (Roche Applied Science, Indianapolis, IN). Seventy-two hours post-transfection, cell culture supernatants were harvested and filtered through 0.45 μm-filters. For titration, human LNCaP, 293T and murine NIH3T3 cells were infected with increasing amounts of GFP-carrying XMRV. Two days after infection, viral titers were determined by enumerating the GFP-positive cell populations by flow cytometry. XMRV-GFP titers reached 2.4×10^6 and 2.5×10^5 infectious units/ml (IU/ml) in LNCaP and 293T cells, respectively (Fig. 1A). As expected, murine NIH3T3 cells were not permissive to XMRV-GFP (Fig. 1A).

No evidence of anti-XMRV activity in HIV protease inhibitors (PIs)

First, we examined the influence of PI treatment on the late phase of XMRV replication. The GFP-carrying XMRV was produced in the presence of commonly used PIs including Ritonavir (Kempf et al., 1995; Molla et al., 1996), Saquinavir (Martin et al., 1991) or Indinavir (Vacca et al., 1994). As a control, an equivalent amount of DMSO was added in the culture supernatant. In order to rule out the possibility that impaired cell viability resulted in the reduced XMRV production or infectivity, we evaluated the toxicity of PIs in 293T and LNCaP cells by MTT assay (Millipore, Billerica, MA). Indinavir and Saquinavir treatment of XMRV producing cells did not strongly affect the resulting XMRV infectivity (Fig. 1B), suggesting the lack of antiviral activity against XMRV. In contrast, Ritonavir reduced the XMRV-GFP titer approximately 3-fold (Fig. 1B). Since treatment of 293T cells with Ritonavir at the final concentration of 30 nM did not show strong toxicity (Fig. 1C), we focused on Ritonavir for its possible anti-XMRV activity. Treatment of XMRV-producing cells with increasing amounts of Ritonavir revealed that 150 nM, but not 6 and 30 nM, of Ritonavir strongly blocked the XMRV production (Fig. 1D). The effects on XMRV were well-correlated with its dose-dependent toxicity in 293T cells (Fig. 1E). In order to address whether the reduced XMRV production was due to the inhibition of viral protease activity or the toxicity of Ritonavir on 293T cells, we tested the influence of Ritonavir treatment on the maturation of XMRV Gag polyproteins. As a positive control, we
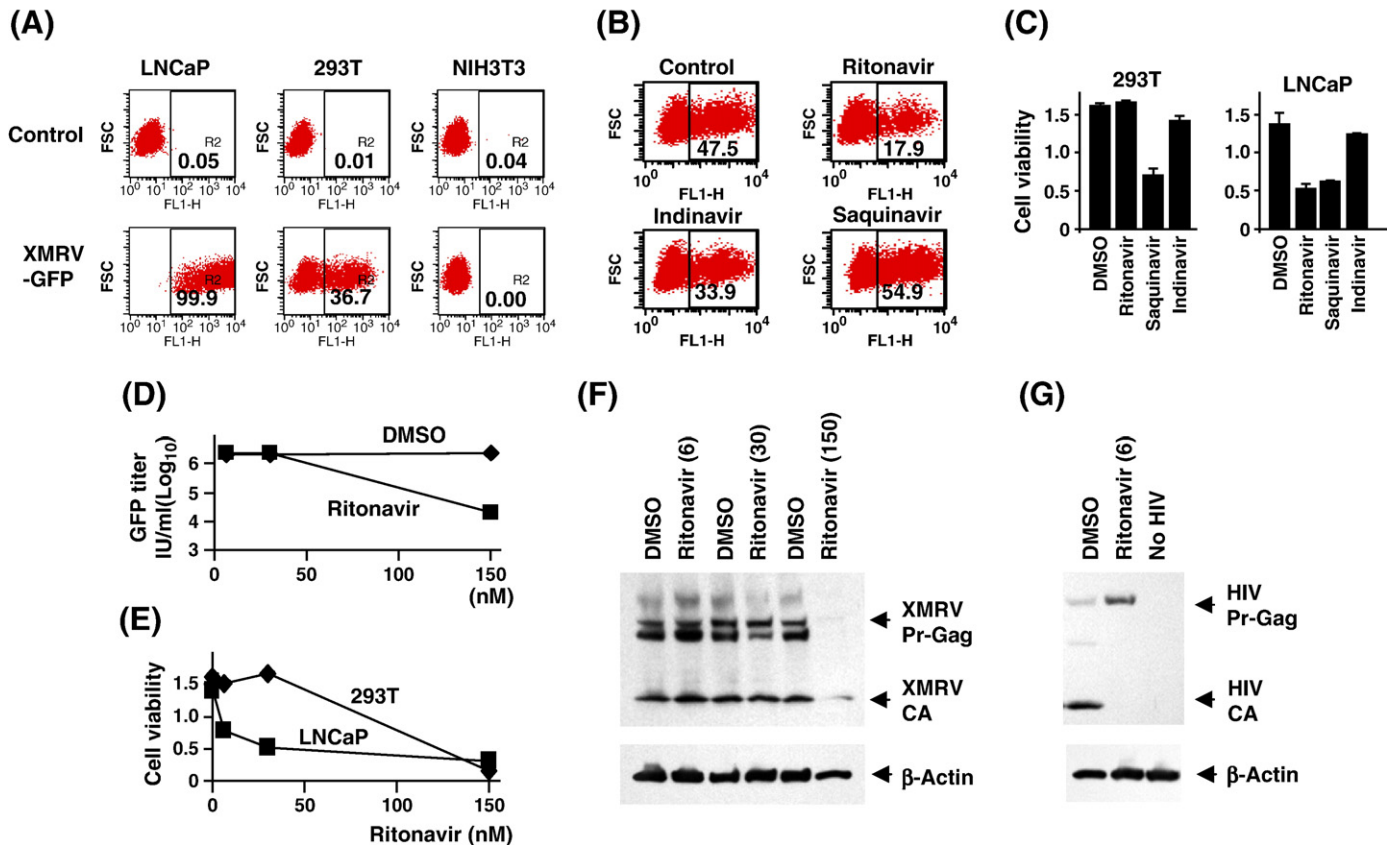

