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Benjamin J. Fowler  
*University of Kentucky*

Bradley D. Gelfand  
*University of Kentucky*

Younghee Kim  
*University of Kentucky*

Nagaraj Kerur  
*University of Kentucky*

Valeria Tarallo  
*University of Kentucky*

*See next page for additional authors*

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Authors
Benjamin J. Fowler, Bradley D. Gelfand, Younghee Kim, Nagaraj Kerur, Valeria Tarallo, Yoshio Hirano, Shoba Amarnath, Daniel H. Fowler, Marta Radwan, Mark T. Young, Keir Pittman, Paul Kubes, Hitesh Agarwal, Keykavous Parang, David R. Hinton, Ana Bastos-Carvalho, Shengjian Li, Testuhiro Yasuma, Takeshi Mizutani, Reo Yasuma, Charles Wright, and Jayakrishna Ambati
Nucleoside reverse transcriptase inhibitors possess intrinsic anti-inflammatory activity

Benjamin J. Fowler1,2, Bradley D. Gelfand1,3,4, Younghee Kim1, Nagaraj Kerur1, Valeria Tarallo1,5, Yoshio Hirano1, Shoba Amarnath6, Daniel H. Fowler6, Marta Radwan7, Mark T. Young7, Keir Pittman8, Paul Kubes8, Hitesh K. Agarwal9, Keykavous A. Parang9, David R. Hinton10, Ana Bastos-Carvalho1, Shengjian Li1, Tetsuhiro Yasuma1, Takeshi Mizutani1, Reo Yasuma1, Charles Wright1, and Jayakrishna Ambati1,2

1Department of Ophthalmology & Visual Sciences, University of Kentucky, Lexington, Kentucky 40536, USA
2Department of Physiology, University of Kentucky, Lexington, Kentucky 40536, USA
3Department of Microbiology, Immunology, and Human Genetics, University of Kentucky, Lexington, Kentucky 40536, USA
4Department of Biomedical Engineering, University of Kentucky, Lexington, Kentucky 40536, USA
5Angiogenesis Lab, Institute of Genetics and Biophysics, CNR, Naples, Italy
6Experimental Transplantation and Immunology Branch; National Cancer Institute; National Institutes of Health; Bethesda, Maryland, USA
7School of Biosciences, Cardiff University, Cardiff CF10 3AX, United Kingdom
8Immunology Research Group, University of Calgary, Calgary, Alberta T2N 4N1, Canada
9Chapman University School of Pharmacy, 9401 Jeronimo Rd., Harry and Diane Rinker Health Science Campus, Irvine, CA, 92618, United States
10Departments of Pathology and Ophthalmology, Keck School of Medicine of the University of Southern California, Los Angeles, CA 90033

Abstract

Nucleoside reverse transcriptase inhibitors (NRTIs) are mainstay therapeutics for HIV that block retrovirus replication. Alu (an endogenous retroelement that also requires reverse transcriptase for its life cycle)-derived RNAs activate P2X7 and the NLRP3 inflammasome to cause cell death of the retinal pigment epithelium (RPE) in geographic atrophy, a type of age-related macular degeneration. We found that NRTIs inhibit P2X7-mediated NLRP3 inflammasome activation independent of reverse transcriptase inhibition. Multiple approved and clinically relevant NRTIs prevented caspase-1 activation, the effector of the NLRP3 inflammasome, induced by Alu RNA. NRTIs were efficacious in mouse models of geographic atrophy, choroidal neovascularization,
NRTIs are widely used to treat HIV. Age-related macular degeneration (AMD) is a leading cause of blindness in the elderly population worldwide (1, 2). In geographic atrophy, the late stage of the prevalent and untreatable dry form of AMD, overabundance of non-coding Alu RNAs is implicated in cell death of the retinal pigment epithelium (RPE) (3–5). Since Alu sequences are non-coding retrotransposons that, like HIV, rely on reverse transcriptase for their life cycle (6), we hypothesized that NRTIs could block Alu RNA-induced cytotoxicity. Multiple NRTIs are typically administered orally to HIV patients at a total NRTI dose of up to 15 mg/kg/day (7) (equivalent dose in mice: 185 mg/kg/day; (8)). A clinically relevant dose (single daily oral administration of 150 mg/kg) of the NRTI stavudine, (d4T), which is U.S. FDA-approved for the treatment of HIV infection, prevented Alu-induced RPE degeneration (4, 5) in wild-type mice (Fig. 1A). A lower dose of oral d4T (50 mg/kg/day) also prevented Alu-induced degeneration, as did twice daily intraperitoneal administration of 50 mg/kg of the NRTI azidothymidine (AZT) (Fig. 1B and fig. S1).

