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Despite the success of antiretroviral therapy (ART), eradication of HIV-1 from brain reservoirs remains elusive. HIV-1 brain reservoirs include perivascular macrophages that are behind the blood-brain barrier and difficult to access by ART. Macrophages express transcription factor FOXO3a and the TNF superfamily cytokine TRAIL, which are known to target HIV-1-infected macrophages for viral suppression. ONC201 is a novel and potent FOXO3a activator capable of inducing TRAIL. It can cross the blood-brain barrier, and has shown an antitumor effect in clinical trials. We hypothesized that activation of FOXO3a/TRAIL by ONC201 will reduce the size of HIV-1 brain reservoirs. Using primary human monocyte-derived macrophages, we demonstrated that ONC201 dose-dependently decreased HIV-1 replication levels as determined by HIV-1 reverse transcriptase activity assay and Western blots for p24. Consistent with data on HIV-1 replication, ONC201 also reduced integrated HIV-1 DNA in infected macrophages in two-step Alu-based nested PCR. In addition, the antiviral effect of ONC201 is applicable to different natural HIV strains and to lymphocytes, microglia and latently infected cell line. A combination of ONC201 and AZT achieved a longer and synergistic viral suppression in HIV-1 infected

macrophages. The anti-HIV-1 effect of ONC201 was further validated *in vivo* in NOD/scid-IL-2Rγ_c^{null} mice. After intracranial injection of HIV-1-infected macrophages into the basal ganglia, we treated the mice daily with ONC201 through intraperitoneal injection for 6 days. ONC201 significantly decreased p24 levels in both the macrophages and the brain tissues, suggesting that ONC201 suppresses HIV-1 *in vivo*. As for the mechanisms, ONC201 treatment activated FOXO3a and induced TRAIL expression in macrophages while blocking TRAIL or knockdown of FOXO3a with siRNA reversed ONC201-mediated HIV-1 suppression, suggesting that ONC201 inhibits HIV-1 through FOXO3a and TRAIL. Furthermore, the antiviral effect of ONC201 is associated with apoptosis and autophagy. Based on these *in vitro* and *in vivo* studies, ONC201 can be a promising drug candidate to combat persistent HIV-1 infection in the brain reservoirs.

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Human immunodeficiency virus (HIV)

HIV-1 associated neurocognitive disorders (HAND)

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CHAPTER 1

Introduction

1.1 Macrophage and microglia in HAND

Despite the success of antiretroviral therapy (ART), up to 70% of HIV-infected people are still suffering from neurocognitive and motor dysfunctions collectively known as HIV-associated neurocognitive disorders (HAND) (1). The high prevalence of HAND can be attributed to the long lifespan of HIV-infected patients (2), the drug resistance of newly-mutated strains (3, 4), the low penetration of the ART drugs on the blood-brain barrier (BBB) (5) and persistent viral production from reservoir cells (6, 7). Notably, in the central nervous system (CNS), only certain types of cells, macrophage and microglia are regarded as reservoir cells for HIV-1. Macrophage and microglia can be preferentially infected by M-tropic HIV strains, which are predominant for virus transmission in vivo (8-11). The infected macrophages and microglia are exploited by HIV-1 to spread virus to circulation and other organs for further infecting CD4+ T cells (12, 13). They also secrete a variety of proinflammatory cytokines, affecting the nearby microenvironment and causing neuroinflammation (14). However, their most unique role in CNS is serving as viral reservoirs. Compared with other susceptible cells, macrophage and microglia have low turnover rates and are particularly more resistant to the cytopathic effect of HIV-1 (15, 16). These characteristics allow macrophage and microglia to keep integrated HIV-1 DNA for month, which could be one of the mechanism of latency in this cells (17).

Latency, aside from low penetration of ART drugs on BBB, is another major mechanism for the persistence of HIV-1 reservoirs in CNS. Infected cells that do not produce infectious virions out of cells are viewed as in a state of latency.

However, once stimulated by certain factors, they can start producing infectious virions again. Whether there is “real” latency in macrophages and microglia is still controversial because only very few macrophages (0.05%) are infected in lymph nodes of HIV patients under HAART and their isolation and characterization for HIV latency is difficult (18). Nevertheless, latency at least can be established in MDMs *in vitro* (19). Generally, there are two types of latency: pre-integration latency and post-integration latency (20). Pre-integration latency may be due to low reverse transcription efficiency and inhibition of nuclear transport of the pre-integration complex (20, 21); while post-integration latency may be due to epigenetic gene silencing (22), transcription gene silencing and post transcriptional gene silencing (23-25). Although new generation of ART drugs are under development for better penetration in CNS, they can only target HIV virions for preventing new infection on susceptible cells. However, HIV-1 enters the CNS in the first week of acute infection and already establishes latency there before ART drugs reach (18, 26), which makes a complete eradication of HIV in CNS reservoirs by ART impossible without help of other strategies.

Two entirely opposite strategies have been proposed to assist ART in managing the HIV-1 reservoir cells in latency. “Block and lock” strategy aims to block the reactivation of latent viruses and permanently lock them in the state of latency (27), while “flushing out” strategy aims to reactivate the latent virus in latently infected cells, leaving those cells under attacks from host immune system and ART drugs (20). The drugs that promote HIV-1 reactivation are

called latency-reversing agent (LRA) (28, 29). Histone deacetylases (HDACs) participate in restraining HIV-1 proviral DNA expression (30), thus serving as a potential target for “flushing out” strategy. However, HDAC inhibitors such as vorinostat did not decease the HIV-1 proviral DNA in CD4+ T cells and rectal tissues, even after successfully reactivating HIV-1 from latency in patients under ART treatment (31, 32). An adapted or alternative strategy for “flushing out” is “prime, shock and kill”, which aims to sensitize the latently-infected reservoir cells for apoptosis and death (33). So far only an antifungal drug, ciclopirox, and an iron chelator, deferiprone, have shown an antiviral effect through inducing apoptosis in HIV-1 infected H9 cells and peripheral blood monocytes (34). However, there was no evidence demonstrating their safety inside the body. Notably, the agents that induce apoptosis in tumor cells may have the similar effect in infected reservoir cells. These apoptosis inducing agents include Bcl2 inhibitors (35), survivin inhibitors (36) and PI3K/AKT inhibitors (37). Further investigations on these agents in the process of apoptosis within virally-infected cells are warranted.

In the development of new drugs that target HIV-1 infected reservoir cells for apoptosis, it is necessary to understand the mechanisms underlying the HIV-1 induced apoptosis in the infected cells during HIV-1 pathogenesis. One of mechanisms for host immune system to clear virally-infected cells is through the induction of apoptosis. In addition, cytopathic effect of virus also causes the death of infected cells though apoptosis. For macrophages, considerable apoptosis has been found during Ebola virus infection, which is part of Ebola

pathogenesis (38). However, whether HIV-1 induces apoptosis in macrophages has been controversial. Noursadeghi et al. believed “no direct cytopathic effect of HIV infection in monocyte-derived macrophages (MDM) (39), as shown by an extensive body of literature (40)”. They also observed no induced apoptosis or necrosis in MDM after establishment of uniform infection of HIV-1_{BaL} (40). On the other hand, Cui et al. observed significant apoptosis and cell death in MDM in vitro after HIV-1 infection for 5-7 days, indicating macrophages still undergo apoptosis after the long-time “no uniform” HIV-1 infection (41). This controversy highlights the mixed and complicated process of apoptosis potentially induced by HIV-1 in macrophages. During HIV-1 infection, the virus-encoded proteins regulate both pro- and anti-apoptotic factors in host cells, leading to a modified destiny of the infected cells in the purpose of survival and proliferation (42). As a result, those apoptosis-resistant macrophages and microglia form HIV-1 reservoirs in the CNS, which is the major obstacle for the current ART that aims to eradicate HIV-1 from CNS.

1.2 TRAIL

From the limited knowledge of HIV-1 induced apoptosis in infected macrophage and microglia, TRAIL plays a critical role in this process. TRAIL, short for TNF-related apoptosis-inducing ligand, is a type II integral membrane protein. As a member of the TNF superfamily, TRAIL is structurally and functionally related to FAS ligand (43, 44). TRAIL exists in two forms: surface-bound one on the cell membrane and soluble one secreted to the extracellular

space (45). There are five receptors for TRAIL: TRAIL receptor one (R1) and receptor two (R2) have death domains (46-48); TRAIL-R3 and TRAIL-R4 have similar structures but do not possess these domains (49, 50); the fifth TRAIL receptor is soluble and also called osteoprotegerin (51). Thus, in order to induce apoptosis, TRAIL need to bind to TRAIL-R1 or -R2, while TRAIL-R3 and -R4 act as decoy receptors to block this effect. TRAIL can homo-oligomerize with its death receptors to recruit the initiators of apoptosis cascade, such as procaspase-8.

Under physiological conditions, TRAIL is actively transcribed in many human tissues except that its expression is absent in brain tissue. Neurons (52), astrocytes (53-55), macrophage and microglia (56) express TRAIL at minimal levels in the normal conditions. However, all types of brain cells express one or a combination of TRAIL receptors (57, 58). Thus, cells in the CNS are sensitive to TRAIL, as massive and non-selective cell death can be induced by TRAIL treatment on a brain slice culture (57). Under pathological conditions such as HIV-1 infection, tat protein and immune activation by interferon-γ (IFN-γ) and lipopolysaccharide (LPS), TRAIL expression is substantially upregulated in macrophage and microglia (56, 59, 60). Meantime, these conditions may undermine the high resistance of macrophage and microglia to TRAIL-induced apoptosis (61).

The role of TRAIL in HIV pathogenesis has been perplexing. On the one hand, treating HIV-1 infected CD4+ T cells and macrophage with high levels of TRAIL agonists preferentially killed HIV-1 infected cells and shrank the size of

viral reservoir ex vivo (62). On the other hand, TRAIL is highly expressed in cells from HIV-1 infected patients (63), and the levels of TRAIL in plasma are higher in HIV-1 infected patients compared with heathy individuals while ART decreases the levels of those soluble TRAILs (64). However, infected cells still cannot be eradicated in such natural conditions. These results demonstrate that HIV-1 infected cells may survive the elevated level of TRAIL in infected patients but still could not tolerate supraphysiologic levels of TRAIL brought by pharmacological intervention. This finding encourages researchers to continue searching for new TRAIL inducers while investigating the mechanisms by which TRAIL induce apoptosis in HIV-1 infected cells.

The signaling pathway of TRAIL in HIV-1 infected macrophage and microglia is partially elucidated. Generally, HIV-1 infection inhibits Akt phosphorylation while inducing the expression of TRAIL and its death receptor, which subseqently leads to cell apoptosis. Specifically, first, one of the mechanisms for HIV-1 infection to induce TRAIL expression has been reported. Step 1: HIV-1 infection induces the expression of IRF-1 and IRF-7 and activates STAT1; Step2: IRF-1 and IRF-7 promote Type I IFNs production and subsequent STAT1 activation; Step3: Type I IFNs and STAT1 activation further enhance the expression of IRF-1 and IRF-7; Step4: accumulated STAT1 and IFNs induce the transcription of TRAIL; together, a positive feedback loop is formed to upregulate TRAIL expression (65). Second, the mechanism for TRAIL to induce apoptosis in HIV-1 infected macrophages has been investigated. HIV-1 infection reduces Akt-1 phosphorylation in macrophages while TRAIL-

mediated apoptosis is dependent on the inhibition of Akt-1 phosphorylation (66). Third, *in vitro* HIV-1 infection was associated with increased levels of TRAIL-R1, which might be one of reasons that TRAIL preferentially induces cell death of HIV-1 infected macrophages without hurting uninfected macrophages (62).

1.3 FOXO3a

TRAIL ligand is under transcriptional control of a transcription factor, FOXO3a, which is a member of Forkhead Class O (FOXO) family. This family has four members: FOXO1, FOXO3 (also called FOXO3a), FOXO4 and FOXO6. They all share a conserved DNA-binding domain (the “Forkhead box”) and are characteristically regulated by the insulin/PI3K/Akt signaling pathway. By transcribing multi-category genes, FOXO family members play critical roles in a variety of cellular activities, including cell death (*Bim-1, FasL*), reactive oxygen species detoxification (*Catalase, MnSOD*), DNA repair (*GADD45, DDB1*), cell cycle arrest (*p27, p130, GADD45, Cyclin G2*), glucose metabolism (*G6Pase, PEPCK*) and energy homeostasis (*AgRP, NPY*) (67).

The regulation of FOXO3a is mainly responsive to environmental conditions. Insulin or growth factors trigger the PI3K/Akt activation to phosphorylate FOXO3a at three conserved residues, prompting the translocation of FOXO3a from the nucleus to the cytoplasm and therefore reducing FOXO3a-mediated transcription. Under oxidative stress, other protein kinases such as JNK and Mst1 phosphorylate FOXO3a at other sites, resulting in the import of FOXO3a from cytoplasm to the nucleus and therefore inducing FOXO3a-dependent

transcription (67). In addition, FOXO3a can be regulated through acetylation, deacetylation, monoubiquitination, polyubiquitination under other environmental conditions including nutrient deprivation and hypoxia (67-69). FOXO3a functions as a coordinator of cell cycle arrest, stress resistance and apoptosis to respond to the volatile environment, as it regulates both pro-apoptotic and anti-apoptotic genes at the same time (70).

In the immune system, FOXO3a is the predominant FOXO member expressed in peripheral lymphoid organs and mediates lymphoid homeostasis (71). FOXO3a regulates helper T cell activation and tolerance by inhibiting NF-κB activity, and FOXO3a deficiency causes spontaneous lymphoproliferation with inflammation of several organs in the absence of apparent apoptotic defects (71-73). FOXO3a mediates the protective effect of glucocorticoid-induced leucine zipper (GILZ) on T lymphocytes from IL-2 withdrawal-induced apoptosis (74). Convergence of TCR and cytokine signaling inactivates FOXO3a and drives the survival of CD4+ central memory T cells (75). Inactivation of FOXO3a is also required for optimal B-cell proliferation (76). However, the knowledge of the exact function and detailed regulation of FOXO3a in macrophage and microglia is still limited. For HIV-1 infected macrophages, HIV-1 infection inhibits Akt-1 to activate FOXO3a, thereby inducing apoptosis and cell death (41). Thus, FOXO3a may also act as a key factor regulating the apoptosis of macrophage.

1.4 ONC201

ONC201, also known as TIC10, is a small molecule compound that has a chemical nomenclature of 7-benzyl-4-(2-methylbenzyl)-1,2,6,7,8,9-Hexahydro-imidazo [1,2-a]pyrido [3,4-e] pyrimidin- 5(1H)-one. It is the founding member of the imipridone class of compounds that share a unique imidazo pharmacophore (Fig. 1.1A). It was first identified as a candidate anti-cancer lead compound for the induction of TRAIL in a colorectal cancer cell line (77, 78). The specific anti-tumor activity of ONC201 has been demonstrated in over 15 solid tumors and hematological malignancies either in single agent treatment or in combination regimens in preclinical models (see review at (79)).

Being selected as the lead compound for clinical application, ONC201 has several advantages. ONC201 is orally active and can cross BBB. In the experiment on rodents, it penetrated intact BBB and accumulated in brain with higher concentrations than in plasma (78). So far, 14 clinical trials are testing ONC201 on brain tumors (Clinicaltrials.gov). ONC201 also shows outstanding safety profile both *in vitro* and *in vivo* (80). It does not induce apoptosis in normal cells possibly due to blunted ISR activation and the absence of death receptor 5 (DR5) induction (78). In the first human Phase I clinical trial on ONC201, a dose of 625 mg administered orally every three weeks was well-tolerated (81).

ONC201 was discovered by a “phenotypic cell-based screen, rather than a target structure-based approach” (79). Thus, its direct binding target has not been confirmed yet. A novel target prediction algorithm as a Bayesian machine-learning approach analyzed millions of data points and predicted that ONC201

should have a highly specific affinity with dopamine receptor D2 (DRD2) (unpublished data, (79)). This result has been supported by both *in vitro* and clinical data. Experimental GPCR profiling and reporter assays showed that ONC201 antagonized the D2-like, but not D1-like, subfamily of dopamine receptors (79). In Phase I clinical trial of ONC201, pharmacodynamic assays demonstrated induction of prolactin as a serum biomarker of DRD2 antagonism in human subjects receiving ONC201 treatment (81). However, DRD2 has not been placed in the signaling pathway of ONC201 yet, due to lack of data linking it to induction of TRAIL.

In cancer cells, ONC201 induces the transcription of TRAIL and the following apoptosis through two pathways. The first pathway is Akt/ERK/FOXO3a/TRAIL/DR5 signaling axis. ONC201 indirectly inactivate pro-survival kinases Akt and ERK, which dephosphorylate transcription factor FOXO3a at Ser253 and Ser294, respectively. The unphosphorylated FOXO3a leaves cytoplasmic 14-3-3 proteins which once sequester it in cytoplasm, and then translocates to nucleus where it binds to the TRAIL promoter and induces the transcription of TRAIL. Soluble TRAIL and surface-bound TRAIL could bind to DR5 on the membrane of TRAIL-producing cells or nearby cells for induction of apoptosis. The second pathway is ER stress/ISR/eIF2a/ATF4/CHOP/DR5 and TRB3 signaling axis. ONC201 induces ER stress. Then the integrated stress response (ISR) increases phosphorylation of eukaryotic initiation factor 2 (eIF2a), which promotes translation of ATF4. ATF4 is the transcription factor for

CHOP and TRB3. CHOP targets DR5 to induce apoptosis while TRB3 is necessary for Akt inactivation, which is the bridge between two pathways (79).