Fig. 1. No evidence of anti-XMRV activity in HIV protease inhibitors. (A) LNCaP, 293T and NIH3T3 cells were infected with GFP-carrying XMRV. Two days after infection, GFP-positive cell populations were analyzed by flow cytometry. (B) GFP-carrying XMRV was produced in the presence of 30 nM of Ritonavir, Indinavir and Saquinavir, or equivalent amount of DMSO (Control). Influence of drug treatment on viral infectivity was determined by infecting 293T cells with the GFP-carrying XMRV produced in the presence of antiviral compounds. (C) 293T and LNCaP cells were treated with 30 nM of a PI for 72 h, and cell viability was determined by MTT assay and shown as the average of absorbance (630 nm) of triplicated experiments with standard deviation. (D) GFP-carrying XMRV was produced in 293T cells in the presence of increasing amounts of Ritonavir and the resulting viral titers were determined by FACS. Equivalent amounts of DMSO were used as controls. (E) 293T and LNCaP cells were treated with 6, 30 and 150 nM of Ritonavir for 3 days, and subjected to MTT assay. (F) 293T cells were transfected with a XMRV proviral DNA plasmid, treated with various concentrations (6, 30, 150 nM) of Ritonavir or equivalent amount of DMSO. Cell lysates were analyzed to detect XMRV precursor Gag (Pr-Gag) and proteolytically cleaved mature capsid protein (CA). As a loading control, β-actin was detected by anti-β-actin antibody. (G) 293T cells were transfected with a HIV-1 provirus plasmid, pNL4-3. HIV-1-producer cells were treated with 6 nM of Ritonavir, and cell lysates were analyzed for HIV-1 precursor Gag (Pr-gag) and mature capsid (CA) proteins.

used an infectious molecular clone of HIV-1, pNL4-3. Although 6 nM of Ritonavir potently blocked the cleavage of HIV-1 Gag polyproteins into mature Capsid protein, 6 or 30 nM of Ritonavir did not affect XMRV Gag maturation patterns. Treatment with 150 nM of Ritonavir strongly reduced the levels of XMRV Gag in the producer cells. This is most likely due to the toxicity from the high concentration of Ritonavir in 293T cells (Fig. 1E), because the treatment did not change XMRV Gag maturation patterns and mature XMRV capsid was detected in the producer cell lysate. We concluded that XMRV is resistant to the licensed HIV-1 protease inhibitors, Ritonavir, Saquinavir and Indinavir.