Alu RNA induces RPE cell death via activation of Caspase-1 by the innate immune complex known as the NLRP3 inflammasome (5, 9). Western blotting of RPE lysates from d4T-treated mice confirmed that d4T blocked Caspase-1 activation (fig. S2A). Caspase-1, in turn, cleaves pro-interleukin (IL)-18 into its mature form, which induces RPE degeneration via IRAK4 phosphorylation (5); d4T also blocked IL-18 maturation and IRAK4 phosphorylation in vivo (fig. S2A). Consistent with the concept that d4T prevents RPE degeneration upstream of IL-18 by blocking Caspase-1 activation, the protective effect of d4T in Alu-treated mice was overridden by subretinal injection of recombinant mature mouse IL-18 (fig. S2B). d4T also prevented Alu-induced Caspase-1 activation (10) and IRAK4 phosphorylation in primary human (Fig. 1C) and wild-type mouse RPE cells (fig. S2C) without reducing Alu RNA levels (fig. S2D). Other clinically relevant NRTIs, including lamivudine (3TC) and abacavir (ABC), similarly blocked Caspase-1 cleavage induced by Alu RNA (Fig. 1D).

To test whether reverse transcriptase inhibition was required for inflammasome blockade by d4T, we synthesized a novel 5′-O-methyl-modified version of d4T (me-d4T) (figs. S3–S5). Only the triphosphate forms of nucleoside analogs inhibit reverse transcriptase; the methyl modification at the 5′ position prevents phosphorylation and thus formation of nucleoside triphosphate (11). As predicted, me-d4T, unlike d4T, did not inhibit lentiviral vector transduction of GFP (fig. S6, A and B). Moreover, the triphosphate metabolite of some dideoxy nucleoside analogs causes depletion of mitochondrial DNA (12); we found that d4T, but not me-d4T, reduced mtDNA levels in RPE cells (Fig. 2A). Remarkably, despite its inability to inhibit polymerases, me-d4T still blocked Caspase-1 activation and IRAK4 phosphorylation by Alu RNA in RPE cells (Fig. 2B). We confirmed that me-d4T also prevented Alu-induced RPE degeneration in wild-type mice (Fig. 2C). These data suggest that d4T can block Caspase-1 activation and RPE degeneration independent of reverse transcriptase inhibition.
We also tested whether NRTIs blocked NLRP3 inflammasome activation by LPS/ATP, which is not known to signal via reverse transcriptase (13). d4T inhibited LPS/ATP-induced Caspase-1 maturation in primary mouse bone marrow-derived macrophages (Fig. 3A). To demonstrate that LPS/ATP-induced inflammasome activation can be inhibited without reverse transcriptase inhibition, we utilized a cell system that is incapable of NRTI phosphorylation, which is required for reverse transcription inhibition. Although d4T metabolism is largely unaffected in thymidine kinase-deficient (Raji/TK−) cells, AZT phosphorylation is severely impaired compared to thymidine kinase-expressing (TK+) parental cells (figs. S7–S10) (14). One consequence of LPS/ATP-induced Caspase-1 activation is the proteolytic cleavage and maturation of interleukin-1β (IL-1β). Even though AZT was not phosphorylated in TK− cells, it still inhibited LPS/ATP-induced Caspase-1 and IL-1β maturation (Fig. 3B), indicating that AZT did not inhibit IL-1β and Caspase-1 maturation via reverse transcriptase inhibition.

In support of the idea that NRTIs specifically impaired Caspase-1 activation, levels of pro-Caspase-1/IL-18 were not considerably changed as shown in (Figs. 1, C and D, fig. S2A, and Fig. 3, A and B), although AZT did diminish pro-IL-1β protein levels (Fig. 3B). Moreover, d4T did not reduce expression of NLRP3 or IL1B mRNAs in RPE cells treated with Alu RNA (fig. S11). d4T also did not affect mRNA priming of non-inflammasome genes (Il6, Il12a, Tnf) by LPS (fig. S12) in BMDMs. Consistent with this finding, and that LPS signaling occurs via IRAK4 phosphorylation, d4T did not reduce IRAK4 phosphorylation after LPS stimulation (fig. S13).