ONC201 has an isomer which is the "inactive version" of ONC201 (Fig. 1.1B). ONC201 isomer was introduced to the research community since it was structurally wrong-assigned as the bioactive ONC201 during early stage development of ONC201 (82). ONC201 isomer was found to be inactive, which did not reduce the viability of cancer cells (82). It is often used as a reference compound to compare with ONC201.

1.5 conclusion

Macrophages and microglia are the HIV-1 reservoir cells in the CNS, contributing to the pathogenesis of HAND. Current ART is not able to eradicate HIV-1 from these latently infected long-lived cells to terminate HIV-1 infection. One mainstream strategy to manage reservoir cells is “prime, shock and kill”. However, the requirement of selective killing infected cells without damaging norm tissues prompts us to understand the mechanism of HIV-1-induced apoptosis in macrophages and microglia. So far it has been reported that HIV-1 induces apoptosis in macrophages by activating transcription factor FOXO3a and promoting the expression of proapoptotic TRAIL. Thus, FOXO3a-TRAIL pathway may serve as a drug target to clear the HIV-1 infected macrophages and microglia (Fig. 1.2).

Among all the TRAIL-based drugs, ONC201 as a small molecule compound shows high performance on safety profile, therapeutic index, biodistribution and

stability. It is orally active and can cross BBB, allowing it to reach a high therapeutic concentration around the niche of reservoir cells in the CNS. Although its direct binding target is not confirmed, ONC201 has been shown to indirectly activate FOXO3a and induce the expression of TRAIL. ONC201 has been tested in Phase I and Phase II in-human clinical trials as an antitumor drug and have shown a preferential apoptosis-inducing effect on tumor cells with minimal cytotoxicity on normal cells. However, it has never been studied in the field of infectious disease. Further exploring the effect of ONC201 on HIV-1 infected reservoir cells may endow this drug with a new application in clinics.

1.6 Figures

Figure 1.1 Molecular structure of ONC201 and its inactive isomer.

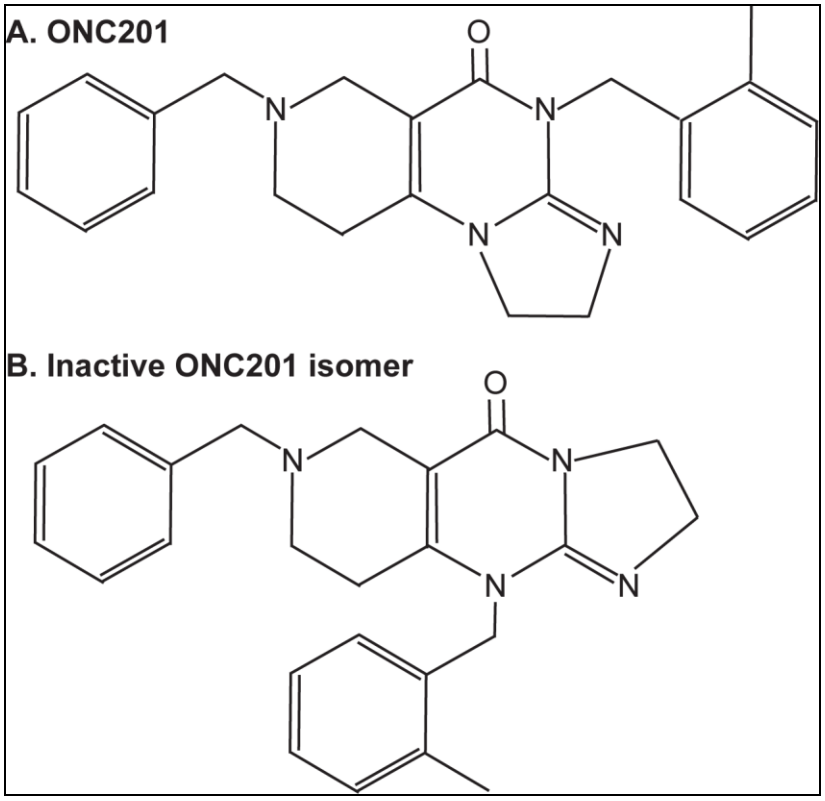
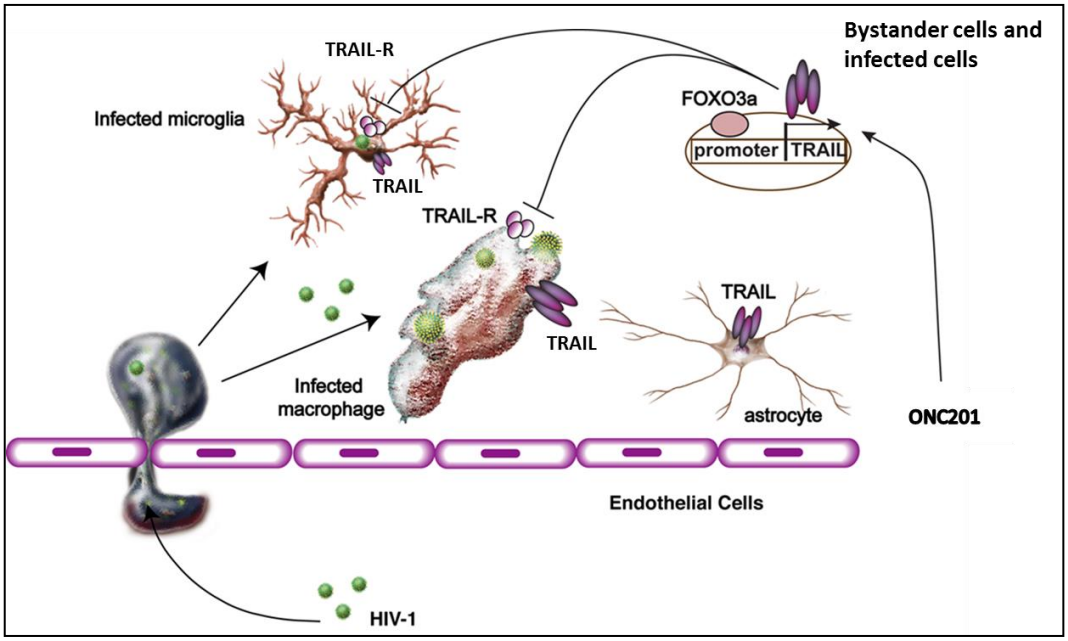


Figure 1.2 Overview of the proposed drug targets (FOXO3a and TRAIL) of ONC201 in HIV-1 CNS reservoirs.



CHAPTER 2

ONC201 Inhibits HIV-1 Infection *in vitro* and *in vivo.*

2.1 Abstract

HIV-1 continues to form reservoirs in the lymph system, gut and central nervous system, representing a significant challenge for viral eradication. FOXO3a, a transcription factor critical for immune homeostasis, is known to inhibit latent HIV-1 reservoirs. Recent drug development has provided ONC201 as the first potent and stable small molecule FOXO3a activator capable of crossing blood-brain barrier. We hypothesize that targeting FOXO3a through ONC201 will inhibit HIV-1 in its reservoir cell types. Human primary microglia, monocyte-derived macrophages and peripheral lymphocytes were infected with HIV-1_{ADA}, HIV Clade B or HIV Clade C *in vitro*. The toxicity of ONC201 was determined by MTS cell viability assay. HIV-1 replication levels were measured by HIV-1 reverse transcriptase activity assay, ELISA and Western blots for p24, and RT-PCR for HIV-1 gag. HIV-1 integration was determined by two-step Alu-based nested PCR. We found even at the highest soluble concentration, ONC201 did not change the cell viability of macrophages and lymphocytes, indicating ONC201 is safe for uninfected cells. ONC201, but not its inactive isomer, potently inhibited HIV-1 replication and reduced integrated HIV-1 DNA in infected cells in a dose-dependent manner, suggesting that the antiviral activity is specific to ONC201. For *in vivo* study, HIV-1-infected macrophages were intracranially injected into the basal ganglia of NOD/scid-IL-2Rγ_c^{null} (NSG) mice. Daily intraperitoneal injection of ONC201 for 6 days significantly decreased p24 levels in macrophages, suggesting that ONC201 suppresses HIV-1 in vivo. In addition, to determine whether ONC201 synergizes with current anti-HIV-1

treatment, we treated macrophages with ONC201 along with reverse transcriptase inhibitor azidothymidine (AZT). Addition of ONC201 increased the potency of AZT and achieved longer viral suppression during viral rebound. Therefore, ONC201 can be a promising drug candidate to combat persistent and latent HIV-1 infection in the current cART era.

2.2 Introduction

HIV continues to be a major global public health issue affecting more than 1.1 million people in the USA and 36.7 million people worldwide (83, 84). Although application of antiretroviral therapy (ART) dramatically reduces the rate of HIV-1 replication in infected patients, it is not able to eradicate HIV-1 from cellular reservoirs in the lymphatic system, gut, and central nervous system (CNS). Although many types of cells can be infected by HIV-1, cells that form HIV reservoirs are typically long-lived, including memory $CD4^+$ T cells (85), brain perivascular macrophages, and microglia (86). In the CNS, macrophages and microglia are the main cell types that can be productively infected by HIV-1. The eradication of HIV reservoirs in the CNS is particularly challenging. Substantial numbers of patients are still affected by HIV-1 associated neurocognitive disorders (HAND) after apparently successful ART. Furthermore, many ART drugs are not able to cross the blood-brain barrier (BBB) and reach therapeutic concentrations within the CNS reservoirs. These long-lived HIV-1 reservoirs can produce replication-competent HIV-1, which may spread to other tissues and fuel the emergence of drug-resistant viral strains. Thus, strategies targeting these CNS reservoir cells for HIV-1 eradication are urgently needed.

Vulnerabilities of HIV-1-infected brain macrophages and microglia include the FOXO3a pathway and its transcriptional target TRAIL. FOXO3a is a conserved transcription factor downstream of PI3K/Akt-1 pathway (87, 88). In the absence of environmental signals or growth factors, FOXO3a is in an unphosphorylated state and remains transcriptionally active. Growth factors

activate the PI3K/Akt-1 signaling pathway, phosphorylating FOXO3a and abrogating its transcriptional activity (89). FOXO3a is predominantly expressed in peripheral lymphoid organs but the specific immunomodulatory role of FOXO3a remains largely undefined (71). In memory CD4+ T cells under HIV-1 infection, multiple signals activate IκB kinase, which phosphorylates FOXO3a, decreasing its ability to induce expression of various pro-apoptotic genes (90). Our previous publications have demonstrated that FOXO3a targets HIV-1-infected macrophages for apoptosis (41). Furthermore, we have determined that FOXO3a is phosphorylated (inactive) during neuroinflammation and expression of FOXO3a inhibits reactive astrogliosis in a neuroinflammatory mouse model (91). Intriguingly, the transcriptional targets of FOXO3a include TRAIL, which is a member of the TNF superfamily and is also known as TNF Superfamily Member 10 (43, 44). TRAIL interacts with at least five unique receptors. TRAIL receptor 1 (R1) and receptor 2 (R2) have death domains and induce cellular apoptosis following ligand binding (46-48). TRAIL-R3, TRAIL-R4, and the fifth receptor named osteoprotegerin do not possess death domains and instead act as decoy receptors (49-51). Originally thought to target only tumor cells (43, 44), TRAIL has been shown to reduce viral burden in HIV-1-infected macrophage and resting memory $CD4^+$ T cells (66, 92). While its receptors are expressed in macrophage and microglia, TRAIL is expressed in much lower levels in the CNS compared with the lymphoid tissues (58, 65, 93, 94). The deficiency of FOXO3a signaling during HIV-1 infection and lack of TRAIL expression in the CNS may inadvertently facilitate the forming of HIV-1 brain reservoirs. Therefore, targeting

the FOXO3a-TRAIL pathway is a novel strategy to combat HIV reservoirs within the CNS.

Targeting FOXO3a-TRAIL pathway has been difficult due to a lack of drug candidates. However, recent drug development provides ONC201 as a potent and stable small molecule that can activate FOXO3a and transcriptionally induce TRAIL expression (77, 78, 95). ONC201 is orally active and can cross the BBB (78). It has shown an efficacious antitumor effect and is currently being tested in clinical trials as a promising anticancer agent (79). ONC201 also has an inactive isomer which is naturally the optimal control to compare with ONC201 in the research. In this study, we have investigated the anti-HIV-1 activity of ONC201. We demonstrate that ONC201 inhibits the HIV-1 infection both *in vitro* in monocyte-derived macrophages and *in vivo* in NOD/scid-IL-2Rγ_c^{null} (NSG) mouse brains engrafted with HIV-1-infected macrophages. This newly found activity of ONC201 suggests that it can serve as a drug candidate to combat persistent HIV-1 infection in the CNS.

2.3 Materials and Methods

Ethics statement and mice

Primary monocyte-derived macrophages (MDM), peripheral blood lymphocytes (PBL) and microglia were used in full compliance with the University of Nebraska Medical Center and National Institutes of Health ethical guidelines, with the Institutional Review Board (IRB) #: 162-93-FB. For animal experiments, all mice were housed in the Comparative Medicine Animal Facilities at the University of Nebraska Medical Center. All procedures were conducted in accordance with the protocol (15-040-06) approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center. Fourteen four-week old male NOD.Cg-Prkdc^{scid}*Il 2rg*^{tm1Wjl}/SzJ mice were purchased from Jackson Laboratories and HIV-1-infected human MDM (5$\times$ 10^5 cells, 3 μl) were injected intracranially (IC) to the basal ganglion of the mice. The coordinates for inoculation is: 0.5 mm posterior to bregma, 3.5 mm lateral and a depth of 3.6 mm. ONC201 (MedKoo Bioscience, Morrisville, NC) was administrated daily through intraperitoneal (IP) injection at 50 mg/kg. Animals were sacrificed 7 days after the IC injection.

Isolation and culture of primary MDM, PBL and microglia, and U1 cells

Human monocytes and peripheral blood lymphocytes were isolated by counter-current centrifugal elutriation from peripheral blood mononuclear cells obtained through leukopheresis from healthy donors (96).

Primary monocytes were cultured as adherent monolayers at a density of 1.1 x 10^6 cells/well in 24-well plates or 0.25 x 10^6 cells/well in 96-well plates and

differentiated for 7 days in differentiation medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co., St. Louis, MO) supplemented with 10% human serum, 50 µg/ml gentamicin (GIBCO Invitrogen Corp.), 10 µg/ml ciprofloxacin (Thermo Fisher Scientific, Waltham, MA), and 1000 U/ml recombinant human macrophage-colony stimulating factor (M-CSF). After 7 days in differentiation, the cells became MDM and M-CSF was removed since the culture can produce M-CSF through autocrine secretion.

Primary PBL (1 x 10^6 cells) were stimulated with 20 ng/mL PMA (Sigma Chemical Co., St. Louis, MO) and 1 µM ionomycin (Sigma Chemical Co., St. Louis, MO) in 10 mL of Roswell Park Memorial Institute (RPMI) 1640 (GE Healthcare Life Sciences, Logan, UT) for 5 hours and then moved to RPMI 1640 media supplemented with 10% fetal bovine serum, 2 mM glutamine (Sigma Chemical Co., St. Louis, MO), 50 ng/mL recombinant IL-2 (R&D Systems, Minneapolis, MN) and antibiotics for further growth.

Human fetal microglia were prepared from a mixed culture of fetal brain tissue-derived microglia-astrocytes as previously described (97, 98). Enriched microglia were plated and cultured in the presence of M-CSF for an additional 7 days before experiments.

U1 cell line was bought from NIH AIDS reagent program (Catalog Number:165, Lot Number:100218) and cultured in RPMI 1640 containing 2.0 mM L-glutamine, 90%; heat-inactivated fetal bovine serum, 10% as recommended.

HIV-1 Infection of MDM and ONC201 treatment

MDM were infected with HIV-1_{ADA}, a macrophage-tropic strain of HIV-1, Clade B or Clade C at a multiplicity of infection (MOI) of 0.1 virus/target cell (96). Briefly, viral stocks were diluted in MDM medium (same with the differentiation medium but without M-CSF) for overnight incubation with MDM. On the second day, medium was removed and substituted with MDM medium and either DMSO (Sigma), ONC201, or ONC201 isomer (MedKoo Bioscience). For selected experiments, a recombinant human TRAIL R2/Fc Chimera Protein (R&D Systems, Minneapolis, MN) was added to the cultures along with ONC201. The cultures were re-treated with the same drugs in fresh MDM medium every 2 days. At 4 days post-infection, the cultures were treated with the same drugs in 0.5 ml/well fresh serum-free Neurobasal™ medium (Thermo Fisher Scientific) for 24 hours before all samples were collected.

Assessment of cell viability

MDM and glioblastoma (ATCC, CRL-1620) viability was determined by a colorimetric CellTiter 96® AQueous One Solution Assay (Promega, Madison, WI) based on the manufacture's instruction. Assays were performed by adding 20 µl of the CellTiter 96® AQueous One Solution Reagent, which contained a tetrazolium compound[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES), directly to each of 100 µl MDM medium in 96-well plates. After 2-hour incubation at 37°C in a humidified 5% CO_2 atmosphere, the absorbance of each well was recorded at 490 nm using a 96-well plate reader (BioTek, Winooski, VT).

