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Authors
Heredia, Alonso
Davis, Charles
Amin, Mohammed N
et al.

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Targeting Host Nucleotide Biosynthesis with Resveratrol Inhibits Emtricitabine (FTC)-resistant HIV-1

Alonso Heredia, **Charles Davis**, **Mohammed N Amin**, **Nhut M Le**, **Mark A Wainberg**[1], **Maureen Oliveira**[1], **Steven G Deeks**[2], **Lai-Xi Wang**, and **Robert R Redfield**

Institute of Human Virology, University of Maryland School of Medicine, Baltimore, Maryland [1]McGill University AIDS Centre, Lady Davis Institute, Jewish General Hospital, Montreal, Quebec, H3T1E2 Canada [2]San Francisco VA Medical Center and University of California, San Francisco, California

Abstract

Objective—The M184V mutation in the HIV-1 reverse transcriptase (RT) gene is frequent (> 50 %) in patients, both in resource-rich and resource-limited countries, conferring high-level resistance (> 100-fold) to the cytosine analog RT inhibitors 3TC and FTC. The RT enzyme of M184V HIV-1 mutants has reduced processivity, resulting in reduced viral replication, particularly at low nucleotide (dNTP) levels. We hypothesized that lowering intracellular dNTPs with Resveratrol (RV), a dietary supplement, could interfere with replication of M184V HIV-1 mutants.

Design and Methods—Evaluation of the activity of RV on infection of primary peripheral blood lymphocytes (PBLs) by wild type and M184V mutant HIV-1. We assayed both molecular clones and primary isolates of HIV-1, containing M184V alone and in combination with other RT mutations. Viral infection was quantified by p24 ELISA and by quantitative real-time PCR analysis. Cell viability was measured by MTT assays.

Results—In virus infectivity assays, RV did not inhibit replication of wild-type NL43 (RV EC_{50} > 10 μM), but it inhibited NL43 184V mutant (RV EC_{50} = 5.8 μM). These results were confirmed by real-time PCR analysis of early and late products of reverse transcription. RV inhibited molecular clones and primary isolates carrying M184V, alone or in combination with other RT mutations (RV EC_{50} values ranging 2.5–7.7 μM).

Conclusions—RV inhibits HIV-1 strains carrying the M184V mutation in RT. We propose RV as a potential adjuvant in HIV-1 therapy, particularly in resource limited settings, to help control FTC-resistant M184V HIV-1 mutants.

Send correspondence to: Alonso Heredia, Institute of Human Virology, 725 W. Lombard St, Baltimore, MD 21201., Phone: 410-7064594, Fax: 410-7064992, aheredia@ihv.umaryland.edu.

POTENTIAL CONFLICTS OF INTEREST

The authors report no conflict of interest.

Authors contributions: AH, CD, MA and RRR designed the experiments; AH, MA, MO, and NML performed the experiments; AH, LW, MA SGD, MAW and RRR contributed to data analysis, discussion of results and writing of the manuscript.

Keywords

HIV-1; drug resistance; cytosine analogs; NRTI; FTC; M184V mutation; antiretroviral therapy; resource-limited setting

INTRODUCTION

Resveratrol (RV), a natural ingredient found in certain plants and plant products, inhibits ribonucleoside reductase, the key enzyme step that converts deoxy-nucleotides (dNTPs) from their ribose precursor [1]. RV also arrests the cell cycle at the S/G2 transition, prolonging the S phase and increasing nuclear nucleoside utilization [2].

We have previously reported that RV, which lacks activity against wild-type HIV-1 or against HIV-1 carrying thymidine or adenosine analog resistance mutations, enhances the antiviral activity of nucleoside analog RT inhibitors (NRTIs) [3, 4]. RV antiviral enhancement is highest (up to 10-fold) with the adenosine analogs didanosine (DDI) and tenofovir (TDF), consistent with RV preferential depletion of dATP (natural competitor of DDI and TDF) [5]. Importantly, RV restores the TDF sensitivity of TDF-resistant HIV-1 [4].