Alu RNA (9) and LPS/ATP (15) activate the NLRP3 inflammasome via the ATP receptor P2X7. d4T did not block extracellular release of ATP induced by Alu RNA in primary human RPE cells (fig. S14A). We therefore hypothesized that d4T blocks P2X7 or a P2X7-dependent pathway. Upon ATP binding, cell-surface P2X7 forms non-selective cation channels that can mediate inflammasome activation (16). However, d4T did not significantly modulate P2X7 cation channel function as monitored by patch clamp analysis of HEK293 cells expressing either the mouse or rat P2X7 receptor (fig. S14, B and C).

P2X7 activation also increases cell permeability to molecules of up to ~ 1,000 Da (17). We found that d4T and the known P2X7 antagonist A438079 inhibited P2X7-dependent uptake of YO-PRO-1 iodide (M.W. 629 Da) induced by the selective P2X7 agonist bzATP in human P2X7-expressing HEK293 cells (Fig. 4A and fig. S15). Interestingly, d4T only partially inhibited YO-PRO-1 uptake, whereas Caspase-1 activation by Alu RNA was completely blocked by a peptide (10°Panx) targeting a P2X7-associated channel that inhibits P2X7-dependent dye uptake and LPS/ATP-induced inflammasome activation, but not cation flux (18) (Fig. 4B). These data are consistent with the concept that P2X7 activation leads to activation of multiple cell permeabilization pathways (19), and that inhibition of some but not all of these pathways by d4T is sufficient to fully block Caspase-1 activation. Although a high concentration of 10°Panx peptide (20) was reported to induce cytotoxicity, the lower concentration used here did not (fig. S16A). Moreover, 10°Panx-treated human RPE cells were still capable of synthesizing other cytokines such as IL-6 (fig. S16B). Interestingly, Alu RNA activation of Caspase-1 was unimpaired in Panx1−/− mouse RPE cells (fig. S16C), which parallels a previous report that Pannexin-1 is not required for Caspase-1 activation by
LPS/ATP (15). The Panx peptide can have non-specific steric effects that impact cell permeability (21), possibly via overlapping mechanisms with P2X7-dependent pathways. Collectively, our data indicate that Caspase-1 activation in RPE cells by Alu RNA occurs via a P2X7-dependent, Pannexin-1-independent pathway.

Conversely, Alu RNA-induced Caspase-1 activation in RPE cells was not inhibited by calmidazolium (fig. S16C), which has been shown to inhibit rat P2X7-mediated cation flux but not dye uptake (22), and to inhibit mouse P2X7-mediated cation flux but not cell death (23). We also found that calmidazolium blocked Caspase-1 activation by the cation-specific inflammasome agonist nigericin in these cells (fig. S16D), which supports the idea that the mode of action of calmidazolium involves the inhibition of cation flux (necessary for nigericin-induced inflammasome activation, but not for P2X7-induced inflammasome activation). Furthermore, the intracellular C-terminus of P2X7 governs P2X7-associated dye uptake, and a version of d4T that is not cell permeable (24) did not block Caspase-1 activation by Alu RNA in RPE cells (fig. S16, F and G). Consistent with antagonism downstream of P2X7 but preceding inflammasome activation, d4T blocked Alu-induced mitochondrial ROS (mtROS) production (fig. S17, A and B) (5).

Supportive of the idea that d4T inhibits NLRP3 inflammasome activation via P2X7, d4T did not prevent Caspase-1 activation in primary mouse BMDMs treated with nigericin or crystalline monosodium urate, NLRP3 agonists that do not signal via P2X7 (fig. S18, A and B) (25). Furthermore, d4T did not inhibit AIM2 inflammasome activation by poly dA:dT or NLRC4 inflammasome activation by flagellin (fig. S18, C and D). These findings suggest that d4T specifically inhibits P2X7-dependent inflammasome activation, since cytosolic flagellin and poly dA:dT activate these other inflammasomes independent of P2X7 (26, 27).