HIV-1 reverse transcriptase (RTase) activity assay

HIV-1 replication levels were determined by HIV-1 RTase activity assay as described previously (96). RT activity was determined in triplicate samples of cell culture fluids. For this assay, 10 µl of supernatant was incubated in a reaction mixture of 0.05% Nonidet P-40, 10 µg of poly(A)/ml, 0.25 µg of oligo(dT)/ml, 5 mM DTT, 150 mM KCl, 15 mM MgCl2, and [3H]TTP in Tris-HCl buffer (pH 7.9) for 24-h at 37 °C. Radiolabeled nucleotides were precipitated with ice-cold 10% trichloroacetic acid on UniFilter-96 GF/C (FilterMate Harveser, Waltham, MA) in an automatic cell harvester (FilterMate Harveser, Waltham, MA) and washed with 95% ethanol. Radioactivity was determined by liquid scintillation spectroscopy.

Western blots

MDM or tissues of injection sites were homogenized with a PowerGen Homogenizer (Thermo Fisher Scientific) and then lysed by M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA). Protein concentrations were determined by Bradford protein assay (Bio-Rad, Hercules, CA). Cell lysates were subjected to SDS-PAGE to separate proteins and electrophoretically transferred to Polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Membranes were incubated overnight at 4 °C with polyclonal antibodies for FOXO3a (Cat#, 75D8, Cell Signaling Technology, Danvers, MA), phosphorylated FOXO3a (Cat#, 9466s, Cell Signaling Technology, Danvers, MA), HIV-1 p24 (Ref#, M0857, DAKO Corp, Carpinteria, CA), human CD68 (Ref#, M0876, DAKO Corp, Carpinteria, CA),

and β-actin (Sigma-Aldrich, St. Louis, MO), followed by horseradish peroxidase-linked secondary anti-rabbit or anti-mouse secondary antibodies (Cell signaling Technology, Danvers, MA). Antigen-antibody complexes were visualized by Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). For quantification of the data, films were scanned with a CanonScan 9950 F scanner (Canon Inc., Tokyo, Japan) and images were analyzed using the public domain NIH Image J program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).

RNA and DNA extraction and real time RT-PCR analysis of HIV-1 gag

Total RNA was isolated with TRIzol Reagent (Thermo Fisher Scientific) and RNeasy Mini Kit according to the manufacturer's protocol (Qiagen Inc., Valencia, CA). Following RNA extraction, DNA was isolated from the remaining organic phase using the DNeasy Blood and Animal Spin-Column kit according to manufacturer's instructions (Qiagen). Assays-on-demand primers for TRAIL (ID#, Hs00921974_m1), FOXO3a (ID#, Hs00818121_m1), 18S rRNA (Ref#, 4352930#) and GAPDH (ID#, Hs01922876_u1) were purchased from Thermo Fisher Scientific. For quantification of HIV-1 gag, primers and probe were purchased from Thermo Fisher Scientific with the following sequences: forward, 5' ACA TCA AGC CAT GCA AAT-3'; reverse, 5'-ATC TGG CCT GGT GCA ATA GG-3'; probe, 5'-FAM-CAT CAA TGA GGA AGC TGC AGA ATG GGA TAG A-TAMRA-3'. Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed in a volume of 10 µl using the one-step quantitative TaqMan Real-time RT-PCR system (Applied Biosystems Inc.). For analysis of HIV-1 gag

DNA, real-time PCR was carried out directly on the DNA samples using the TaqMan Universal Master Mix and a StepOne Plus Real-Time PCR System (Thermo Fisher Scientific). Relative FOXO3a, TRAIL, CD4, CCR5 and HIV-1 gag levels were determined and standardized with GAPDH or 18S rRNA internal control using comparative ΔΔCT method. All primers used in the study were tested for amplification efficiencies and the results were similar.

Analysis of HIV-1 proviral DNA integration

Two-step nested PCR assays were used for quantitative HIV-1 DNA integration analysis as previously described (99, 100). The first round was performed in a 25 µl reaction mix to pre-amplify the genomic DNA from infected macrophages. The following primers were used: Alu forward, 5'-GCC TCC CAA AGT GCT GGG ATT ACA G-3'; and HIV-1 gag reverse, 5'-GCT CTC GCA CCC ATC TCT CTC C-3'. The PCR reaction contained 10x EX Taq Buffer, dNTP mix (2.5 mM), EX Taq (all from TaKaRa Bio Inc., Otsu, Shiga, Japan), ddH_2O, 100 nM Alu forward primer, 600 nM gag reverse primer, and 100 ng of genomic DNA. The DNA Engine Peltier Thermal Cycler (Bio Rad, Hercules, CA) was programmed to perform a 2-minute hot start at 94°C, followed by 30 steps of denaturation at 93°C for 30 second, annealing at 50°C for 1 minute, and extension at 70°C for 2 minutes. The second round real-time quantitative PCR was performed by using 5.6 µl of the reaction mix from the pre-amplification step. The amplified DNA was standardized with an internal GAPDH control. The sequences of the primers and probe were as follows: LTR forward, 5'-GCC TCA ATA AAG CTT GCC TTG A-3'; LTR reverse, 5' -TCC ACA CTG ACT AAA AGG

GTC TGA-3'; probe: 5'-FAM-GCG AGT GCC CGT CTG TTG TGT GAC TCT GGT AAC TAG CTC GC-BHQ-3'.

P24 and TRAIL ELISAs

Supernatants collected at 5 days post-infection were analyzed for p24 and TRAIL levels by in-house ELISAs (paired antibodies, R&D Systems, Minneapolis, MN) as previously described (101).

Free-floating immunohistochemistry and image analyses

Animals were euthanized under deep anesthesia and perfused with phosphate-buffered saline (PBS) and then with 4% paraformaldehyde (PFA) in PBS. The brains were removed and immersed in freshly depolymerized 4% PFA in PBS for 48 h and then cryoprotected by 30% sucrose for 48 h. The fixed, cryoprotected brains were frozen and sectioned in the coronal plane at 30 microns using a Cryostat (Leica Microsystems Inc., Bannockburn, IL), with sections collected serially in PBS as previously described (102). Brain sections were then incubated overnight at 4 °C with primary antibodies, followed by secondary antibodies (Molecular Probes, Eugene, OR, 1:1000) for 1 h at 25 °C. Primary antibodies included P24 (DAKO, 1:1000) and human CD68 (DAKO, 1: 1000). All antibodies were diluted in 5% goat serum in PBS. Cells were counterstained with DAPI (Sigma-Aldrich, 1:2000) to identify the nuclei. Images were taken using a Zeiss Meta 710 confocal microscope (Carl Zeiss MicroImaging, LLC) (20× object, tile scan 4 × 4 mode). Section images from eight mice were imported into Image-ProPlus, version 7.0 (Media Cybernetics, Silver Spring, MD) for quantification of p24/DAPI and CD68/DAPI double

positive staining. The assessors were blinded during image acquisition and quantification.

Statistical analysis

Data are expressed as means ± SD unless otherwise specified. Statistical analysis was performed by using one-way ANOVA, followed by the Tukey's-post-test for paired observations, or two-way ANOVA when two independent variables are considered. The two-tailed Student's t test was used to compare two groups. Significance was determined by a p value < 0.05. All experiments were performed with cells from at least three donors to account for any donor-specific differences. Assays were performed at least three times in triplicate or quadruplicate within each assay.

2.4 Results

Anti-tumor agent, ONC201, is safe for uninfected macrophages and lymphocytes

Because ONC201 was originally identified as a TRAIL-inducing anti-tumor agent (78), we first sought to confirm its activity in tumor and transformed cells. We treated A-172 human glioblastoma cells, Hela cells and 293T cells with doses of ONC201 or its isomer ranging from 0.03 to 30 μM for 72 hours and then determined the cell viability through an MTS assay (Fig. 2.1). Treatment with ONC201, but not ONC201 isomer induced dose-dependent cytotoxicity in glioblastoma cells, Hela cells and 293T cells, confirming the bioactivity of ONC201.

Next, to determine the safe dose of ONC201 for macrophages and lymphocytes, we treated MDM and PBL with either ONC201 or its isomer for 5 days at concentrations ranging from 0.03 to 30 μM. Treatment with either ONC201 or its isomer did not significantly change cell viability of MDM and PBL at all concentrations tested, as measured by MTS assays (Fig. 2.2). Even at the highest soluble concentration, 30 μM, ONC201 is safe for human primary macrophages and lymphocytes.

ONC201 activates FOXO3a and induces TRAIL expression in macrophages.

There are two forms of FOXO3a: the phosphorylated form is inactive and kept in the cytoplasm; once dephosphorylated, it moves to the nucleus and becomes active, inducing pro-apoptotic genes. To assess the status of FOXO3a

in HIV-1 infected macrophages after ONC201 treatment, the levels of total FOXO3a, phosphorylated FOXO3a and β-actin were measured by Western blots (Fig. 2.3). After quantification, we found the phosphorylation of FOXO3a, determined by the ratio of phosphorylated FOXO3a over total FOXO3a, was decreased after ONC201 treatment compared with treatments of the isomer and DMSO controls. This result indicates that FOXO3a is dephosphorylated and activated by ONC201 in HIV-1 infected MDM. Next, the soluble TRAIL in the culture supernatant was measured by ELISA. After adjusted to intracellular protein concentrations, the result showed dose-dependent increased levels of soluble TRAIL after ONC201 treatment compared with those after treatment of the isomer or DMSO controls (Fig. 2.4). Thus, ONC201 activated FOXO3a and induced the downstream expression of TRAIL in HIV-1 infected macrophages.

The findings in HIV-1 infected MDM provide us with the information of overall changes of FOXO3a phosphorylation and TRAIL expression in infected cells with the same settings to the following antiviral tests. However, these changes might only be a consequence of differentiated HIV infection but not a direct consequence of ONC201 treatment. Thus, the effect of ONC201 in uninfected MDM also needed to be assessed. For this sake, MDM were treated with ONC201 or its isomer at 3 to 30 μM for 5 days without HIV-1 infection. Analysis of phosphorylation of FOXO3a and expression of TRAIL was the same as that in infected MDM. We found that in uninfected MDM, ONC201 treatment also reduced the phosphorylation of FOXO3a and increased the amount of soluble TRAIL, which is consistent with the findings in HIV-1 infected MDM (Fig.

2.5 and 2.6). This result confirms that ONC201 treatment is mainly responsible for the activation of FOXO3a and induction of TRAIL expression in HIV-1 infected MDM.

ONC201 inhibits HIV-1 infection of macrophages in vitro

To explore the potential antiviral effect of ONC201 on macrophages, we infected MDM with HIV-1_{ADA}, a macrophage-tropic strain of HIV-1, for 24 hours and then treated cells with doses of ONC201 or its isomer ranging from 0.03 to 30 μM. At 5-day post treatment when HIV-1_{ADA} replication peaks in MDM, supernatants were collected and subjected to HIV-1 RTase activity assay. This assay determines the levels of reverse transcriptase activity in cultural supernatants and is a semi-quantitative measurement of HIV-1 replication levels (103). Measured by RTase activity assay, the levels of HIV-1 viral replication were significantly decreased in the ONC201-treated groups in a dose-dependent manner from 3 to 30 μM, compared with those in the isomer-treated groups as control (Fig. 2.7). Therefore, ONC201 has a specific antiviral activity against HIV-1 in human MDM.

To further confirm the antiviral effect of ONC201 in MDM, we determined the levels of both intracellular and extracellular HIV-1 capsid protein p24 through Western blots on cell lysates and ELISA on culture supernatants respectively. Treatment with ONC201 significantly decreased the amount of p24 in the infected MDM on both intracellular and extracellular levels (Fig. 2.8). Consistent with the p24 data, treatment with ONC201 significantly decreased the levels of HIV-1 gag RNA and DNA when compared with those in the isomer or DMSO

groups (Fig. 2.9A and B). Furthermore, we determined the HIV-1 integration through a two-step Alu-based nested PCR method and found that treatment with ONC201 significantly decreased the level of integrated HIV-1 DNA in MDM when compared with those in isomer or DMSO groups (Fig. 2.9C). Together, ONC201 appears to reduce multiple viral intermediates and products in HIV-1 lifecycle.

To confirm this antiviral effect of ONC201 on other HIV strains in macrophages, we infected MDM with two HIV Clade B strains, G0048CPX and 2562BG, and one HIV Clade C strain, 2873MVC, respectively and then treated cells with doses of ONC201 or its isomer for 14 days. For all three strains, ONC201 treatment at the concentration of 10 and 30 μM reduced the viral replication at 10-day post infection when compared with isomer treatment, which is consistent with the results in HIV-1_{ADA} (Fig. 2.10). In addition, ONC201 treatment at the concentration of 3 μM also decreased viral replication for HIV Clade C strain, though the same treatment did not affect viral replication for other two HIV Clade B strains. These results showed that the anti-HIV-1 activity of ONC201 is applicable to different natural HIV strains that can infect MDM.

ONC201 inhibits HIV-1 infection in lymphocytes, microglia and latently infected cell line in vitro

Lymphocytes and microglia are also HIV-1 reservoir cells in the CNS. To test the antiviral activity of ONC201 in these cells, we infected human PBL and microglia with HIV-1 for 7 and 1 day respectively, followed by 5-day treatment of ONC201 or its isomer. Analysis of HIV-1 replication in PBL and microglia by

RTase activity assay demonstrated similar results to those seen in MDM, that ONC201 treatment significantly reduced HIV-1 replication at the concentrations over 3 μM in these two cell types, indicating ONC201 is an effective agent to limit HIV-1 replication in all three major reservoir cell types in CNS (Fig. 2.11).

Pro-monocytic U1 cells, latently infected with HIV-1, can be stimulated by TNF to reactivate the virus from latency. To study the effect of ONC201 on the reactivated HIV-1 in latently infected cell line, we treated U1 cells with ONC201 or its isomer in the presence of TNF and measured the viral production by RTase activity assay and ELISA. Two assays both showed that ONC201 treatment decreased the level of reactivated HIV-1 in the culture supernatant (Fig. 2.12). This inhibitory effect of ONC201 were seen at the concentrations as low as 0.3 μM, suggesting a higher antiviral efficacy of ONC201 in these latently infected cells compared with that in MDM.

ONC201 synergies with AZT in HIV-1 inhibition in vitro

Azidothymidine (AZT) is one of the most frequently used reverse transcriptase inhibitors *in vitro*. To determine whether the combination of AZT and ONC201 could further improve the anti-HIV-1 effect of both drugs, HIV-1 infected MDM were incubated with doses of AZT along with either ONC201 or its isomer. We found that addition of ONC201 shifted the inhibitory curve of AZT leftward compared with addition of its isomer, with the half maximal effective concentration (EC50) decreasing from 0.15 μM to 0.015 μM (Fig. 2.13). This potentiation indicates that ONC201 synergies with AZT in HIV-1 inhibition.

AZT has a potent inhibition on HIV-1 replication in macrophages. However, viral replication usually rebounds in days after the AZT treatment discontinues. To test whether ONC201 treatment could prolong the viral suppression established by AZT, HIV-1 infected MDM were incubated with AZT along with either DMSO, ONC201 or its isomer for 6 days. The treatment was ceased at 7-day post infection (dpi). We observed that the viral rebound occurred at 11 dpi in DMSO and isomer treated groups while no viral rebound was seen in ONC201 treated group even at 15 dpi, indicating addition of ONC201 to AZT treatment achieves longer viral suppression after the treatment was interrupted (Fig. 2.14).

ONC201 has an anti-HIV-1 effect on macrophages in vivo

To further determine whether ONC201 has an anti-HIV-1 effect *in vivo*, we used an established HIV-1 infected NOD/SCID/IL2Rγ_c^{null} (NSG) model (104-106). HIV-1 infected human MDM were intracranially injected into basal ganglion of the NSG immunocompromised mice. At 24-h post MDM engraftment, the mice were administrated daily with either ONC201 (50 mg/kg) or vehicle control via intraperitoneal (IP) for six days. At 7-day post MDM engraftment, the mice were sacrificed for HIV-1 detection (Fig. 2.15).

To visualize the HIV-1-infected cells in basal ganglion, we first performed immunohistochemistry in serial para-coronal brain slices encompassing the injection track with DAPI (Fig. 2.16A and D) and p24 (Fig. 2.16B and E). Both DAPI and p24 clearly marked the injection tracks and their signals were in close proximity to each other in the injection tracks (Fig. 2.16C and F). To confirm that the p24-positive cells are CD68-positve macrophages, we performed CD68

staining. Because CD68 and p24 antibodies are from the same species (mouse) and alternative antibodies from other species worked poorly in the co-immunostaining (data not shown), we individually labeled the injection site with either CD68 or p24 along with DAPI. CD68 staining revealed a substantial number of macrophages remained in the injection track in both DMSO and ONC201 treatment groups (Fig. 2.16G and H). We quantified the p24-positive and CD68-positive cells in consecutive slides through a previously established stereological method (107, 108). As expected, we observed that ONC201 administration significantly decreased p24-positive cells as a percentage to total CD68-positive cells (Fig. 2.16I), compared with those in the vehicle control group. These data suggest that ONC201 reduces the number of HIV-1-infected macrophages in the *in vivo* NSG mouse model.