Azidothymidine (AZT) inhibits XMRV infection through inhibition of viral reverse transcription

Next, we examined the anti-XMRV activity of licensed antiretroviral drugs including nucleoside reverse transcriptase inhibitors (NRTIs; AZT, 3TC, Tenofovir and D4T; Coates et al., 1992; Ruprecht et al., 1986; Tsai et al., 1995), non-nucleoside reverse transcriptase inhibitors (NNRTI; Efavirenz and Nevirapine; Merluzzi et al., 1990; Young et al., 1995) and an integrase inhibitor (118-D-24). LNCaP cells were infected with XMRV-GFP in the presence of 30 nM of anti-retroviral drugs. Among NRTIs tested, AZT inhibited XMRV infection up to 25-fold, while 3TC, Tenofovir and Efavirenz showed minimal or no inhibitory effects on XMRV infection (Fig. 2A). Similarly, NNRTI and

integrase inhibitors did not strongly affect XMRV infection (Fig. 2A). Marginal toxicity was observed in the LNCaP cells treated with 30 nM of the anti-retroviral drugs (Fig. 2B). To further understand the AZT-mediated block of XMRV infection, we examined the relationship between inhibition of viral infectivity and drug-induced toxicity using cells treated with 0, 6, 30 or 150 nM of AZT. As shown in Fig. 2C, 6 nM of AZT inhibited XMRV infection by 20-fold. In contrast, 150 nM of AZT was required to get 50% reduction in cell viability (Fig. 2D), demonstrating AZT as a potent anti-XMRV drug. To further examine the influence of AZT treatment on XMRV reverse transcription, we designed quantitative PCR probe/primers based on XMRV *gag*, *pol* and *env* genes and examined the levels of reverse transcripts in the XMRV-infected cells. LNCaP cells were infected with benzonase-treated XMRV in the presence of 30 nM of AZT, and total cellular DNA was isolated by QIAGEN DNA blood kit at 8 and 24 h post infection. The copy numbers of viral reverse-transcripts were determined by ABI Real Time PCR system using following primers/probes: *gag* (forward primer, 5′-CAGTTGCTCTTAGCGGGTCT-3′; reverse primer, 5′-TTAC-CTTGGCCAAATTGGTG-3′; probe "Roche library #51", 5′-FAM-ggcag-gag-3′), *pol* (forward primer, 5′-TAAAGGCGGCGACAACTC-3′; reverse primer 5′-GGTTTTGAGAACGCTGGACT-3′; probe "Roche library #150", 5′-FAM-gaacagca-3′); *env* (forward primer, 5′-CCTAGTGGCCACCAAA-CAAT-3′; reverse primer, 5′-GGCCCCAAGGTCTGTATGTA-3′; probe "Roche library #1", 5′-FAM-gctccagg-3′). As shown in Fig. 2E, AZT treatment reduced the copy numbers of XMRV cDNA in the infected
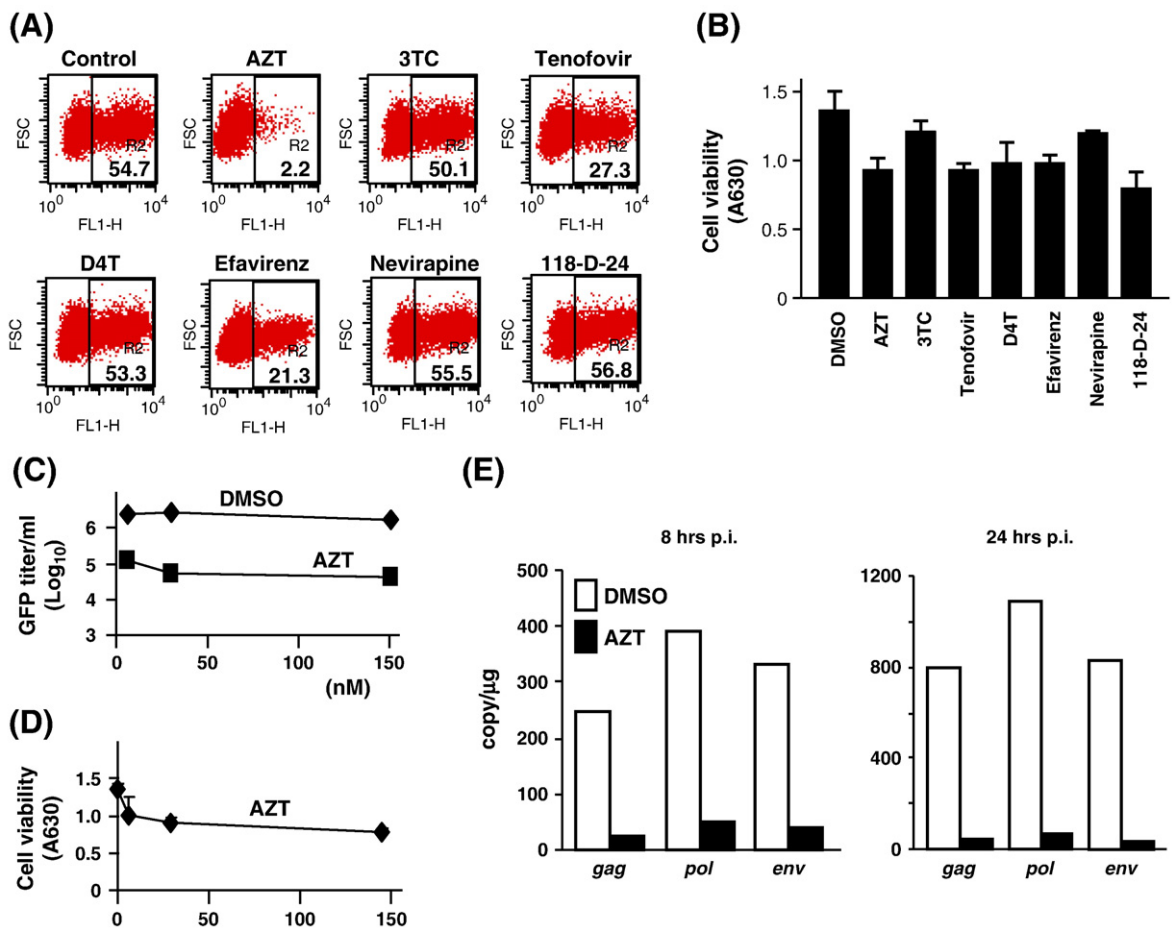