To explore the therapeutic relevance of NRTIs beyond geographic atrophy, we hypothesized that NRTIs might be broadly useful in other P2X7-driven animal models of disease. Murine graft-versus-host disease (GVHD) is mediated by P2X7 (28); consistent with these results, irradiated BALB/c mice reconstituted with allogeneic (C57BL/6) bone marrow and T cells showed improved survival when treated with d4T compared to saline-treated controls (Fig. 4C). We also found that at day 3 after transplant, d4T-treated mice had lower serum levels of IFN-γ, TNF-α, and IL-6 proteins compared to saline treatment (fig. S19). The increased abundance of these cytokines in serum is characteristic of allogeneic T cell transfer in murine models and thought to play a role in acute GVHD pathogenesis (29–31). Supporting the idea that d4T targets P2X7, serum levels of these three cytokines were also decreased in an acute GVHD model employing P2X7-deficient host mice (28). Moreover, studies in a variety of other systems indicate that P2X7 regulates the expression of these cytokines (32–34). Further supporting the activity of d4T at the level of P2X7, in the P2X7-driven model of liver inflammation in which neutrophils are recruited from the circulation to a site of sterile injury (35), intravenous d4T reduced early neutrophil migration to the focus of hepatic necrosis (Fig. 4D and fig. S20). Finally, since P2X7 activation is known to increase tumor angiogenesis (36), we investigated whether NRTIs reduced choroidal neovascularization (CNV), which characterizes the “wet” form of AMD. In the laser-induced mouse model of CNV we found that d4T and me-d4T reduced CNV volume in wild-type mice (fig. S21A), but not in P2X7-deficient mice (fig. S21B). These data suggest...
that NRTIs might be therapeutic for both dry and wet AMD, and provide further evidence that these drugs work at the level of P2X7 in these systems.

NRTIs are a diverse, widely used, inexpensive class of small molecules, with extensive pharmacokinetic and safety data collected over several decades of human use. Our work, by illustrating a novel mechanism of action of NRTIs, paves a clear path for the broad repurposing of this drug class to address major unmet medical needs. Our data indicate that NRTIs could have dual therapeutic use in AMD in treating both geographic atrophy and neovascular AMD.

Since inflammasome inhibition by NRTIs can be achieved without their phosphorylation, the use of me-d4T or other phosphorylation-incompetent nucleoside analogs to treat disease could avoid dose-limiting toxicities associated with NRTI-triphosphate-mediated polymerase inhibition. It is not known whether long-term NRTI use is protective against developing AMD; however, as the population of aging HIV-positive individuals continues to grow, it might be possible to determine this predicted effect.

Interestingly, recent work has shown caspase-1 activation by HIV in abortively infected T-cells (37) and a role for NLRP3 in sensing HIV in macrophages and monocytes (38); such studies support the importance of inflammasome regulation by NRTIs. Also of note, HIV patients have increased plasma levels of the inflammasome effector IL-18 (39), which decreases after treatment with NRTI-containing highly active anti-retroviral therapy (40). Thus, while it is unclear whether suppression of viral replication by NRTIs or other components of HAART leads to the reduction of plasma IL-18 levels in these patients, our findings raise the possibility that inflammasome inhibition by NRTIs independent of reverse transcriptase inhibition could be responsible, at least in part, for modulation of HIV-induced cytokine expression.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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References and Notes


Fig. 1. NRTIs block Alu-induced RPE degeneration and Caspase-1 activation

(A, B) Fundus photographs (top row) and flat mounts stained for zonula occludens-1 (ZO-1; red) (bottom row) of mice injected sub-retinally with control (pNull) or Alu RNA-expressing (pAlu) plasmids and (A) once daily oral administration of d4T (150 mg/kg/day) or (B) twice daily intraperitoneal administration of AZT (100 mg/kg/day). In fundus photographs degeneration outlined by blue arrowheads. RPE degeneration prevented in (A) 5/6 (d4T) vs. 0/6 (vehicle) eyes; \( P = 0.015 \) and (B) 8/9 (AZT) vs. 0/8 (vehicle) eyes; \( P = 0.0004 \) by Fisher’s exact test (pAlu vs. pAlu + d4T or AZT). Scale bars, 50 μm. See also fig. S1.