In order to better quantitatively determine the levels of HIV-1 inhibition, we homogenized the tissues that contained injection sites and subjected the tissue lysates to Western blots (Fig. 2.17A). The quantification data revealed that p24 levels significantly decreased in the ONC201 group compared with the control group (Fig. 2.17B). In contrast, CD68 levels were comparable in all of the brain lysates. As a result, the ratio of p24 to CD68 significantly decreased after ONC201 treatment (Fig. 2.17C), which is consistent with the p24 and CD68 staining data. Together, these data in HIV-1-infected NSG mice demonstrated that ONC201 is able to inhibit HIV-1 infection of macrophages *in vivo.*

2.5 Discussion

Termination of long-term HIV persistence by eliminating viral reservoirs is essential to combat AIDS in the post-ART era (109-111). In the present study, ONC201 was tested for its potential antiviral effect on the HIV-1 infected CNS reservoir cells. We demonstrated that ONC201 has little toxicity in uninfected human macrophages and lymphocytes and exert an anti-HIV-1 effect on infected macrophages, lymphocytes, microglia and U1 cell line *in vitro*. This antiviral activity of ONC201 is applicable not only to macrophage-tropic HIV strain but also to other broadly spread HIV strains. When combined with ONC201, AZT achieves a synergic antiviral effect and a longer viral suppression without viral rebound. In a murine HIV Encephalitis model, ONC201 treatment significantly reduced HIV-1 levels in transplanted macrophages, indicating the antiviral effect of ONC201 against HIV brain reservoirs *in vivo*.

Although variants of recombinant TRAIL and the longer-lived TRAIL receptor agonist antibodies have been developed and applied in clinical trials (112-118), those protein-based therapeutics are costly and the therapeutic concentrations remain difficult to maintain. Therefore, increasing endogenous TRAIL protein production via pharmacological means is an attractive approach. ONC201 was originally screened from the National Cancer Institute (NCI) Diversity Set II as one of the small molecules capable of up-regulating endogenous TRAIL gene transcription (77). There are other potent TRAIL-inducing compounds such as TIC9 reported from the screening. However, ONC201 seems to uniquely potentiates tumor cell death while sparing normal

cells and lacks genotoxicity in normal fibroblasts, which could be the result of a relatively milder induction of death receptor 5 compared with those inducted by TIC9 (77). This characteristic of ONC201 allows it to specifically target HIV-1 infected macrophages and be further considered for clinical applications.

ONC201 has been well-known in cancer research for its capacity to induce sustained TRAIL upregulation and apoptosis in tumor cells both *in vitro* and *in vivo* (77, 78). Our study for the first time demonstrates that ONC201 inhibits HIV-1 replication in macrophages both *in vitro* and *in vivo*. The evidence for viral inhibition after ONC201 treatment is multifold. First, the HIV-1 RTase activity assay is a relatively accurate measurement of HIV-1 virion production in the supernatants (103). In our study, the result of this measurement was confirmed by ELISA on the HIV-1 p24 in the same supernatants. Second, two-step Alu-based nested PCR determines the level of HIV-1 integration by measuring HIV-1 LTR DNA as the proviral DNA integrated in the host genome (119). ONC201 is able to lower the levels of HIV-1 integration into the genome. Third, ONC201 also decreases intracellular p24, GAG RNA, and GAG DNA in HIV-1-infected MDM.

Furthermore, this antiviral effect of ONC201 is not limited to HIV-1_{ADA} in macrophages, but also applicable to other HIV strains and other reservoir cell types. HIV-1_{ADA} is a macrophage-tropic HIV-1 strain. After laboratory adaption, its ability to infect macrophage is further enhanced so that its infection in macrophage typically peaks at 5-day post infection. This characteristic allows HIV-1_{ADA} to be one of the optimal HIV strains to study HIV-1 infection in

macrophages. Apart from HIV-1_{ADA}, HIV Clade B and C strains can also infect macrophages. In our study, the infection of two HIV Clade B strains in MDM peaked at around 10-day post infection whereas the HIV Clade C strain may still have not reached its highest infection at 14-day post infection. For 2873MVC and 2562BG strains, the antiviral effect of ONC201 started to be observed at 10-day post infection, and a nearly full viral suppression by ONC201 was seen at 14-day post infection, showing an even more potent antiviral effect than that in HIV-1_{ADA}. Based on the inhibitory curves of ONC201 in these HIV strains, the EC50 of ONC201 for HIV-1_{ADA} and HIV Clade C is around 3 μM while the EC50 for HIV Clade B is between 3 and 10 μM. This suggests that ONC201 is of higher efficacy for HIV Clade C than for HIV Clade B.

Microglia and lymphocytes are other major cell types of HIV-1 reservoir. Microglia, though usually regarded as the resident macrophages in CNS, possess distinct phenotype compared with MDM. Fate-mapping studies of macrophage populations in the body have determine that microglia are not derived from the bone marrow but originate from erythromyeloid precursors early in the embryonic development (120, 121). The effective concentrations of ONC201 found in human primary microglia are similar to those in MDM, suggesting that both CNS reservoir cells can benefit from the same therapeutic doses of ONC201 in clinical application. Memory $CD4^+$ T cells form the HIV-1 reservoirs outside CNS. ONC201 inhibits HIV-1 infection in infected PBL and has no cytotoxicity on uninfected PBL at the effective concentrations, suggesting the application of ONC201 as an anti-HIV-1 agent may not be limited to CNS but

be general to the circulation and other organs. Thus, future *in vivo* study of ONC201 on lymphocytes in peripheral blood, spleen, gut and lymph nodes is needed.

Despite its specific effect on HIV-1 replication, at the current working concentration (30 μM), ONC201 is not able to completely eradicate HIV-1 from the cultures. This could be due to the expression of TRAILshort in MDM that confers TRAIL resistance upon ONC201 treatment (122), or lack of optimal trimeric TRAIL conformation that is most potent for the antiviral effect (123). To achieve an even more potent antiviral effect, a higher concentration of ONC201 might be needed. However, ONC201 is only soluble in DMSO but not in water. In our study, ONC201 is soluble in DMSO up to 25 mM. In order to achieve ONC201 concentrations above 30 μM in MDM cultures, at least 0.1% final concentration (v/v) of DMSO will be used, which may cause cytotoxicity, potentially affecting the observation and interpretation of the specific antiviral effect of ONC201. Thus, we used 30 μM as the highest test concentration for ONC201 in the experiments. To further increase the solubility of ONC201 in DMSO and water, modifications on the structure of ONC201 or a nanoparticle-mediated delivery of ONC201 should be considered for the future study.

For a complete HIV-1 eradication, another strategy is to use a combination of anti-HIV-1 drugs targeting different steps in the process of viral infection. AZT is one of the classic drugs in Highly Active Antiretroviral Therapy (HAART) that aims to prevent HIV resistance. As a thymidine analogue, AZT selectively inhibits the reverse transcriptase of HIV-1. Although AZT itself can effectively

inhibit HIV-1 replication in macrophages in vitro, viral rebound occurs days after drug withdrawal. In our study, ONC201 left-shifted the inhibitory curve of AZT in HIV-1 infected MDM, showing a potentiation on the antiviral effect of AZT. When combined with ONC201, AZT achieved a longer HIV-1 suppression after drug withdrawal. The mechanism underlying this synergy needs to be further investigated.

One of the most commonly used *in vitro* models to study HIV-1 latency in macrophage is U1 cells. As a subclone of U937, which is a pro-monocyte obtained from diffuse histiocytic lymphoma, U1 is latently infected with HIV-1 (124). It contains two integrated copies of the viral genome but shows minimal constitutive expression of virus (124). Under certain stimulated conditions, such as TNF and PMA treatment, latent virus can be reactivated and secreted into the medium (125). With analysis of both p24 and HIV-1 reverse transcriptase activity in the medium, we observed a potent viral inhibition by ONC201 in TNF-stimulated U1 cells, suggesting an antiviral effect of ONC201 in macrophages containing latent virus. However, this effect may not be attributed to the cytotoxicity of ONC201 in U1 cells, because we did not observe a significant decrease of cell viability in stimulated U1 cells with ONC201 treatment (data not shown). Instead, the ability of ONC201 to inactivate Akt-1 could play a major role in this process. Previous study on the mechanism of viral reactivation in U1 cells has shown that the activation of Akt in host cells is critical in the reactivation of latent virus (125). Thus, Akt activators such as disulfiram were used to reactivate the latent virus in the strategy of “shock and kill” (126), while

Lopinavir/Ritonavir limits HIV-1 reactivation via blocking the Akt pathway in the strategy of “block and lock” (125). ONC201 also inactivates Akt, thus possibly preventing the latency virus in TNF-stimulated U1 cells from reactivation in the strategy of “block and lock”. However, the real scenario of HIV-1 latency in macrophages may differ from the artificial latency established in chronically infected U1 cells. Further study on the Akt pathway in HIV-1 latency is needed for better interpreting the antiviral effect of ONC201 in latently infected cells.

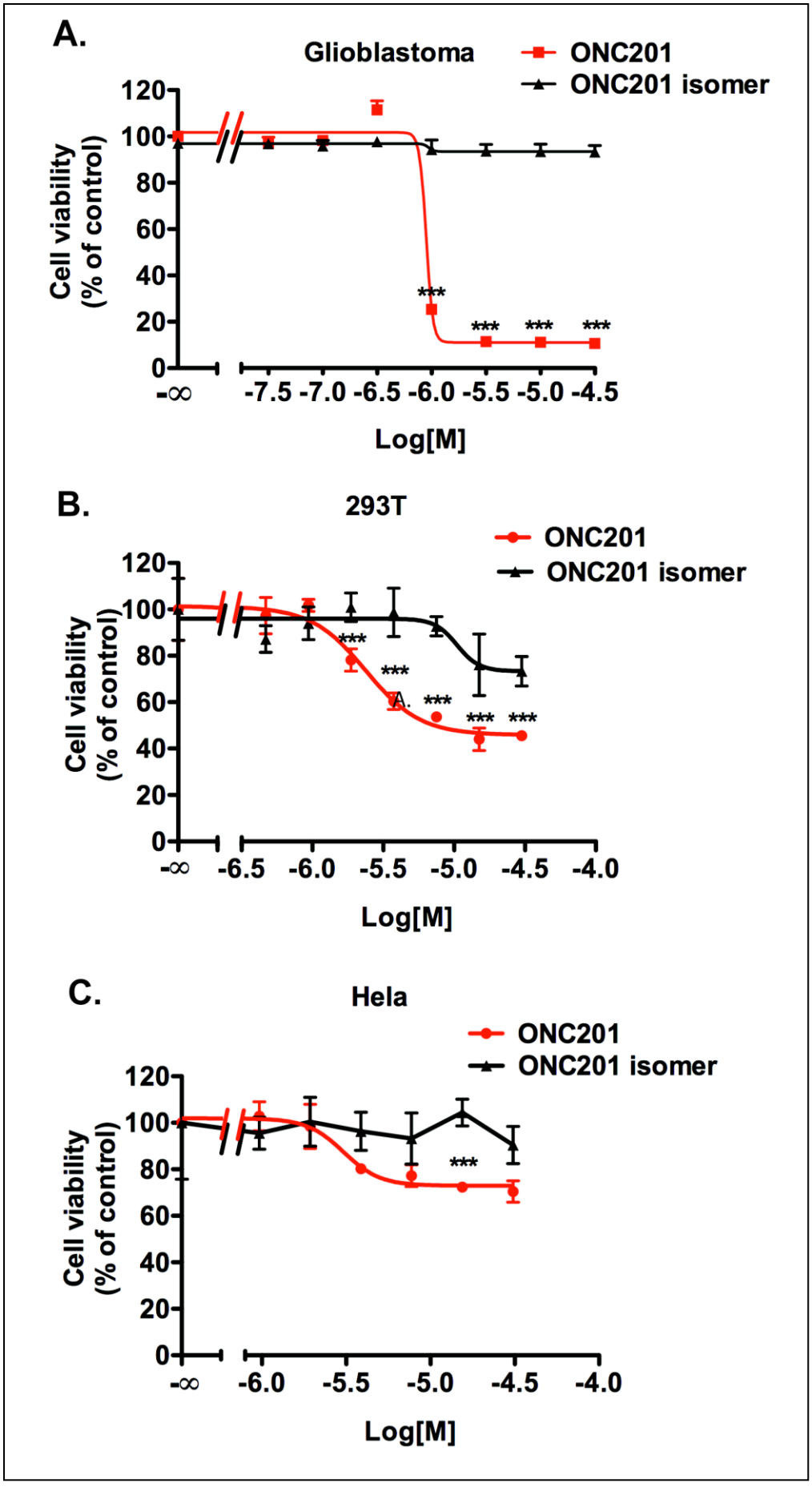
As a small molecule, ONC201 shows an outstanding capability of BBB penetration, which makes it particularly attractive for combating CNS viral reservoirs. For brain tumors such as glioblastoma, ONC201 penetrates the intact BBB with 5 times higher concentrations in brain tissue relative to plasma in rodents (78). Therefore, after a successful dose escalation phase I trial (127), many ongoing clinical trials (clinicaltrials.gov) focus on brain tumors. In the present study, ONC201 is injected through IP into the NSG mice and its antiviral effect against the HIV-1-infected macrophages in the brains indicate that it is able to cross the BBB and achieve the therapeutic outcome in the case of CNS HIV-1 infection.

Immune-activating agents, such as interleukin 2 and anti-CD3 antibody, or for epigenetic modulation, such as HDACs inhibitors, often failed to purge the virus *in vivo* (31, 128-131). Therefore, new therapeutic agents are warranted to combat persistent HIV-1 infection. Our study demonstrates for the first time that ONC201, a FOXO3a activator and TRAIL inducer, inhibits HIV-1 replication in macrophages both *in vitro* and *in vivo*. Notably, ONC201 is the founding

member of the imipridone class of compounds that share a unique imidazo pharmacophore. New compounds continue to be developed in this class (132, 133). These new group of compounds provide us with more drug candidates to modulate immunity and fight viral infections.

2.6 Figures

Figure 2.1 ONC201 inhibits the proliferation of tumor and transformed cells. Glioblastoma cell line A172 (A), 293T cell line (B) and Hela cell line (C) were plated in 96-well plates in triplicate and then incubated with doses of ONC201 or its isomer ranging from 0.03 to 30 μM. Cell viability was determined by the MTS assay. Results shown are from representative experiments performed with three different plates of cells. Data were analyzed by two-way ANOVA: * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$.


A.
Glioblastoma
ONC201
ONC201 isomer
Cell viability (% of control)
120
100
80
60
40
20
0
-∞
-7.5 -7.0 -6.5 -6.0 -5.5 -5.0 -4.5
Log[M]
***
B.
293T
ONC201
ONC201 isomer
Cell viability (% of control)
-∞
-6.5 -6.0 -5.5 -5.0 -4.5 -4.0
Log[M]
C.
Hela
ONC201
ONC201 isomer
Cell viability (% of control)
-∞
-6.0 -5.5 -5.0 -4.5 -4.0
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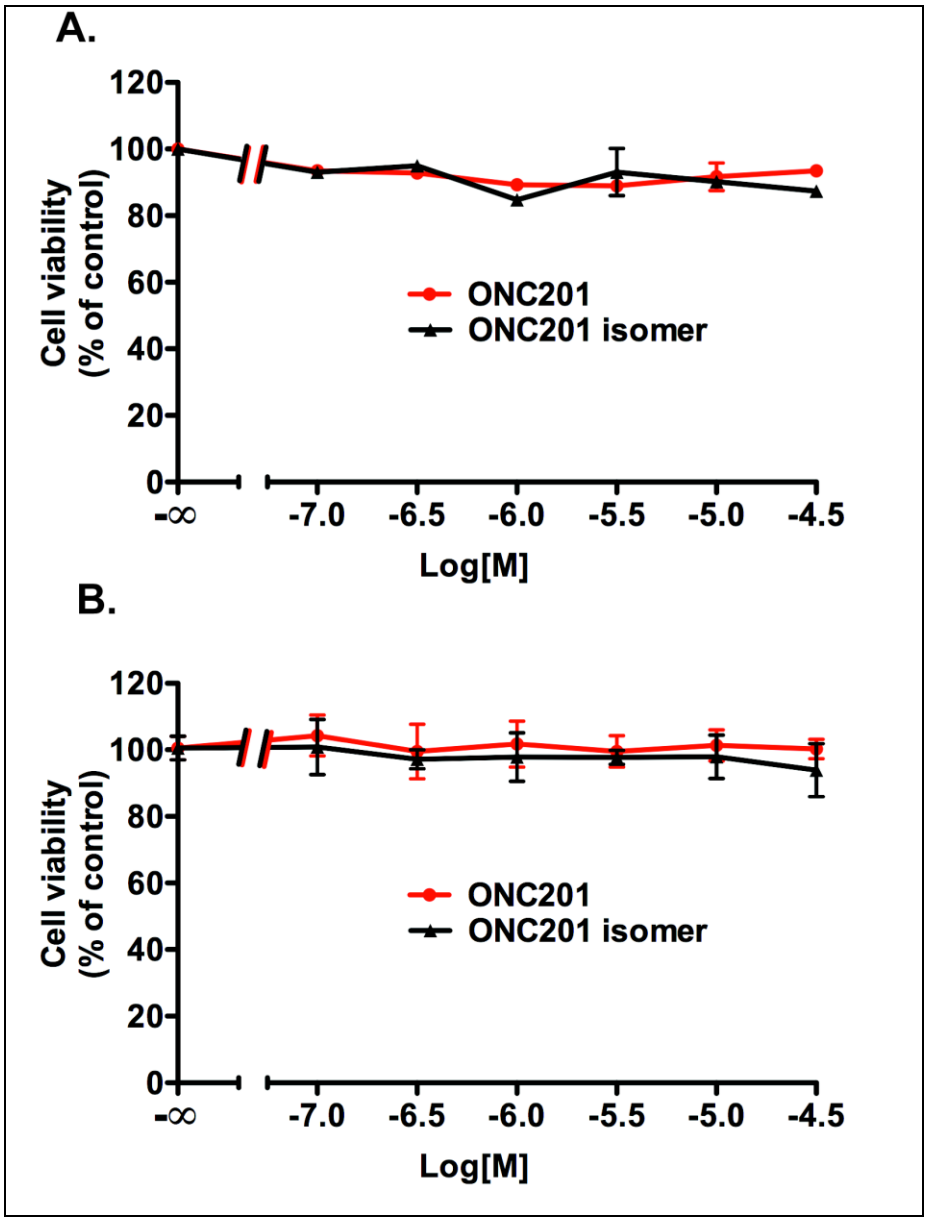


Figure 2.2 ONC201 does not change the cell viability of uninfected macrophages and lymphocytes. Human monocyte-derived macrophages (MDM) (A) and peripheral blood lymphocytes (PBL) (B) were plated in 96-well plates and incubated with doses of ONC201 or its isomer ranging from 0.03 to 30 μM. At 5-day post ONC201 and isomer treatments, cell viability was determined by the MTS assays. Data were analyzed by two-way ANOVA, and results shown are the means ± SD of 3 experiments.