The cytosine analogs lamivudine (3TC) and emtricitabine (FTC) select for the M184V mutation, which confers high-level drug resistance (100 to 1000-fold) [6]. However, the gain of function in the mutated 184V RT enzyme is offset by a decreased processivity (i.e.; average number of nucleotides incorporated each time RT engages a primer) and thereby reduced replicative capacity of the mutant virus [7]. Decreased processity of the mutant RT is further reduced at low dNTP levels [8, 9]. Thus, we tested the hypothesis that reduction of dNTP levels with RV might impair the replication of HIV-1 carrying the M184V mutation. Our data demonstrate that RV by itself has activity against viruses with the M184V mutation, unlike what has been shown with wild type HIV-1 or with HIV-1 carrying thymidine/adenosine analog resistance mutations [3, 4].

METHODS

Viruses and Drugs

The HIV-1 molecular clone NL4-3 and its derivatives carrying RT sequences amplified from the plasma of patients with drug resistant HIV-1 were obtained from Dr. Robert Shafer (Stanford University School of Medicine, Stanford, CA) through the NIH AIDS Repository (Germantown, MD). Primary isolates 4742, BG05, BG15 and their corresponding FTC-resistant mutants were provided by Dr. Mark Wainberg [10]. Multi-drug resistant primary isolates were provided by Dr. Steven Deeks. Emtricitabine (FTC) was obtained from the NIH AIDS Repository, and RV (*trans* form) was purchased from Sigma (St Louis, MO).

Cells and infectivity assays

Peripheral blood lymphocytes (PBLs) were separated from buffy coats of HIV-1 seronegative donors (New York Blood Center, NY) by density centrifugation over Ficoll-

Hypaque (Sigma). For infection, PBLs, cells were stimulated with 2.5 μg/ml phytohemagglutinin (PHA; Boehringer Mannheim, Indianapolis, IN) for 3 days. Stimulated PBLs were infected by incubation with virus at a multiplicity of infection (MOI) of 0.001 for 2 hours. PBLs were then washed three times with PBS and cultured in 5% CO2 at 37° C, in RPMI/10% FBS supplemented with 10 units/ml IL-2 (Boehringer Mannheim) and drugs. PBLs were seeded in 96-well flat-bottom plates at a density of 2×10^5 PBLs/200 μl. Following 3 days of culture, half of the medium was replaced with fresh medium containing IL-2 and drugs. On day 7, HIV-1 p24 antigen production in the culture supernatant was assayed by ELISA (Coulter, Hialeah, FL).

MTT assays

Cell viability was measured by the colorimetric MTT test using a commercial kit (Roche). This test is based on the reduction of the yellow colored MTT [3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to blue formazan by mitochondrial dehydrogenases. The quantity of formazan produced (absorbance at 490 nm) is directly proportional to the number of living cells. Briefly, cell aliquots were seeded in 96-well plates (100 μl) and incubated with 10 μl of MTT solution for 4 h at 37°C. A solubilization solution (50 μl) was added and plates incubated overnight at 37°C. MTT conversion to formazan by mitochondrial dehydrogenase was assayed by optical density at 490 nm measured in an ELISA plate reader.

Real-Time PCR

DNA was isolated from HIV-1 infected cells using Miniblood kit (Qiagen, Germantown, MD) following the manufacturer's recommendations. PCR amplification was performed using Quantitect SYBR Green PCR Kit (Qiagen), in reactions containing 100 ng of DNA and primers to detect early or late HIV-1 reverse-transcribed DNA. Detection of early transcripts was done with primer pairs 5'-GCTCTCTGGCTAACTAGGGAAC-3' and 5'-TGACTAAAAGGGTCTGAGGGAT-3' (R/U5 region), and late transcripts with 5'-TGGCATGGGTACCAGCACA-3' and 5'-CTGGCTACTATTTCTTTTGCTA-3' (R/gag region). Samples were also amplified with primers for the housekeeping gene α–tubulin. Amplifications were done in a LightCycler (Biorad, Hercules, CA) at an annealing temperature of 56°C. Amplified products were analyzed by denaturation/renaturation to verify the specific Tm. The PCR cycle at which the signal entered the exponential range was used for quantification, and HIV-1 copy numbers corrected for those of α–tubulin. Standard curves for HIV-1 and α–tubulin copy numbers were generated by analyzing serial dilutions of plasmids carrying the corresponding sequences.