Fig. 2. Influence of RT inhibitors on XMRV infection. (A) LNCaP cells were infected with GFP-carrying XMRV in the presence of 30 nM of AZT, 3TC, Tenofovir, D4T, Efavirenz, Nevirapine and 118-D-24. As a control, equivalent amount of DMSO was used. Three days after infection, GFP-positive cell populations were analyzed by FACS. (B) 293T cells were maintained in the presence of 30 nM of the indicated antiretroviral compounds for 72 h, and cell viability was assessed by MTT assay. (C) LNCaP cells were infected with 20 μl of GFP-carrying XMRV in the presence of 6, 30 and 150 nM of AZT or equivalent amount of DMSO. Three days after infection, viral infectivity was determined by FACS. (D) LNCaP cells were treated with 6, 30 and 150 nM of AZT for 3 days, and subjected to MTT assay. (E) LNCaP cells were infected with benzonase-treated XMRV in the presence of 30 nM of AZT or equivalent amounts of DMSO. Total cellular DNA was isolated at 8 and 24 h post infection. The copy numbers of viral reverse-transcripts were determined by ABI Real Time PCR system.

cells up to 20-fold, which correlated well with the reduced XMRV infectivity upon AZT treatment. We therefore concluded that AZT blocks XMRV infection through inhibition of viral reverse transcription.

Influence of AZT treatment on XMRV replication

AZT strongly blocked single-round XMRV infection without showing marked toxicity (Fig. 2). We therefore assessed whether AZT could inhibit XMRV replication. XMRV-infected LNCaP cells were mixed with uninfected LNCaP cells at day 0, and cultured for 3 weeks in the presence of 30 nM AZT. As a control, co-cultured cells were maintained in the presence of equivalent amount of DMSO. Viral replication was monitored by the secreted virion-associated RT activity (Reverse Transcriptase Assay Colorimetric (Roche), with additional 10 mM $MnCl_2$ in the reaction buffer). Although robust XMRV replication in DMSO-treated cells was evident by the RT activity in the culture supernatants, AZT treatment could maintain the RT production at low levels (0.2 to 0.3 ng RT/ml) for 3 weeks. When

AZT was removed from the culture media at 2 weeks after treatment, rapid increases in the RT activity were observed (Fig. 3A). In contrast, when AZT was added to the DMSO-treated cells at 2 weeks after co-cultivation, no reduction in RT activity was observed in the XMRV-infected culture (Fig. 3A). These observations suggest that AZT does not eliminate the virus or virus-infected cells but blocks new XMRV infection in uninfected cells.