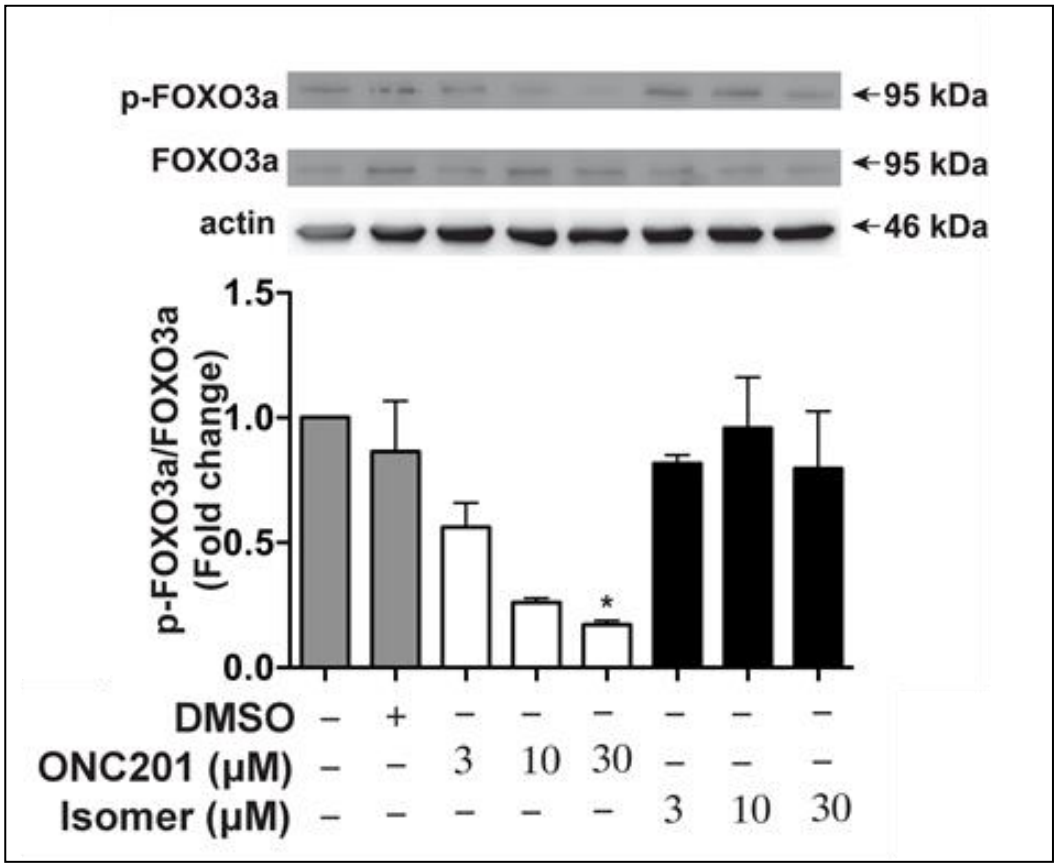


Figure 2.3 ONC201 activates FOXO3a in HIV-1 infected macrophages. Human MDM were plated in 24-well plates and then infected with HIV-1_{ADA} for 24 hours before incubation with ONC201 or ONC201 isomer at 3 to 30 μM for 5 days. Cell lysates were collected and subsequently subjected to SDS-PAGE and immunoblotting for the detection of phospho-FOXO3a and total FOXO3a. Actin was used as the loading control. Densitometric quantifications of phospho-FOXO3a in MDM were presented as a ratio to total FOXO3a and normalized as fold changes to the no treatment control. Values represent SEM of biological replicates. ANOVA analysis: * denotes $p < 0.05$ and ** denotes $p < 0.01$, compared with the DMSO group.

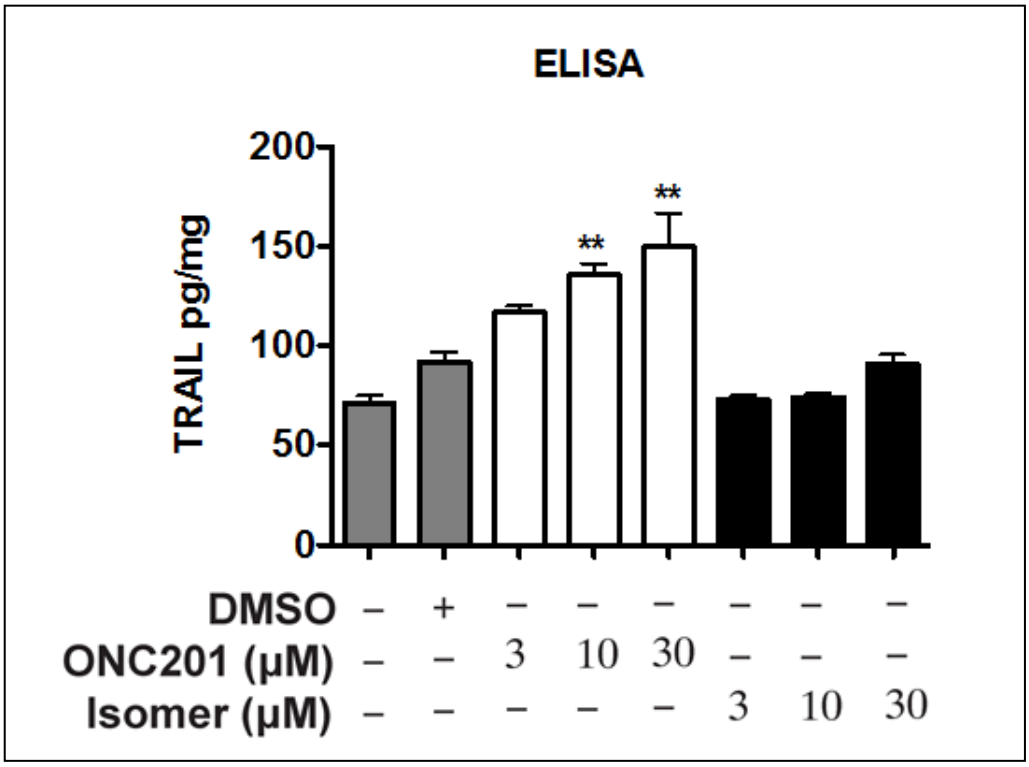


Figure 2.4 ONC201 induces TRAIL expression in HIV-1 infected macrophages. Human MDM were plated in 24-well plates and then infected with HIV-1_{ADA} for 24 hours before incubation with ONC201 or ONC201 isomer at 3 to 30 μM for 5 days. Soluble TRAIL concentration (pg/mg) was determined from cell culture supernatants using ELISA and adjusted to intracellular protein concentrations. Values represent SEM of biological replicates. ANOVA analysis: * denotes $p < 0.05$ and ** denotes $p < 0.01$, compared with the DMSO group.

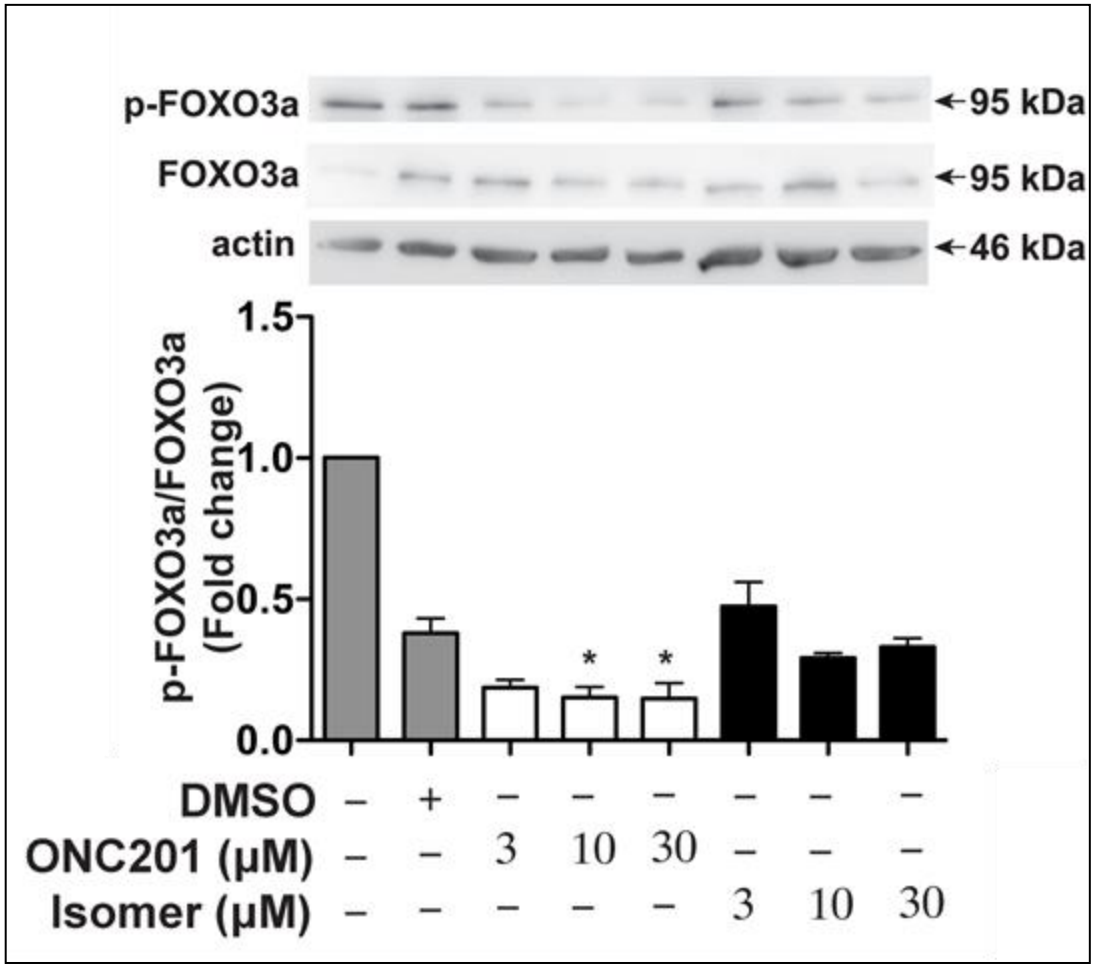


Figure 2.5 ONC201 activates FOXO3a in uninfected macrophages. Human MDM were plated in 24-well plates and then incubated with ONC201 or ONC201 isomer at 3 to 30 μM for 5 days. Cell lysates were collected and subsequently subjected to SDS-PAGE and immunoblotting for the detection of phospho-FOXO3a and total FOXO3a. Actin was used as the loading control. Densitometric quantifications of phospho-FOXO3a in MDM were presented as a ratio to total FOXO3a and normalized as fold changes to the no treatment control. Values represent SEM of biological replicates. ANOVA analysis: * denotes $p < 0.05$ and ** denotes $p < 0.01$, compared with the DMSO group.

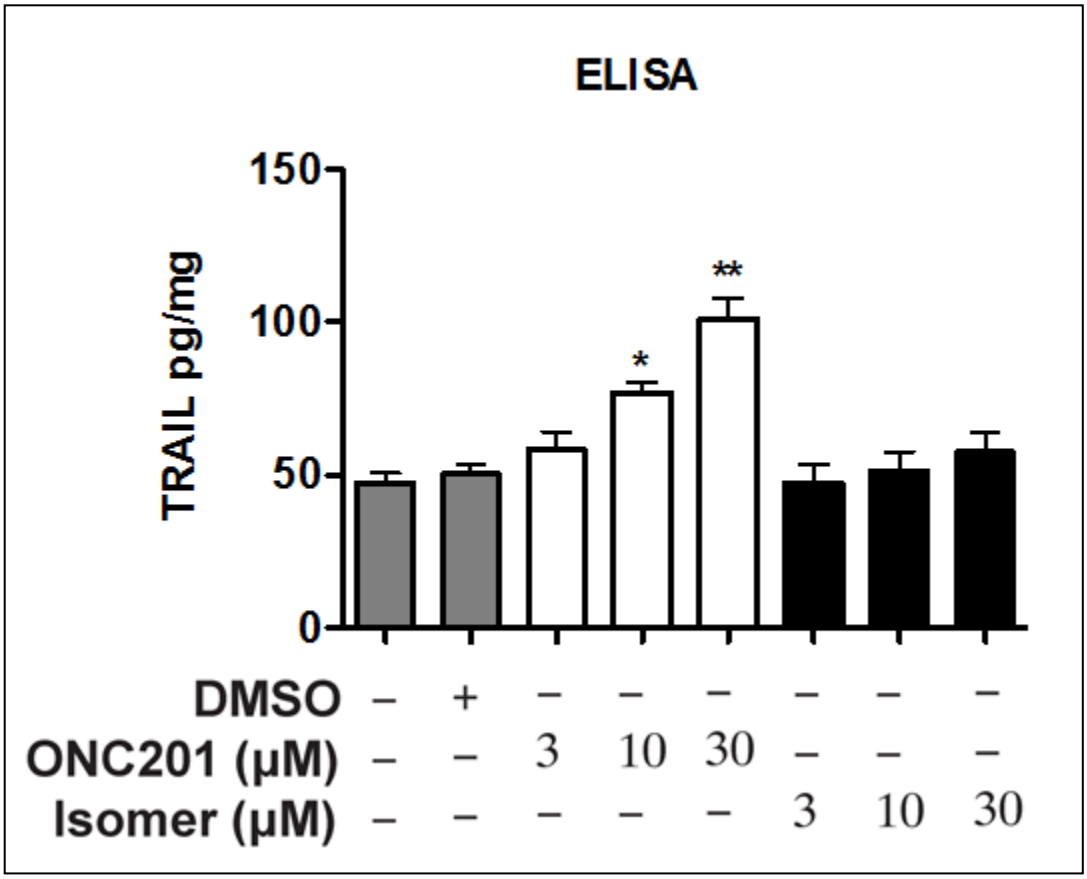


Figure 2.6 ONC201 induces TRAIL expression in uninfected macrophages. Human MDM were plated in 24-well plates and then incubated with ONC201 or ONC201 isomer at 3 to 30 μM for 5 days. Soluble TRAIL concentration (pg/mg) was determined from cell culture supernatants using ELISA and adjusted to intracellular protein concentrations. Values represent SEM of biological replicates. ANOVA analysis: * denotes $p < 0.05$ and ** denotes $p < 0.01$, compared with the DMSO group.

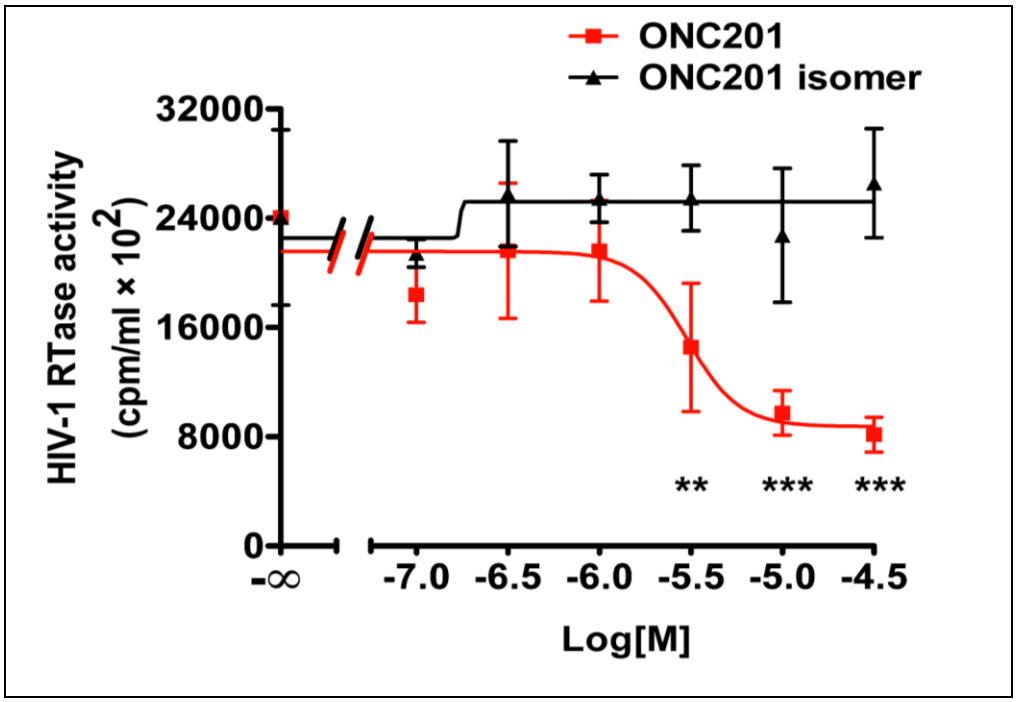


Figure 2.7 ONC201 has an antiviral effect on macrophages. Human MDM were plated in 96-well plates and infected with HIV-1_{ADA} for 24 hours before incubation with doses of ONC201 or its isomer ranging from 0.03 to 30 μM. At 5-day post ONC201 and isomer treatments, supernatants were removed and the replication levels of HIV-1 were monitored by RTase activity assay. Results shown are from representative experiments performed with three different donors. Data were analyzed by two-way ANOVA: * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$.