(C) Western blot of Caspase-1 activation (p20 subunit) or p45 pro-form and IRAK4 phosphorylation in primary human RPE cells transfected with Alu RNA ± d4T (100 μM).
Fold change in densitometry compared to mock. (D) Western blot of Caspase-1 pro (p45) and active (p20) forms in human RPE cells transfected with Alu RNA ± NRTIs (3TC, AZT, ABC) (100 μM). Fold change in densitometry compared to mock. Images representative of n = 3–4 (A, B), n = 6–9 (C, D).
Fig. 2. Methoxy-d4T blocks Alu-induced RPE degeneration and Caspase-1 activation independent of reverse transcriptase inhibition. (A) (B, C)

(A) Real-time quantitative PCR for mitochondrial DNA normalized to chromosomal DNA exon-intron junction sequence of primary mouse RPE cells treated with unmodified d4T or me-d4T (100 μM both drugs). n = 4, * P < 0.05 by one-way ANOVA and Tukey’s post-hoc test.

(B) Western blot of Caspase-1 activation (p20 subunit) and phosphorylated IRAK4 in primary human RPE cells transfected with Alu RNA ± me-d4T (100 μM). Fold change in densitometry compared to mock.

(C) Fundus photographs (top row) and flat mounts stained for zonula occludens-1 (ZO-1; red) (bottom row) from mice treated with me-d4T (twice daily intraperitoneal injection; 50 mg/kg/day) (P = 0.029). In fundus photographs degeneration outlined by blue arrowheads. Representative images of n = 4 (B, C) shown. Scale bars, (C): 20 μm.
Fig. 3. NRTIs block LPS/ATP-induced inflammasome activation

(A) Western blot of pro (p45) and active (p10) Caspase-1 from cell lysate of wild-type BMDM treated with LPS alone or LPS/ATP with or without d4T (50 μM). Fold change in densitometry compared to LPS alone. (B) Western blot of pro- and mature IL-1beta and mature Caspase-1 in cell lysates of Raji TK− and TK+ cells untreated or with LPS ATP with or without AZT (100 μM). Fold change in densitometry compared to no treatment. Representative images of n = 3–4 experiments (A–B). See also figs. S7–S10.
Fig. 4. NRTIs selectively block P2X7 pore function and P2X7-driven models of graft rejection and sterile liver inflammation

(A) P2X7-dependent YO-PRO-1 dye uptake induced by bzATP (100 μM) in HEK293 cells stably expressing the human P2X7 receptor and treated with d4T or A438079 (64 μM for both drugs). Fluorescence values are baseline subtracted from cells without bzATP treatment. * bzATP vs. d4T; # bzATP vs. A438079, P < 0.05 by two-way ANOVA and Student-Newman Keuls post-hoc test (n = 12). See also fig. S15.

(B) Western blot of Caspase-1 activation (p20 subunit) in primary human RPE cells transfected with Alu RNA ±

(A) Short peptide (10Panx; 240 μM) that blocks P2X7 pore function but not cation flux (vs. scrambled peptide: Scr 10Panx; 240 μM).

(C) Administration of d4T starting From Day –1 pre-transplant protects murine recipients from graft versus host disease (GVHD). BALB/c mice were subjected to TBI (950 cGY) and then reconstituted with T depleted bone marrow (TDBM) from B6 donors. Cohorts (n = 10 per cohort) received either BM alone or BM plus bulk CD4⁺ T cells (2.5M) on the same day as the irradiation. Cohorts that received BM and bulk CD4⁺ T cells were additionally treated with either vehicle (saline) or drug (d4T) from day –1 or day 0 for a period of 10 days. Clinical manifestations of GVHD and overall survival were monitored post-BMT. Survival analysis was performed according to the Kaplan-Meier method and survival between groups was compared using the log-rank test. Results were pooled from two independent experiments. See also fig. S19.

(D) LysMeGFP⁺ cells/FOV around a sterile focal hepatic lesion. d4T (400 mg/kg) or PBS administered via jugular vein 20 minutes prior to injury. Intravenous d4T inhibits neutrophil recruitment 60 minutes after injury. n = 4, error bars S.E.M., P < 0.05 by two-way ANOVA and Student-Newman Keuls post-hoc test. See also fig. S20.