In this study, we evaluated 10 licensed anti-HIV-1 retroviral drugs for their activity against XMRV. We found that no protease inhibitors strongly blocks XMRV production. Among RT and integrase inhibitors tested, only AZT could efficiently block XMRV infection and replication through inhibition of viral reverse transcription. Our findings are thus consistent with a recent study demonstrating inhibition of XMRV replication by AZT in human prostate cancer cell line DU145 (Hong et al., 2009).

Despite the structural diversity of the compounds and the use of concentrations that were more than 10-fold higher than those to block HIV-1, XMRV showed no marked susceptibility to HIV-1 protease and integrase inhibitors. This is likely due to the structural
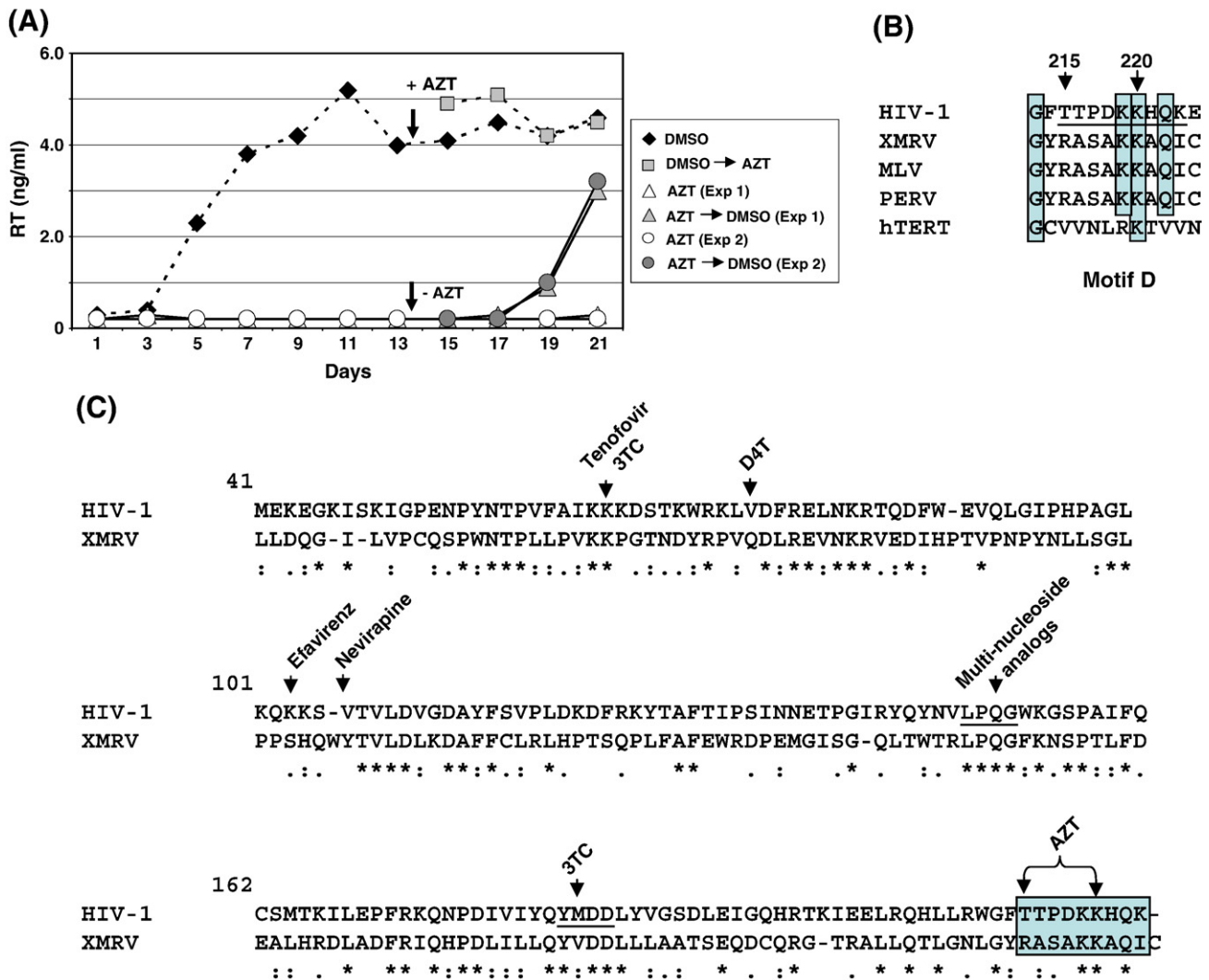