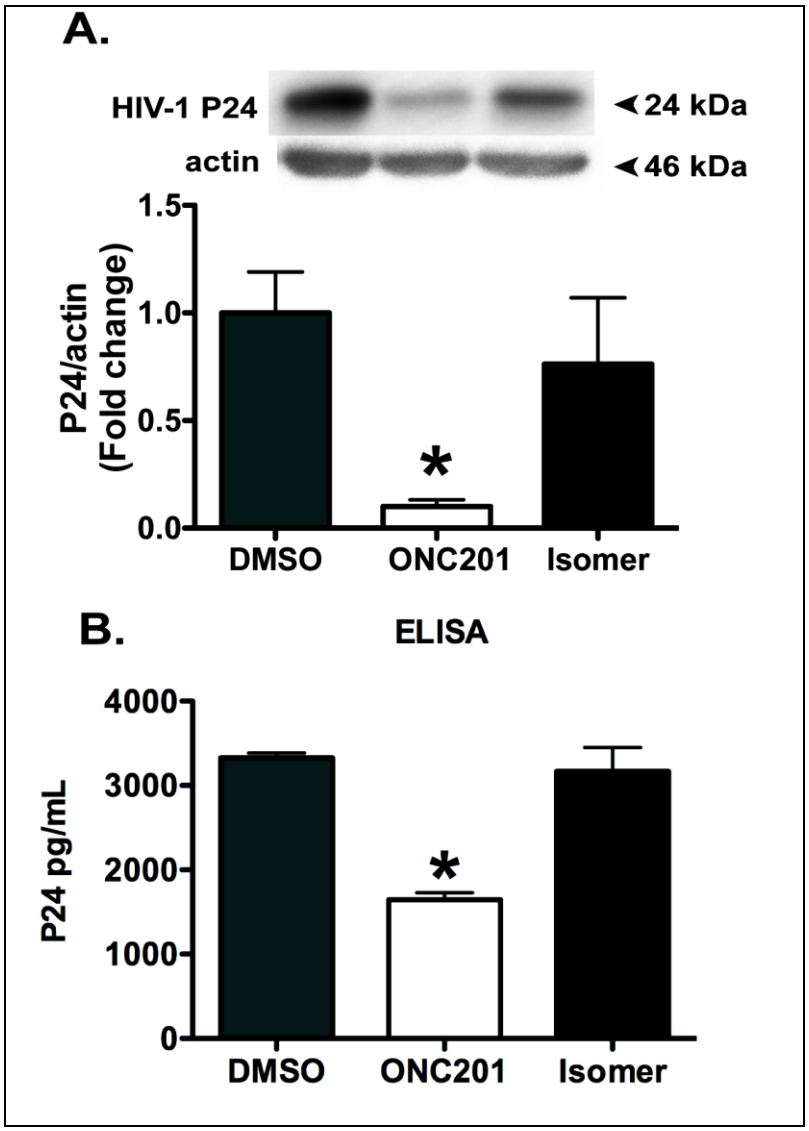


Figure 2.8 ONC201 reduces HIV-1 p24 in HIV-1 infected macrophages. Human MDM were plated in 24-well plates in triplicate and then infected with HIV-1_{ADA} for 24 hours before incubation with ONC201 or ONC201 isomer at 30 μM for 5 days. A) Cell lysates were collected and subjected to SDS-PAGE and immunoblotting for HIV-1 p24. Actin was used as the loading control. B) Supernatants were collected and subjected to ELISA for HIV-1 p24. Values represent SEM of biological replicates. ANOVA analysis: * denotes $p < 0.05$ compared with DMSO group.

Figure 2.9 ONC201 reduces HIV-1 gag RNA, DNA, and integrated LTR DNA in HIV-1 infected macrophages. Human MDM were plated in 24-well plates in triplicate and then infected with HIV-1_{ADA} for 24 hours before incubation with ONC201 or ONC201 isomer at 30 μM for 5 days. RNA (A) and DNA (B, C) were isolated and HIV-1 gag was detected through quantitative real time RT–PCR and real time PCR, respectively. Relative HIV-1 gag levels were determined and standardized with 18S rRNA or GAPDH internal control. Values represent SEM of biological replicates. ANOVA analysis: * denotes $p < 0.05$ compared with DMSO group; ## denotes $p < 0.01$ compared with ONC201 treatment group.

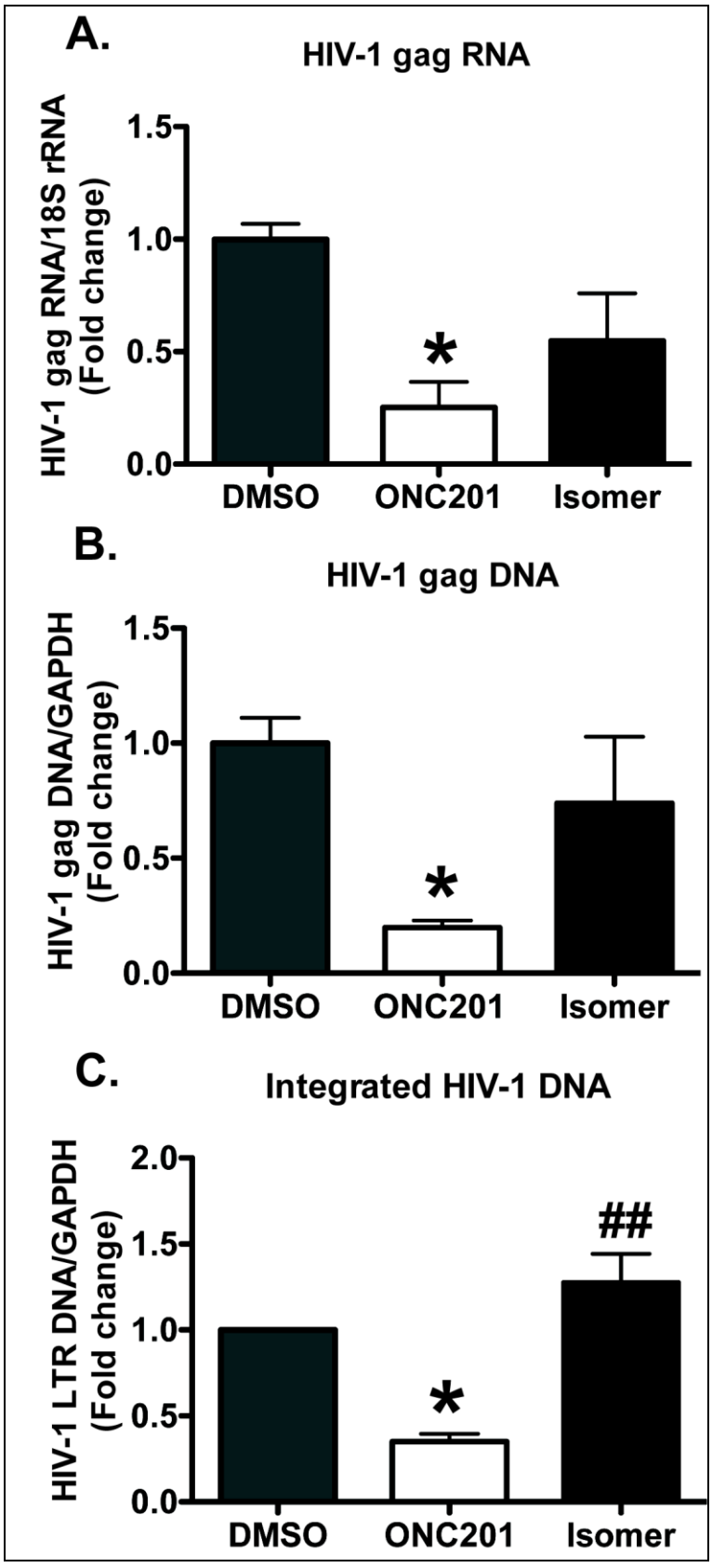
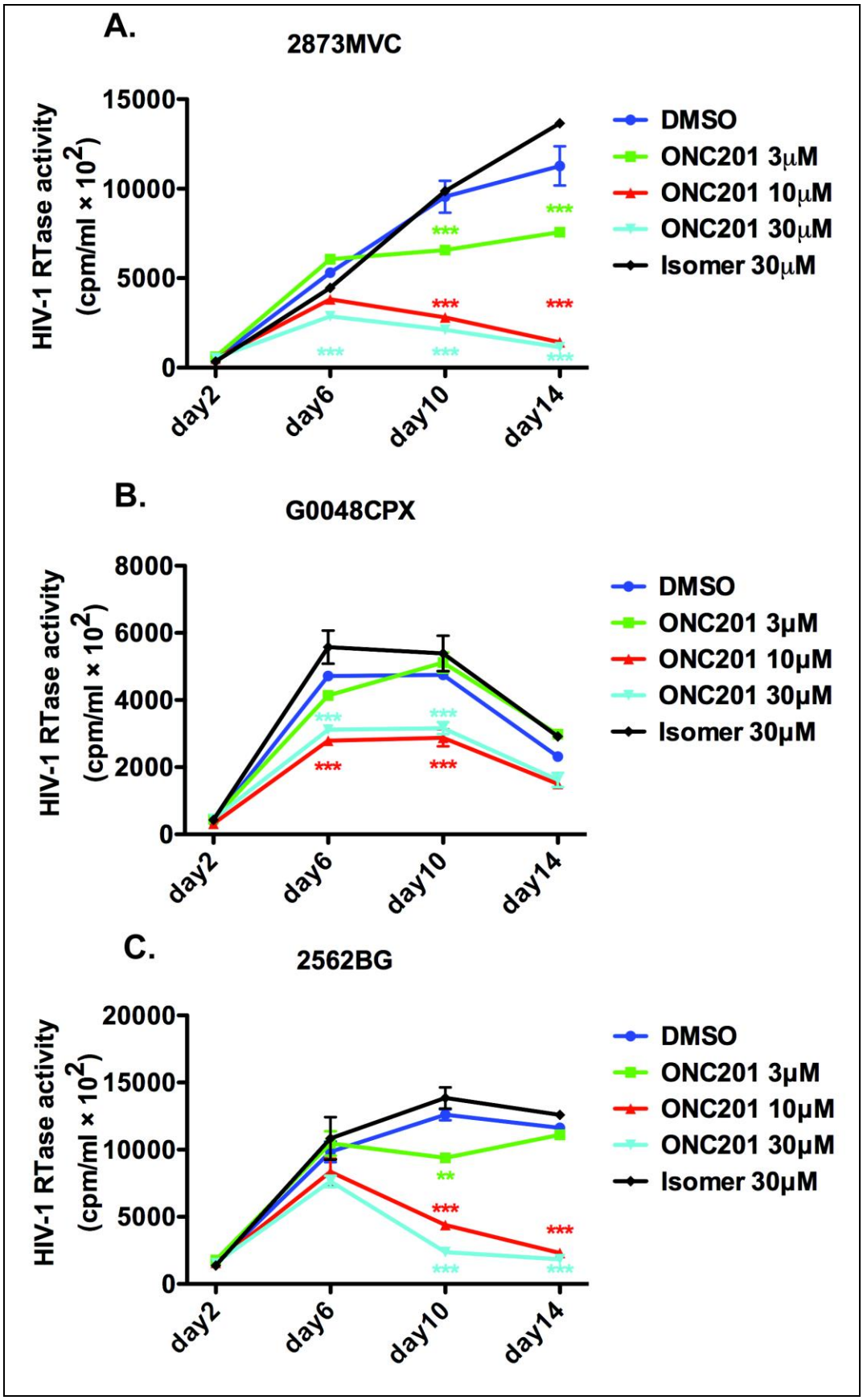

A.
HIV-1 gag RNA
HIV-1 gag RNA/18S rRNA (Fold change)
1.5
1.0
0.5
0.0
*
DMSO
ONC201
Isomer
B.
HIV-1 gag DNA
HIV-1 gag DNA/GAPDH (Fold change)
1.5
1.0
0.5
0.0
*
DMSO
ONC201
Isomer
C.
Integrated HIV-1 DNA
HIV-1 LTR DNA/GAPDH (Fold change)
2.0
1.5
1.0
0.5
0.0
*
##
DMSO
ONC201
Isomer

Figure 2.10 ONC201 has an antiviral effect on different HIV strains. Human MDM were plated in 96-well plates and infected with different HIV strains, i.e. 2873MVC (a HIV Clade C strain, A), G0048CPX (a HIV Clade B strain, B) and 2562BG (a HIV Clade B strain, C) for 24 hours before incubation with doses of ONC201 or its isomer ranging from 3 to 30 μM. At 2-, 6-, 10-, and 14-day post ONC201 and isomer treatments, supernatants were collected and the replication levels of HIV-1 were monitored by RTase activity assay. Values represent SEM of biological replicates. Data were analyzed by two-way ANOVA: * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$.


A.
2873MVC
HIV-1 RTase activity (cpm/ml × 10^2)
15000
10000
5000
0
day2
day6
day10
day14
DMSO
ONC201 3μM
ONC201 10μM
ONC201 30μM
Isomer 30μM
B.
G0048CPX
HIV-1 RTase activity (cpm/ml × 10^2)
8000
6000
4000
2000
0
day2
day6
day10
day14
DMSO
ONC201 3μM
ONC201 10μM
ONC201 30μM
Isomer 30μM
C.
2562BG
HIV-1 RTase activity (cpm/ml × 10^2)
20000
15000
10000
5000
0
day2
day6
day10
day14
DMSO
ONC201 3μM
ONC201 10μM
ONC201 30μM
Isomer 30μM

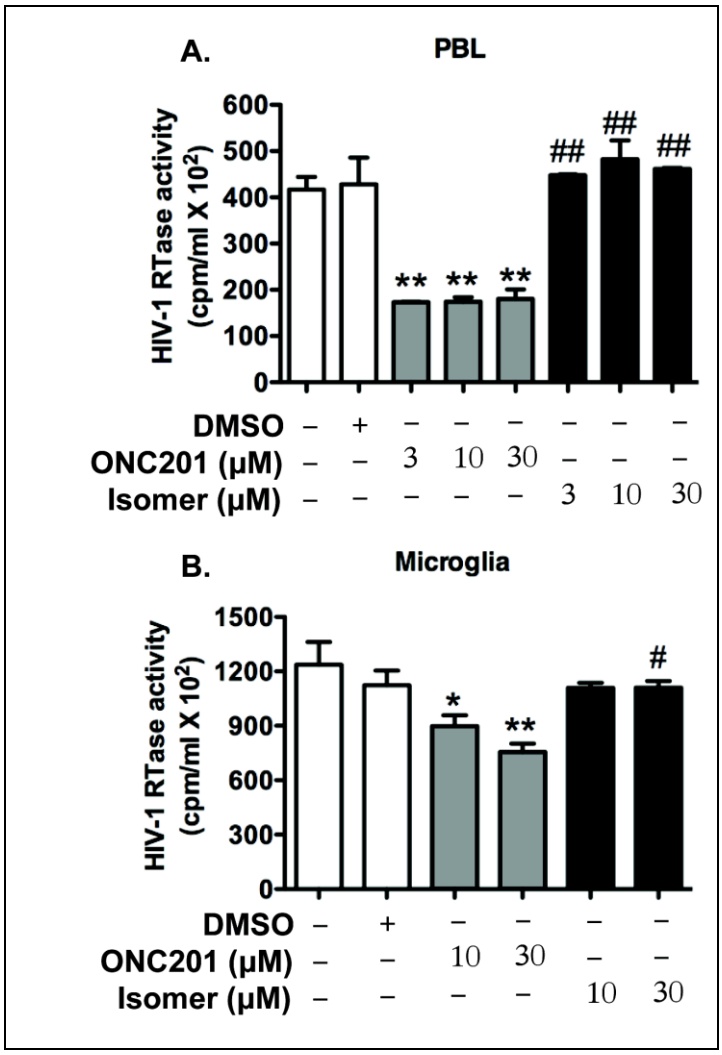


Figure 2.11 ONC201 inhibits HIV-1 replication in lymphocytes and microglia. Human PBL (A), and microglia (B) were infected with HIV-1 ADA for 7 and 1 day, respectively, and then incubated with or without various doses of ONC201 and ONC201 isomer for 5 days. The infection levels of HIV-1 were monitored by RTase activity assay. Results shown are representative experiments performed with three different donors. ANOVA analysis: * denotes $p < 0.05$ and ** denotes $p < 0.01$, compared to DMSO group; # denotes $p < 0.05$, ## denotes $p < 0.01$, compared to the ONC201 treatment group at the same dose.