RESULTS

RV inhibits FTC-resistant HIV-1 carrying the M184V mutation

We evaluated the activity of RV against wild-type NL4-3 and mutant NL4-3/184V infectious molecular clones in PBLs. We conducted these experiments in the absence and presence of 10 μM FTC to confirm the FTC sensitivity phenotype of the tested viruses. As expected, in the absence of RV, 10 μM FTC completely inhibited wild-type NL4-3 but not NL4-3/184V (Fig 1a). Also as expected, RV treatment alone did not have activity against

wild-type NL4-3. In contrast, RV alone inhibited NL4-3/184V (Fig 1a). RV inhibition of NL4-3/184V was slightly increased by FTC. We confirmed the RV inhibitory activity against NL4-3/184V infection of PBLs by performing real-time PCR analysis with HIV-1 primer pairs specific for R/U5 (initial region of reverse transcription) and R/gag (last region of reverse transcription) (Fig 1b). At 72 h after infection, RV did not inhibit DNA synthesis of wild-type HIV-1 (as expected), but 5 μM RV markedly (>10-fold) and 10 μM RV completely inhibited DNA synthesis of NL4-3/184V (both R-U5 and R-gag transcripts). Together, these data suggest that RV depletion of dNTPs is sufficient to reduce the enzymatic activity of 184V RT, which has less processivity than wild-type RT at low dNTPs levels [7–9].

We next evaluated RV against HIV-1 primary isolate pairs, with and without the M184V mutation as the sole mutation in RT, derived from the same patient. These isolates (4742, BG05 and BG15) have been described previously [10]. We chose viruses with M184V as single mutation to avoid confounding of the data by additional mutations that might compensate for the viral growth disadvantage conferred by M184V. Results are shown in Table 1. In each WT/M184V virus pair, RV inhibited M184V mutant (EC_{50} values ranging between 2.5 and 6.7 μM), but not WT (EC_{50} > 10 μM). These results demonstrate that RV has antiviral activity against viruses with the M184V mutation as the sole mutation in RT.

RV inhibits multi-drug resistant viruses carrying the M184V mutation

The M184V mutation is frequently observed in conjunction with RT mutations conferring resistance to other NRTIs. We tested the activity of RV against NL4-3 clones containing the RT region amplified from plasma of patients with multi-drug resistance (Table 1). RV failed to inhibit drug-resistant NL4-3 clones lacking the M184V mutation, but inhibited all drug-resistant clones containing M184V (EC_{50} values ranging between 2.7 and 5.8 μM). To further confirm inhibition of RV against multi-drug resistant HIV-1, we next evaluated RV against isolates from patients with multi-drug resistance (isolates 3212, 6061 and 6017). Similar to the data with NL4-3 molecular clones, RV inhibited the multi-drug resistant isolate with the M184V mutation but not those lacking it. Collectively, the data demonstrate antiviral activity of RV against HIV-1 carrying the M184V mutation, alone or in combination with other mutations in RT.

DISCUSSION

The dietary supplement RV modulates cell proliferation by inhibiting ribonucleotide reductase, preferentially depleting dATP and prolonging the cellular S phase [1, 2]. RV inhibition of cell proliferation is more potent in cancer cells (EC_{50} < 10 μM) than in normal cells or PBLs (EC_{50} >10 μM) [11, 12]. We have previously demonstrated that RV, which had no direct antiviral effects against wild-type HIV-1 or against thymidine or adenosine analog-resistant mutants, enhances the antiviral activity of NRTIs [3, 4]. Clouser et al. have shown that RV and nucleoside analog decitabine are synergistic against HIV-1 [13].

We now demonstrate that treatment with RV alone is sufficient to inhibit HIV-1 mutants with the M184V mutation, present in > 50 % of treated patients [14]. RV inhibited viruses carrying M184V, singly or in combination with other mutations. These data are consistent

with reduced processivity of 184V mutant RT [7], particularly at low dNTP levels [8, 9]. The decreased processivity of 184V RT mutants confers a viral replication disadvantage that has clinical benefit [15]. As such, maintenance of the M184V mutation is considered to be clinically useful to sustain this replication disadvantage even in the presence primary drug resistance. Our data suggest that RV could be used as an adjuvant in the treatment of HIV-1, helping to control replication of drug-resistant M184V mutants. In addition to this anti-HIV activity, adjuvant treatment with RV may be beneficial to HIV-1 patients by decreasing oxidative stress induced by thymidine analogs [16], and by decreasing protease inhibitor toxicity [17].