Fig. 3. (A) Influence of AZT treatment on XMRV replication. LNCaP cells were infected with XMRV stock for a week, and then co-cultured with uninfected LNCaP cells (day 0). The XMRV-infected LNCaP cells were maintained in the presence of 30 nM AZT or DMSO. Culture supernatants were collected every 2 days. Cells were passaged on days 7 and 13. On day 13, AZT treated cells were divided into two culture bottles; one was maintained in 30 nM AZT and the other in DMSO. Similarly, DMSO-treated cells were divided into two bottles and treated with DMSO or 30 nM AZT for additional 8 days. (B) Alignment of portions of HIV-1 and XMRV RT amino acid sequences. Amino acid sequence alignments of various RNA-dependent DNA polymerases including HIV-1, XMRV, MLV, PERV and hTERT. The HIV-1 motif D amino acid residues were underlined. Codons associated with resistance of HIV-1 to AZT (T215Y, K220Q) are indicated by arrows. (C) Alignment of portions of HIV-1 and XMRV RT amino acid sequences. Identical residues are indicated with an asterisk. The HIV-1 polymerase active-site YMDD and the LPQG motif are underlined. Codons associated with resistance of HIV-1 to multi-nucleoside analogs (Q151M), Tenofovir (K65R), D4T (V75I), Efavirenz (K103N), Nevirapine (V106A), 3TC (K65R/M184V) and AZT (T215Y, K220Q) are indicated by arrows.

differences between XMRV and HIV-1 viral enzymes. In this context, it is notable that AZT could inhibit the reverse transcriptase activity of two highly divergent retroviruses. The determinant of the sensitivity of HIV-1 RT to AZT has been extensively studied (Larder et al., 1989). Although many mutations in the HIV-1 RT were reported to be responsible for AZT resistance, the motif D in the palm domain of RT appears to play a critical role for the resistance against AZT (Canard et al., 1999; Lacey and Larder, 1994). Mutations in the amino acid residues Thr-215 and Lys-220 can render HIV-1 RT resistant to AZT through enhanced excision of the chain-terminating AZT-5′-mono-phosphate (Boyer et al., 2001a, 2002; Sarafianos et al., 2002) or through changing the interaction with the incoming nucleotide (Canard et al., 1999; Gao et al., 1992; Lopez-Galindez et al., 1991; Matamoros et al., 2004). Intriguingly, other RNA-dependent DNA polymerases, including retroviral and mammalian enzymes, have a motif D in their palm regions (Armanios et al., 2005; Canard et al., 1999). The amino acid sequence of XMRV motif D shows a modest homology to its HIV-1 counterpart and the amino acid residue corresponding to the critical Lys-220 of HIV-1 RT is conserved (Fig. 3B). The XMRV motif D shows 100% homology to those of other gammaretroviruses, such as MLV and porcine endogenous retrovirus (PERV) (Fig. 3B). As expected, human telomerase reverse transcrip-tase (hTERT) shows limited homology to the retroviral motif D sequences, yet the amino acid residue corresponding to the HIV-1 RT Lys-220 is conserved (Fig. 3B). It is intriguing to note that AZT can also inhibit the reverse transcriptase activities of MLV, PERV and hTERT (Mo et al., 2003; Qari et al., 2001; Ruprecht et al., 1986; Sharpe et al., 1988; Strahl and Blackburn, 1996), although the precise mechanisms of inhibition remain to be determined. We speculate that the motif D sequences, particularly the conserved Lys residues, of retroviral RTs or hTERT determine their sensitivity to AZT. Verifying the possible role of XMRV motif D in the sensitivity to AZT will require further drug susceptibility analysis of site-specific XMRV mutants. Although we did not observe the emergence of an AZT-resistant XMRV mutant during the first 3 weeks of treatment, it will be of interest to assess whether XMRV can escape from AZT by naturally acquiring mutation(s) in the RT upon long-term culture in the presence of AZT.