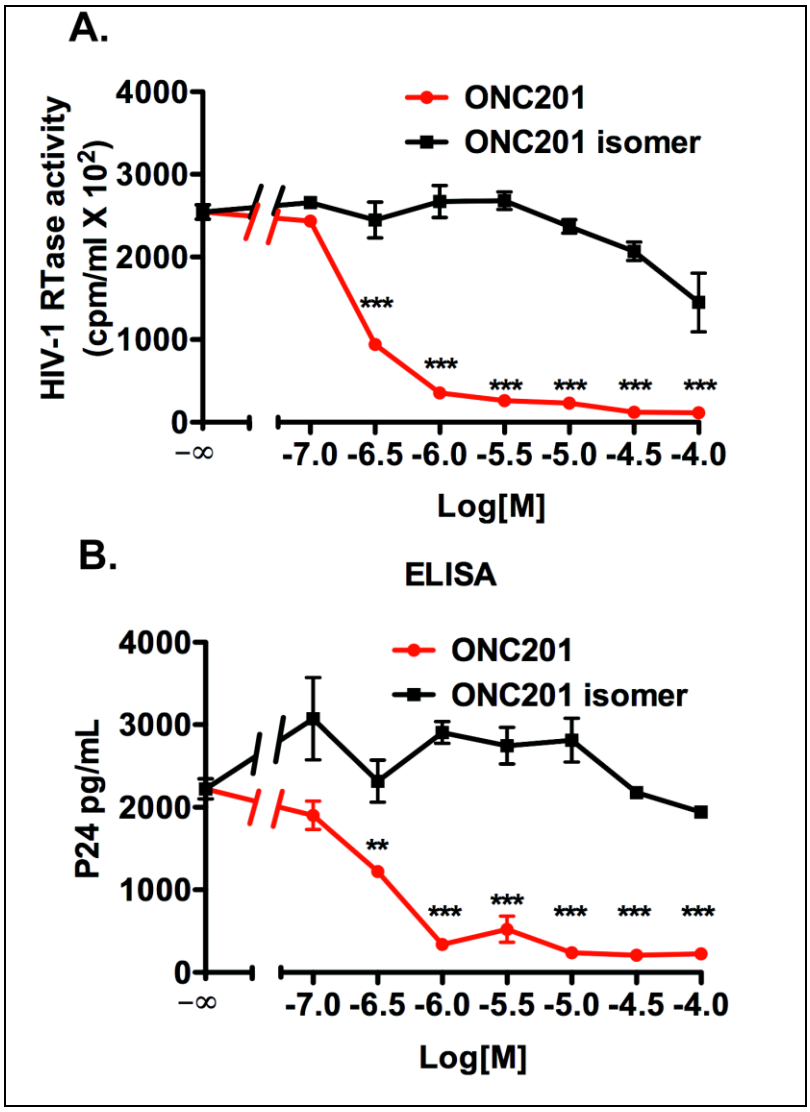


Figure 2.12 ONC201 has an antiviral effect on latently HIV-1-infected cells. U1 cells were plated in 96-well plates and incubated with doses of ONC201 or its isomer ranging from 0.01 to 100 μM in the presence of TNF (10 ng/mL). At 5-day post ONC201 and isomer treatments, supernatants were removed and the replication levels of HIV-1 were monitored by RTase activity assay (A) and ELISA (B). Results shown are from representative experiments performed with three different plates of cells. Data were analyzed by two-way ANOVA: * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$.

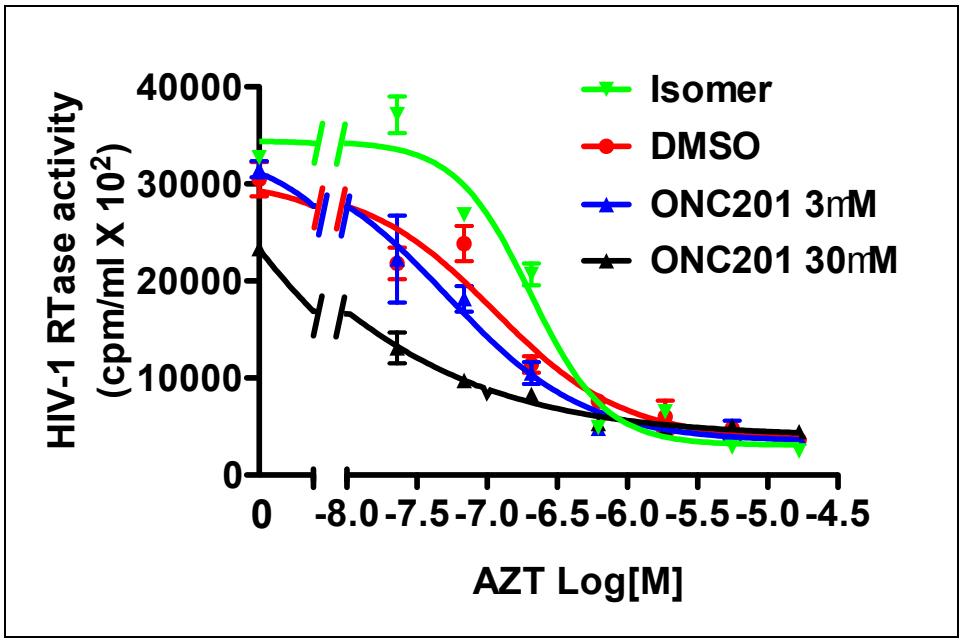


Figure 2.13 ONC201 synergies with AZT in HIV-1 inhibition. HIV-1 infected MDM were incubated with doses of AZT ranging from 0.015 to 15 μM along with either DMSO, ONC201 (3 μM or 30 μM), or ONC201 isomer (30 μM) for 5 days. ONC201 isomer was used as a control for ONC201. The infection levels of HIV-1 were monitored by RTase activity assay. Results shown are from representative experiments performed with three different plates of cells.

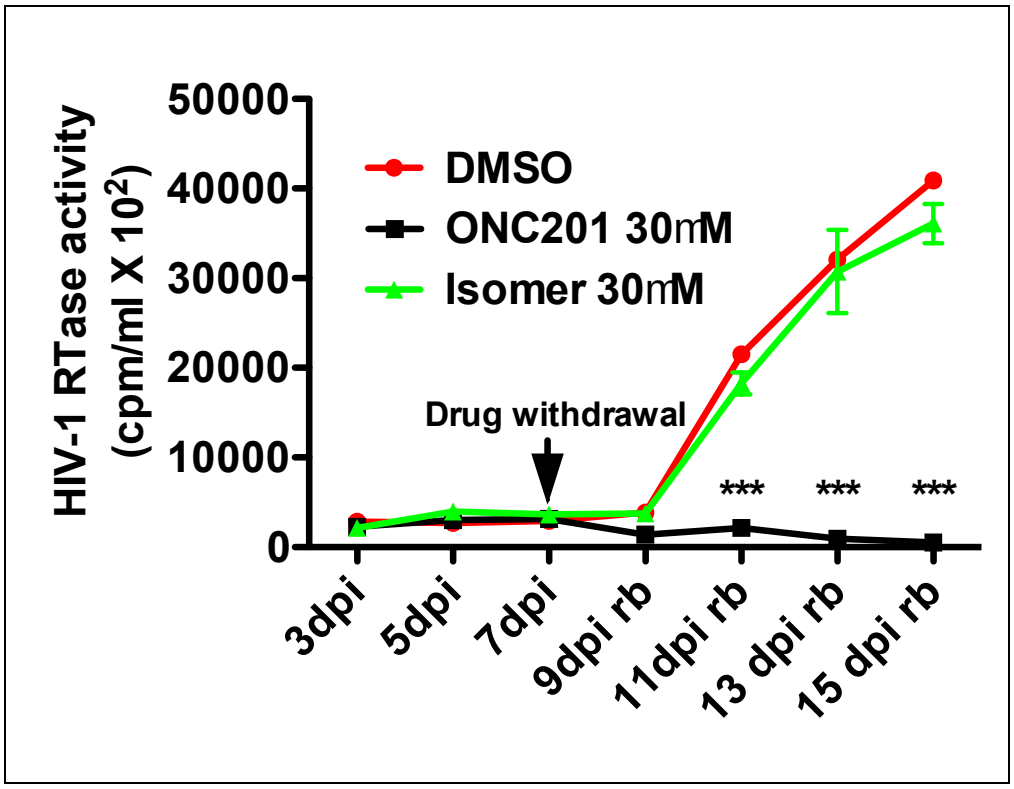


Figure 2.14 Addition of ONC201 to AZT treatment achieves a longer viral suppression during viral rebound. MDM were infected with HIV-1_{ADA} for 1 day and then incubated with 1 μM AZT along with either DMSO, ONC201 (30 μM), or ONC201 isomer (30 μM) for 6 days. The treatment was ceased at 7-day post infection (dpi). The infection levels of HIV-1 were monitored by RTase activity assay during and after treatment. Results shown are from representative experiments performed with three different plates of cells. Data were analyzed by two-way ANOVA: * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$.

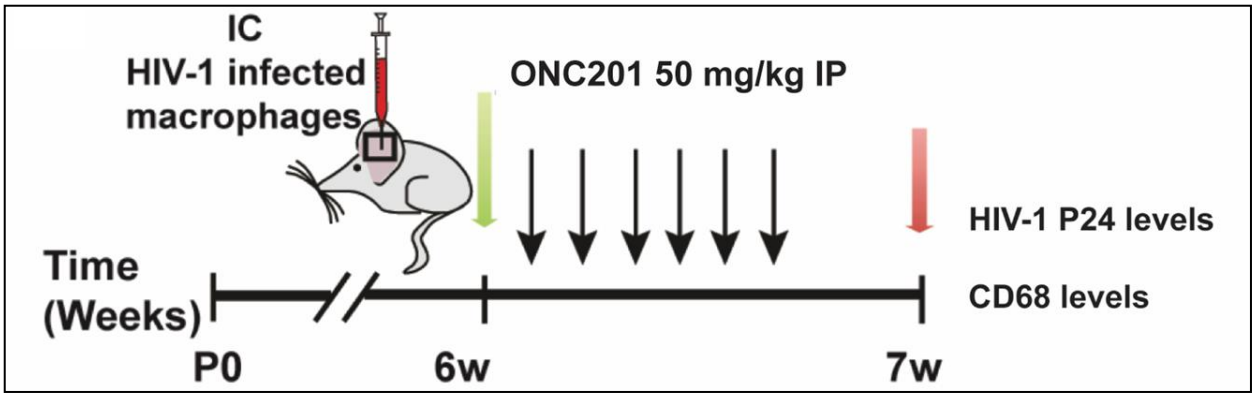


Figure 2.15 Timeline of experimental procedures and sample collection in the HIV-1-infected NSG mouse model. HIV-1-infected human macrophages were intracranially injected into the basal ganglia of NOD/scid-IL-2Rγ_c^{null} (NSG) mice. Mice were administrated daily with ONC201 (50 mg/kg) or solvent control DMSO through intraperitoneal injections for six days. At the seventh day, the mice were sacrificed for either immunostaining or Western blot to measure the levels of HIV-1 P24 and CD68.

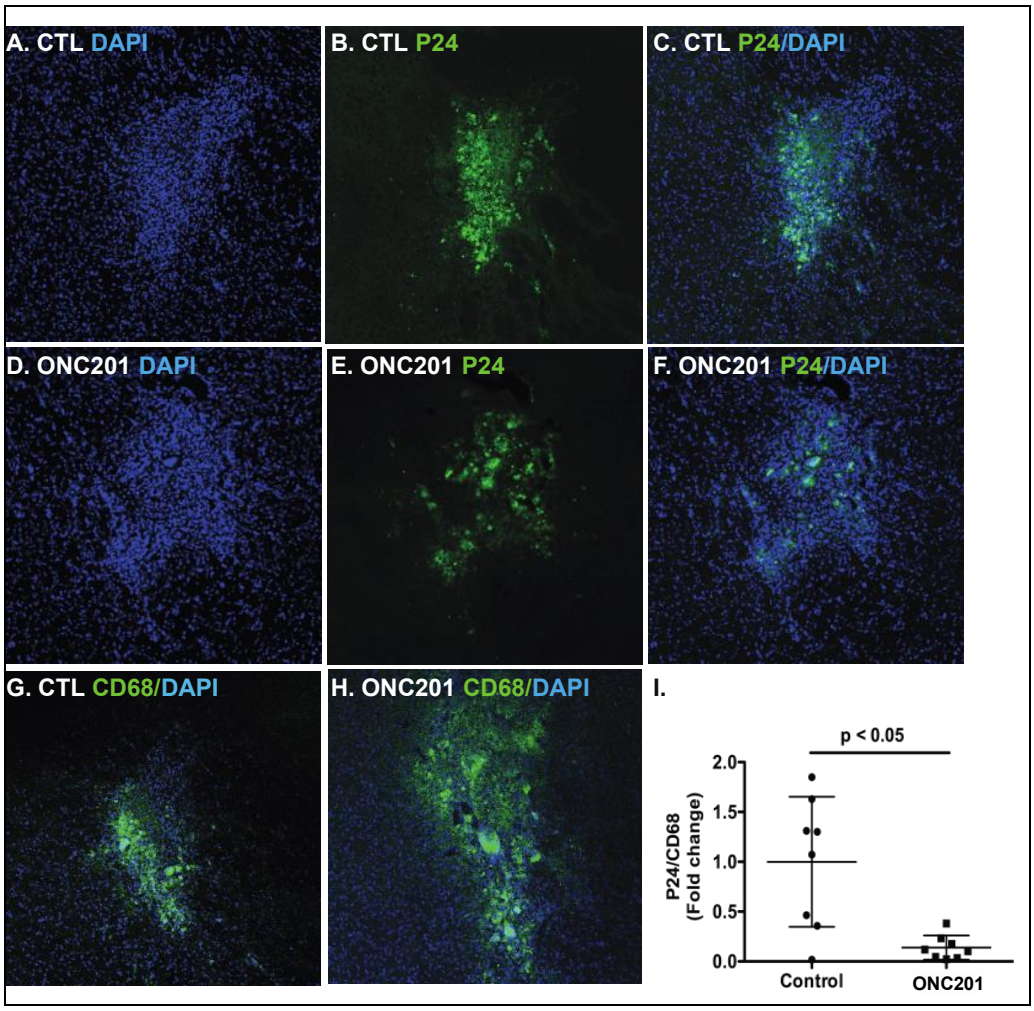


Figure 2.16 ONC201 reduces HIV-1 infection levels in mouse brains xenotransplanted with infected human macrophages (shown by immunostaining). Immunostaining of p24 and CD68 in the injection sites of mouse brains: co-immunostaining of p24 and DAPI (A-F); co-immunostaining of CD68 and DAPI (G-H); quantification of the p24/CD68 ratio per area (I). Data were evaluated statistically by an unpaired t-test, n = 6 per treatment group.

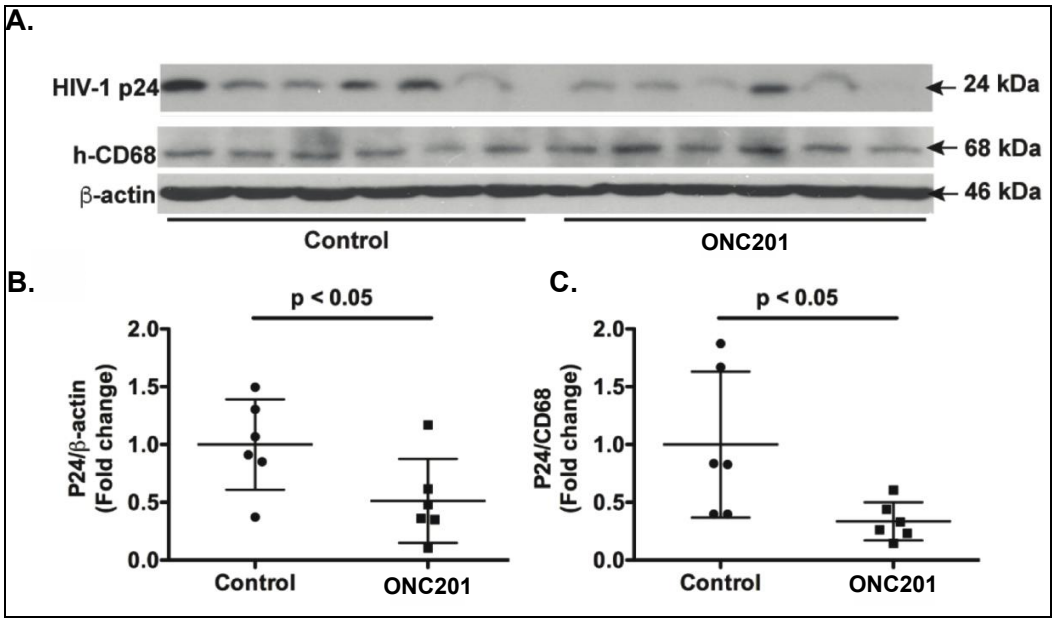


Figure 2.17 ONC201 reduces HIV-1 infection levels in mouse brains xenotransplanted with infected human macrophages (shown by Western blots). Brain tissues that contained the injection sites were homogenized for detection of HIV-1 p24 and human CD68 in Western blots: A) bands; B) quantification of the p24/ β-actin ratio; C) quantification of the p24/ β-actin ratio. Data were evaluated statistically by an unpaired t-test, n = 6 per treatment group.

CHAPTER 3

FOXO3a-TRAIL Pathway Mediates the Antiviral Effect of ONC201.