RV administration has shown no significant toxicity in humans [18, 19]. However, one caveat with its use is low bioavailability, with rapid metabolism into glucuronides and sulfates [20, 21]. Despite low bioavailability, it is intriguing that RV has shown beneficial effects in the treatment of some cancers, diabetes and cardiovascular disease, both in animals and in humans [18, 19]. It is possible that RV metabolites (mainly glucuronides and sulfates) may be converted back to active RV by cellular esterases and sulfatases [20, 21]. It is also possible that enterohepatic recirculation through biliary secretion of metabolites and subsequent deconjugation by gut microflora could explain the *in vivo* activity of RV [22]. In an effort to boost RV bioavailability, several derivatives and delivery approaches are being pursued [13, 23, 24].

Together, adjuvant treatment with RV, or with more bioavailable derivatives, may increase antiretroviral treatment success in patients by helping control replication of highly prevalent FTC-resistant M184V HIV-1 mutants.

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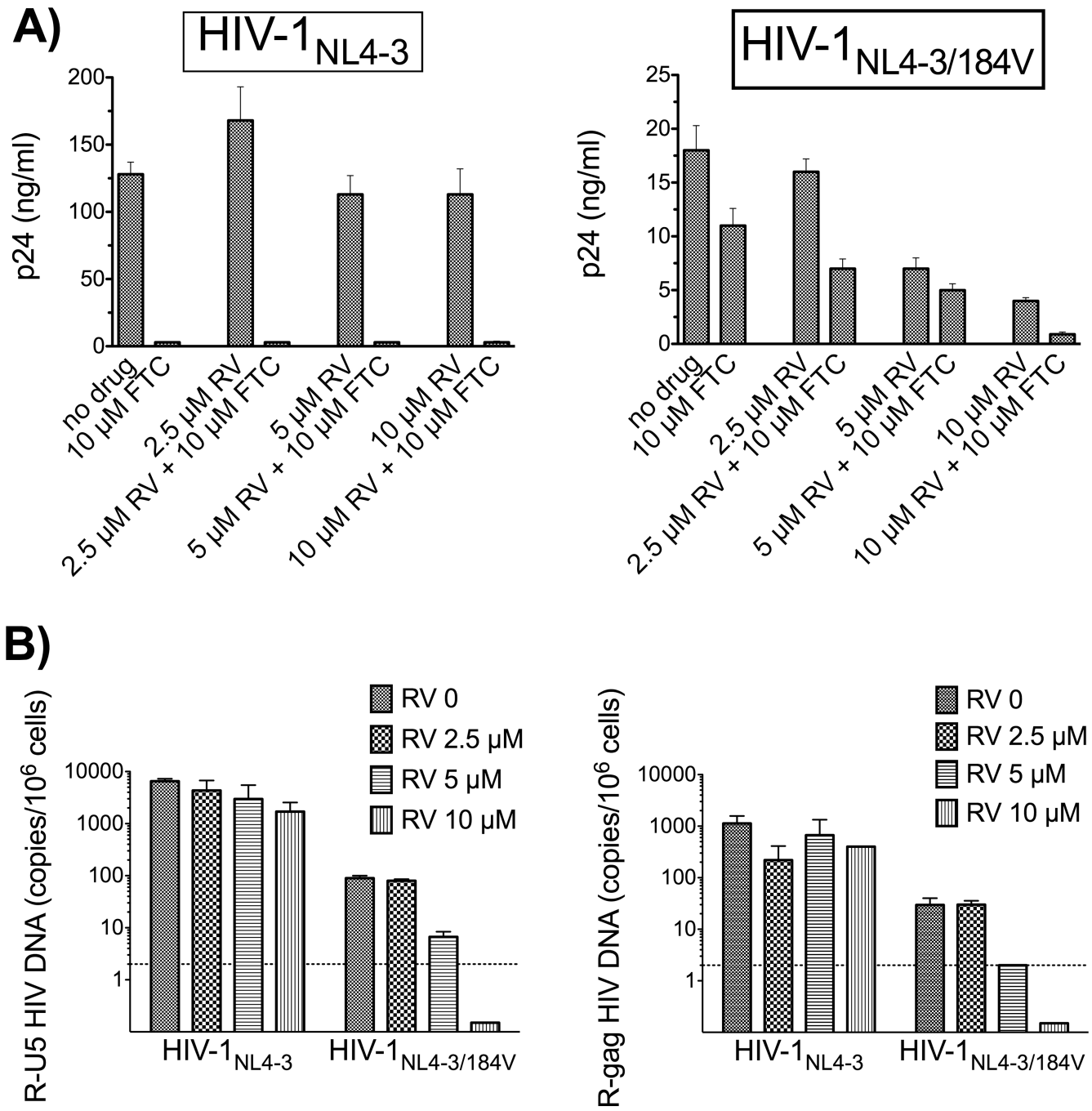