In contrast to AZT, other HIV-1 RT inhibitors did not show notable anti-XMRV activity. To better understand the basis of XMRV susceptibility to the RT inhibitors tested in this study, we compared the amino acid sequences of the XMRV and HIV-1 RTs. Although the mechanisms of HIV-1 resistance to RT inhibitors cannot be explained by single amino acid mutations, we indicated common RT inhibitor resistance-related amino acid residues of HIV-1 RT by arrows (Fig. 3C). Although Q151M mutation in the LPQG motif of HIV-1 RT is associated with resistance to multiple nucleoside analogs (Shirasaka et al., 1995), this motif is fully conserved in XMRV RT (Fig. 3C), thus ruling out the possible role of this domain in the XMRV resistance against many RT inhibitors. Mutations K65R, V75I, K103N, V106A and K65R/M184V in HIV-1 RT are associated with resistance to Tenofovir, D4T, Efavirenz, Nevirapine and 3TC, respectively (Gu et al., 1994; Masquelier et al., 2004; Nitanda et al., 2005; Quan et al., 1999; Ren et al., 2000; White et al., 2004). XMRV RT has a Lys at the residue corresponding to Lys-65 in HIV-1 RT, which does not support a role of this residue in the observed resistance to Tenofovir. It is plausible that the amino acid residues proximal to Lys-65 render the XMRV RT resistant to Tenofovir. In contrast, XMRV has Gln, Ser, Leu and Val at the residues corresponding to 75-Val, 103-Lys, 106-Val and 184-Met, respectively, which may explain the observed XMRV resistance to D4T, Efavirenz, Nevirapine and 3TC.

Although we did not see notable antiviral effects by Indinavir on XMRV production, Powell et al. (1999) reported the inhibition of MLV production by Indinavir. It is possible that the toxicity of Indinavir resulted in the block of MLV production in this particular study (Powell et al., 1999). Alternatively, the variations in the pol gene between MLV and XMRV changed the susceptibility of these two

related retroviruses to Indinavir. In addition, it will be of interest to assess whether didanosine (ddI) can block XMRV infection, as ddI has minimal antiviral effects against MLV (Powell et al., 1999; Boyer et al., 2001b).

Many retroviruses are known to cause cancer. Well-characterized mechanisms for tumorigenesis include the insertional activation of host oncogenes or introduction of acquired host-derived oncogenes in the infected cells (Lund et al., 2002; Peters et al., 1983; Sheiness et al., 1980; Steffen, 1984). Some retroviruses, such as human T cell leukemia virus and Jaagsiekte sheep retrovirus, are known to encode viral proteins which induce abnormal cell growth by activation of growth-promoting genes or by inhibiting tumor suppressor genes (Seiki et al., 1984; Tanaka et al., 1990; Wootton et al., 2005). The role of XMRV in prostate cancer development remains to be determined. However, the initial study identified XMRV-infected cells as stromal cells, rather than carcinoma cells (Urisman et al., 2006), suggesting that XMRV may support prostate cancer development through indirectly promoting abnormal cell growth. If XMRV is established as a contributing agent in prostate cancer, AZT may be able to prevent/slow prostate cancer development in XMRV-infected populations. It is however notable that a recent study have identified a commonly used prostate carcinoma cell line, 22Rv1, to consist primarily of a single clone of cells with multiple integrated copies of XMRV (Knouf et al., 2009), which may suggest an alternative, insertional mutagenesis-mediated prostate cancer development in XMRV-infected subjects. Our result indicates that AZT blocks XMRV infection, but does not eliminate the infected cells (Fig. 3A). If insertional mutagenesis is the primary mode of XMRV-mediated tumorigenesis, AZT treatment is unlikely to block the progression of XMRV-positive prostate cancers. In this case, AZT may be used to prevent/control XMRV infection, particularly in high-risk subjects with a homologous RNase L R462Q mutation. Further understanding of the role of XMRV in prostate cancer development is necessary for the possible use of AZT to control prostate cancers in XMRV-infected patients.

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