Nucleoside reverse transcriptase inhibitors possess intrinsic anti-inflammatory activity

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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 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, graft-versus-host disease, and sterile liver inflammation. Our findings suggest that NRTIs are ripe for drug repurposing in P2X7-driven diseases.

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N ucleoside reverse transcriptase inhibitors (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 un-
LPS/ATP-induced inflammasome activation can be inhibited without reverse transcriptase inhibition, we used a cell system that is incapable of NRTI phosphorylation, which is required for reverse transcriptase 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 to S10) (4). One consequence of LPS/ATP-induced caspase-1 activation is the proteolytic cleavage and maturation of 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 procaspase-1/IL-1β were not considerably changed (Fig. 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 noninflammasome genes (Il6, Il12α, 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 nonselective cation channels that can mediate inflammasome

**Fig. 1. NRTIs block Alu-induced RPE degeneration and caspase-1 activation.** (A and B) Fundus photographs (top row) and flat mounts stained for zonula occludens-1 (ZO-1; red) (bottom row) of mice injected subretinally with control (pNull) or Alu RNA-expressing (pAlu) plasmids and (A) once daily oral administration of d4T (150 mg kg⁻¹ day⁻¹) or (B) twice daily intraperitoneal administration of AZT (100 mg kg⁻¹ day⁻¹). In fundus photographs, degeneration is outlined by blue arrowheads. RPE degeneration was prevented in (A) 5 of 6 (d4T) versus 0 of 6 (vehicle) eyes (P = 0.015) and (B) 8 of 9 (AZT) versus 0 of 8 (vehicle) eyes (P = 0.0004 by Fisher’s exact test) (pAlu versus 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 = 6 to 9 (A and B) and n = 3 to 4 (C and D) experiments.

**Fig. 2. 5′-O-methyl-d4T (me-d4T) Alu-induced RPE degeneration and caspase-1 activation independent of reverse transcriptase inhibition.** (A) Real-time quantitative polymerase chain reaction 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 experiments, *P < 0.05 by one-way analysis of variance (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⁻¹ day⁻¹) (P = 0.029). In fundus photographs, degeneration outlined by blue arrowheads. Representative images of n = 4 experiments (B and C) shown. Scale bars: 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 BMDMs 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-1β 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 to 4 experiments (A and B). See also figs. S7 to S10.

activation (16). However, d4T did not significantly modulate P2X7 cation channel function, as monitored by patch-clamp analysis of human embryonic kidney 293 (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 ~1000 daltons (17). We found that d4T and the known P2X7 antagonist A438079 inhibited P2X7-dependent uptake of YO-PRO-1 iodide (molecular mass 629 daltons) induced by the selective P2X7 agonist bzATP in human P2X7-expressing HEK293 cells (Fig. 4A and fig. S15). d4T only partially inhibited YO-PRO-1 uptake, whereas caspase-1 activation by Alu RNA was completely blocked by a peptide (10Panx) 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 notion 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 10Panx peptide (20) was reported to induce cytotoxicity, the lower concentration used here did not (fig. S16A). Moreover, 10Panx-treated human RPE cells could still synthesize other cytokines such as IL-6 (fig. S16B). Alu RNA activation of caspase-1 was unimpaired in Panx−/− 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 10Panx peptide can have nonspecific steric effects that alter cell permeability (20), 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. S16D), 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 P2X7-mediated cation flux but not cell death (24).
capase-1 activation by the cation-specific inflammasome agonist nigericin in these cells (fig. S16E), 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 capase-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 reactive oxygen species production (fig. S17, A and B) (5).

Supportive of the idea that d4T inhibits NLRP3 inflammasome activation via P2X7, d4T did not prevent capase-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, because 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 (C57/B6) 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 interferon-γ, tumor necrosis factor-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 using P2X7-deficient host mice (28). Moreover, studies in a variety of other systems indicate that P2X7 regulates the expression of these cytokines (32–34). For this 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, because 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 burden 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.

Because NRTI-mediated 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.

Recent work has shown capase-1 activation by HIV in abiotically 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 treatment with NRTI containing highly active antiretroviral therapy (HAART) (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.

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HIV drugs can dampen inflammation, too

Nucleoside reverse transcriptase inhibitors (NRTIs) stop HIV in its tracks by blocking reverse transcription, a process critical for HIV to replicate its genome. Fowler et al. found that in mice, these drugs also block inflammation caused by a large protein complex called the NLRP3 inflammasome. This activity is independent of the drugs’ ability to block reverse transcription. Instead, the drugs block the activity of the ion channel P2X7, which activates the NLRP3 inflammasome. NRTIs improved outcomes in several NLRP3 inflammasome-dependent mouse models of inflammation, including age-related macular degeneration and graft-versus-host disease.

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