Figure 1. Resveratrol (RV) inhibits FTC-resistant HIV-1 NL4-3184V

A) Activated PBLs infected with wild-type NL4-3 (top) or FTC-resistant NL4-3_{184V} (bottom) were cultured in the absence and presence of RV and FTC, alone and combined. Virus production was measured on day 7 by p24 ELISA. Note differences in scale in *y axis* between plots. B) Quantification of HIV-1 transcripts (R-U5 and R-Gag) in PBLs infected with NL4-3 or NL4-3_{184V} and cultured in the presence of various concentrations of RV for 72 h. HIV-1 copy numbers were corrected after amplification with α–tubulin primers. The

dotted line indicates the limit of detection of the PCR assay. In both A and B, data are means ± S.D. of two independent experiments.

Table 1

Resveratrol inhibits HIV-1 with the M184V mutation, alone or in combination with other mutations in reverse transcriptase

Virus[1]	**Drug Resistance Mutations in RT**	**Geometric mean RV EC_{50}, μM (95% CI)**[2]
HIV-1_{4762}	none	>10
HIV-1_{4762}/3TC	**184V**	6.7 (2.34–19.4)
HIV-1_{BG05}	none	>10
HIV-1_{BG05}/3TC	**184V**	2.5 (0.76–8.4)
HIV-1_{BG15}	none	>10
HIV-1_{BG15}/3TC	**184V**	4.3 (1.86–9.86)
NL43	none	>10
$NL43_{7303-3}$	41L,67N,210W,215Y,69D,44D,118I	>10
$NL43_{7324-1}$	41L,67N,70R,215F,219E,69N	>10
$NL43_{7324-4}$	41L,70R,215F,219E,	>10
$NL43_{71361-1}$	65R	>10
$NL43_{184V}$	**184V**	5.8 (5.81–5.82)
$NL43_{7295-1}$	67N,70R,215F,219Q,**184V**,69N	4.1 (2.76–6.03)
$NL43_{8415-2}$	**184V**,65R	3.9 (3.28–4.75)
$NL43_{10076-4}$	41L,215Y,**184V**	3.6 (1.25–10.60)
$NL43_{4755-5}$	41L,67N,210W,215Y,**184V**,69D,44D,118I	5.5 (2.84–10.76)
$NL43_{6463-13}$	41L,67N,210W,215Y,**184V**,118I	3.5 (2.60-4-60)
$NL43_{1617-1}$	70G,**184V**,69K,75I,77L,116Y,151M	3.3 (2.57–4.35)
$NL43_{29129-2}$	41L,67N,210W,215Y,**184V**,	5.2 (4.13–6.60)
$NL43_{52534-2}$	41L,210W,215Y,**184V**,InsSS,74V	2.7 (1.55–4.70)
HIV-1_{3212}	103N,181C	>10
HIV-1_{6061}	41L,67N,70R,210W,215Y,219E	>10
HIV-1_{6017}	41L,67N,69D,70R,103N,**184V**,219Q	7.7 (6.44–9.21)

RV denotes Resveratrol; RT denotes reverse transcriptase.

[1] Viruses with the HIV-1 prefix are primary isolates, whereas those with the NL43 prefix are molecular clones.

[2] EC_{50} values determined by variable slope non-linear regression analysis using GraphPad Prism software. Each virus was run in at least 2 independent assays, with different donors in each assay.