3.1 Abstract

ONC201 has been well-known in cancer research for its capacity to induce sustained TRAIL upregulation and apoptosis in tumor cells *in vitro* and *in vivo*. It inactivates both Akt and ERK to dephosphorylate FOXO3a so that FOXO3a can move to nucleus to induce the transcription of TRAIL in cancer cell lines. Macrophages express FOXO3a and TRAIL, which participate in the apoptosis and autophagy during HIV-1 infection. The activation of FOXO3a and induction of TRAIL by ONC201 in HIV-1 infected macrophages have been confirmed by Western blots and ELISA respectively. We hypothesize that the anti-HIV-1 effect of ONC201 is mediated by FOXO3a-TRAIL pathway. Blocking TRAIL with soluble TRAIL receptor or knockdown of FOXO3a with siRNA reversed ONC201-mediated HIV-1 suppression, indicating that ONC201 suppresses HIV-1 through FOXO3a and TRAIL. The viral inhibition after pretreatment of ONC201, as shown by RTase assay, is associated with apoptosis and cell death, as shown by capspase-3 immunoblots and cell viability assay on the same samples. Meantime, ONC201 induced autophagy in macrophages and blocking autophagy by PI3K inhibitor 3-MA reversed ONC201-mediated HIV-1 suppression, suggesting that autophagy may also play a role in the antiviral effect of ONC201. In addition, ONC201 did not change the expression of HIV-1 receptor and co-receptor, CD4 and CCR5, and HIV-suppressive chemokines, RANTES and MIP-1α, excluding that the antiviral activity of ONC201 is through blocking viral entry or increasing autocrine of HIV-suppressive chemokines.

3.2 Introduction

The persistence of HIV-1 reservoirs in the CNS is always an obstacle for ART. Failing to eradicate virus in those long-lived, low-turnover macrophages and microglia, ART itself cannot ensure a complete end of the infection. On the opposite, the persistently-produced viral proteins and activated immune cells may damage brain tissues, leading to HAND. One strategy, “prime, shock and kill”, has been proposed to manage the infected reservoir cells. According to this strategy, infected cells will be killed by selectively-induced apoptosis through pharmacological interventions. Apoptosis-inducing drugs are abundant in cancer biology since apoptosis is one of the targets for accurately killing cancer cells. It is reasonable that those antitumor agents may also induce apoptosis in HIV-1 infected cells.

ONC201, as a TRAIL inducer, is one of such antitumor agents that has been shown to preferentially kill cancer cells via apoptosis without affecting normal cells(79). It is orally active and BBB-permeable (78). After the first-in-human clinical trial, ONC201 has been confirmed well-tolerated with a recommended dose of 625 administered orally every three weeks for the ongoing phase II clinical trials (81, 127). Recently, we introduced this drug into HIV field because TRAIL also induces HIV-1 infected macrophages for apoptosis (66). We observed that ONC201 inhibited HIV-1 infection in macrophages, microglia and lymphocytes in vitro and in mouse brains xenotransplanted with infected human macrophages in vivo. However, the mechanism underlying this antiviral effect is still unknown.

Based on the current knowledge of the mechanism of action of ONC201 and the mechanism of HIV-1-induced apoptosis in macrophages, ONC201 may inhibit HIV infection through several mechanisms.

First, ONC201 may take advantage of FOXO3a-TRAIL pathway to increase the expression of TRAIL and kill infected cells through TRAIL-induced apoptosis. FOXO3a and TRAIL mediate HIV-1-induced apoptosis in infected macrophages. Knockdown of FOXO3a or blocking TRAIL protected macrophages from HIV-1 induced apoptosis (41, 66). In cancer cells, ONC201 inactivates Akt and ERK to activate FOXO3a and induce the transcription of TRAIL (78). TRAIL binds to TRAIL-R1 or -R2 on the membrane of infected cells to trigger the apoptosis.

Second, ONC201 may induce DR5 to promote apoptosis in infected cells. DR5 is another name for TRAIL-R2. Previous study showed that ONC201 induces ER stress, which activates Activating Transcription Factor 4 (ATF4)/ C/EBP homologous protein (CHOP) through integrated stress response (79). CHOP further targets DR5 to increase its expression (134). In HIV-1 infected macrophages, DR5 is upregulated at the late stage of productive viral infection, which is one of proposed mechanisms for TRAIL's preferential killing profile on infected macrophages (62, 66). ONC201 may further increase the expression of DR5 to sensitize infected macrophages for apoptosis. Nevertheless, it is possible that ONC201 induces apoptosis through other pathways downstream to ER stress.

Third, ONC201 may utilize autophagy to inhibit HIV-1 infection. Autophagy is one of the most ancient defense processes against invading pathogens.

Evidences have shown that autophagy restricts HIV-1 infection in CD4 T cells, monocytes and macrophages. In a clinical study in 2014, researchers found PBMCs from HIV-infected long term non-progressors and elite controllers had significantly higher levels of autophagy than normal progressors, suggesting autophagy plays an important role in the containment of HIV-1 in nonprogressor-infected patients (135). In an *in vitro* study on HIV-1 infected macrophages, two populations of autophagic cells were observed: one highly autophagic and the other weakly autophagic. Surprisingly, viruses could be detected in the weakly autophagic cells but not in the highly autophagic cells (136). The potential mechanism could be that autophagy selectively degrades the HIV-1 Tat by p62/SQSTM1 (137). Furthermore, autophagy-inducers such as Vitamin D3 and Tat–beclin 1 peptide have been tested on HIV-1 infected macrophage and shown to reduce the replication of HIV-1 in an autophagy-dependent manner (138, 139). Notably, FOXO3a and TRAIL are also associated with autophagy. FOXO3 stimulates the transcription of autophagy-related genes (*LC3, Atg12, Atg4, Beclin1, Gabarapl1, Ulk2, Bnip3,* and *Bnip3l*) to increase autophagy and lysosomal proteolysis(140-142). In addition, addition of recombinant TRAIL triggered autophagy in human macrophage (143). Based on these evidences, it is possible that ONC201 induces autophagy and inhibits HIV-1 replication in macrophage through FOXO3a-TRAIL pathway.

Fourth, ONC201 as a DRD2 antagonist may reduce the dopamine-potentiated HIV-1 infection. Human macrophages express mRNA for all dopamine receptor subtypes and protein for DRD1, DRD2, DRD3 and DRD4 in

the plasma membrane (144, 145). Psychostimulants elevate the extracellular dopamine levels in the CNS, which may facilitate HIV-1 infection in macrophage and microglia, since dopamine treatment significantly increased HIV-1 replication in primary human macrophages though a D2-like dopamine receptor (144). Another study showed that exposure to dopamine during infection increased the entry of R5 tropic HIV into macrophages, and dopamine receptors mediated this process (146).

ONC201 may inhibits HIV-1 infection through one or a combination of the potential mechanisms listed above. Since the activation of FOXO3a and induction of TRAIL expression by ONC201 in macrophages has been confirmed in last chapter and we introduced ONC201 into HIV field with the expectation that it should also induce apoptosis as it works in cancer cells, we first tested the first potential mechanism. Knockdown of FOXO3a with siRNAs and blocking TRAIL with soluble TRAIL receptors completely reversed the antiviral effect of ONC201. We also observed a dose-dependently increased apoptosis in HIV-1 infected macrophages after pretreatment of ONC201. In addition, ONC201 induced autophagy in macrophages, and blocking autophagy via 3-MA reversed the antiviral effect of ONC201, suggesting the autophagy might also be involved as a mechanism.

3.3 Materials and Methods

Ethics statement

Primary monocyte-derived macrophages (MDM) were used in full compliance with the University of Nebraska Medical Center and National Institutes of Health ethical guidelines, with the Institutional Review Board (IRB) #: 162-93-FB.

Isolation and culture of primary MDM

Human monocytes were isolated by counter-current centrifugal elutriation from peripheral blood mononuclear cells obtained through leukopheresis from healthy donors (96).

Primary monocytes were cultured as adherent monolayers at a density of 1.1 x 10^6 cells/well in 24-well plates or 0.25 x 10^6 cells/well in 96-well plates and differentiated for 7 days in differentiation medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co., St. Louis, MO) supplemented with 10% human serum, 50 µg/ml gentamicin (GIBCO Invitrogen Corp.), 10 µg/ml ciprofloxacin (Thermo Fisher Scientific, Waltham, MA), and 1000 U/ml recombinant human macrophage-colony stimulating factor (M-CSF). After 7 days in differentiation, the cells became MDM and M-CSF was removed since the culture can produce M-CSF through autocrine secretion.

HIV-1 Infection of MDM and ONC201 treatment

MDM were infected with HIV-1_{ADA}, a macrophage-tropic strain of HIV-1, at a multiplicity of infection (MOI) of 0.1 virus/target cell (96). Briefly, viral stocks were diluted in MDM medium (same with the differentiation medium but without

M-CSF) for overnight incubation with MDM. On the second day, medium was removed and substituted with MDM medium and either DMSO (Sigma), ONC201, or ONC201 isomer (MedKoo Bioscience). For selected experiments, a recombinant human TRAIL R2/Fc Chimera Protein (R&D Systems, Minneapolis, MN) or 3-MA (Sigma Chemical Co., St. Louis, MO) was added to the cultures along with ONC201. The cultures were re-treated with the same drugs in fresh MDM medium every 2 days. At 4 days post-infection, the cultures were treated with the same drugs in 0.5 ml/well fresh serum-free Neurobasal™ medium (Thermo Fisher Scientific) for 24 hours before all samples were collected.

Assessment of cell viability

MDM viability was determined by a colorimetric CellTiter 96® AQueous One Solution Assay (Promega, Madison, WI) based on the manufacture's instruction. Assays were performed by adding 20 µl of the CellTiter 96® AQueous One Solution Reagent, which contained a tetrazolium compound[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES), directly to each of 100 µl MDM medium in 96-well plates. After 2-hour incubation at 37°C in a humidified 5% CO_2 atmosphere, the absorbance of each well was recorded at 490 nm using a 96-well plate reader (BioTek, Winooski, VT).

HIV-1 reverse transcriptase (RTase) activity assay

HIV-1 replication levels were determined by HIV-1 RTase activity assay as described previously (96). RT activity was determined in triplicate samples of

cell culture fluids. For this assay, 10 µl of supernatant was incubated in a reaction mixture of 0.05% Nonidet P-40, 10 µg of poly(A)/ml, 0.25 µg of oligo(dT)/ml, 5 mM DTT, 150 mM KCl, 15 mM MgCl2, and [3H]TTP in Tris-HCl buffer (pH 7.9) for 24-h at 37 °C. Radiolabeled nucleotides were precipitated with ice-cold 10% trichloroacetic acid on UniFilter-96 GF/C (FilterMate Harveser, Waltham, MA) in an automatic cell harvester (FilterMate Harveser, Waltham, MA) and washed with 95% ethanol. Radioactivity was determined by liquid scintillation spectroscopy.

Western blots

MDM or tissues of injection sites were homogenized with a PowerGen Homogenizer (Thermo Fisher Scientific) and then lysed by M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA). Protein concentrations were determined by Bradford protein assay (Bio-Rad, Hercules, CA). Cell lysates were subjected to SDS-PAGE to separate proteins and electrophoretically transferred to Polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Membranes were incubated overnight at 4 °C with polyclonal antibodies for Akt (Cat#, 9272S, Cell Signaling Technology, Danvers, MA), phosphorylated Akt (Cat#, 9271S, Cell Signaling Technology, Danvers, MA), ERK(Cat#, 9102, Cell Signaling Technology, Danvers, MA), phosphorylated ERK (Cat#, 9101S, Cell Signaling Technology, Danvers, MA), caspase-3 (Cat#, 9662S, Cell Signaling Technology, Danvers, MA), cleaved caspase-3 (Cat#, 9664T, Cell Signaling Technology, Danvers, MA), LC3 A/B (Cat#, 4108, Cell Signaling Technology, Danvers, MA), HIV-1 p24 (Ref#, M0857,

DAKO Corp, Carpinteria, CA), human CD68 (Ref#, M0876, DAKO Corp, Carpinteria, CA), and β-actin (Sigma-Aldrich, St. Louis, MO), followed by horseradish peroxidase-linked secondary anti-rabbit or anti-mouse secondary antibodies (Cell signaling Technology, Danvers, MA). Antigen-antibody complexes were visualized by Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). For quantification of the data, films were scanned with a CanonScan 9950 F scanner (Canon Inc., Tokyo, Japan) and images were analyzed using the public domain NIH Image J program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).

RNA and DNA extraction and real time RT-PCR analysis

Total RNA was isolated with TRIzol Reagent (Thermo Fisher Scientific) and RNeasy Mini Kit according to the manufacturer's protocol (Qiagen Inc., Valencia, CA). Following RNA extraction, DNA was isolated from the remaining organic phase using the DNeasy Blood and Animal Spin-Column kit according to manufacturer's instructions (Qiagen). Assays-on-demand primers for TRAIL (ID#, Hs00921974_m1), FOXO3a (ID#, Hs00818121_m1), CD4 (ID#, Hs01058407_m1), CCR5 (ID#, Hs00152917_m1), 18S rRNA (Ref#, 4352930#) and GAPDH (ID#, Hs01922876_u1) were purchased from Thermo Fisher Scientific. For quantification of HIV-1 gag, primers and probe were purchased from Thermo Fisher Scientific with the following sequences: forward, 5' ACA TCA AGC CAT GCA AAT-3'; reverse, 5'-ATC TGG CCT GGT GCA ATA GG-3'; probe, 5'-FAM-CAT CAA TGA GGA AGC TGC AGA ATG GGA TAG A-TAMRA-

3’. Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed in a volume of 10 µl using the one-step quantitative TaqMan Real-time RT-PCR system (Applied Biosystems Inc.). For analysis of HIV-1 gag DNA, real-time PCR was carried out directly on the DNA samples using the TaqMan Universal Master Mix and a StepOne Plus Real-Time PCR System (Thermo Fisher Scientific). Relative FOXO3a, TRAIL, CD4, CCR5 and HIV-1 gag levels were determined and standardized with GAPDH or 18S rRNA internal control using comparative ΔΔCT method. All primers used in the study were tested for amplification efficiencies and the results were similar.

RANTES and MIP-1α ELISAs

Supernatants collected at 5 days post-infection were analyzed for RANTES and MIP-1α levels by in-house ELISAs (paired antibodies, R&D Systems, Minneapolis, MN) as previously described (101).

siRNA transfection

Predesigned siRNA duplexes targeting FOXO3a (ID: 144672) and a negative control siRNA (catalog no. AM4611) were purchased from Thermo Fisher Scientific. At 72 h after HIV-1 infection, cells were transfected with 100 nM siRNA duplexes for 4 h in DMEM culture medium without serum, in the presence of siIMPORTER reagent (EMD Millipore Corporation, Billerica, MA) according to the manufacturer’s instructions. At 72 h post-transfection, cells were harvested and total RNA was extracted for the detection of FOXO3a and TRAIL levels by real-time RT-PCR.

Statistical analysis

Data are expressed as means ± SD unless otherwise specified. Statistical analysis was performed by using one-way ANOVA, followed by the Tukey's-post-test for paired observations, or two-way ANOVA when two independent variables are considered. The two-tailed Student's t test was used to compare two groups. Significance was determined by a p value < 0.05. All experiments were performed with cells from at least three donors to account for any donor-specific differences. Assays were performed at least three times in triplicate or quadruplicate within each assay.

3.4 Results

ONC201 inactivates Akt and ERK in HIV-1 infected macrophages.

After showing the antiviral effect of ONC201 *in vitro* and *in vivo*, we investigated the underlying mechanism of viral inhibition by focusing on the FOXO3a-TRAIL pathway. In cancer cells, ONC201 inactivates two kinases, Akt and ERK, to activate FOXO3a-TRAIL pathway (78). However, whether ONC201 targets the same kinases in HIV-1 infected macrophage is still unknown. To answer this question, MDM were infected with HIV-1_{ADA} for 24 hours and then incubated with DMSO, ONC201 or its isomer for 5 days, which are the same settings as the previous antiviral tests. First, we assessed the status of Akt in HIV-1 infected MDM by measuring the levels of phosphorylated Akt, total Akt, and β-actin though Western blots. After quantification, we found that the phosphorylation of Akt, determined by the ratio of phosphorylated Akt over total Akt, was decreased after ONC201 treatment in a dose-dependent manner compared with treatment of isomer and DMSO controls (Fig. 3.1). Then we assessed the status of ERK in the same cells and found a similar pattern that phosphorylation of ERK decreased as ONC201 concentration increased (Fig. 3.2). These results indicate ONC201 inactivate both Akt and ERK in HIV-1 infected macrophages.

The antiviral effect of ONC201 in HIV-1 infected macrophages is dependent on FOXO3a and TRAIL expressions.

To determine if the antiviral effect of ONC201 is mediated by FOXO3a, knockdown of FOXO3a was performed by siRNA targeting FOXO3a in HIV-1-

infected MDM. A non-targeting control siRNA was used as the control. We assayed FOXO3a and TRAIL expression at 72 hours after siRNA transfection and found that FOXO3a-targeting siRNA reduced the levels of FOXO3a and TRAIL mRNA in HIV-1 infected macrophages (Fig. 3.3A and B), confirming that FOXO3a has indeed been knocked down. In the control groups, ONC201 significantly decreased the HIV-1 replication levels, as measured by HIV-1 RTase activity assay, compared with those in the isomer treatment (Fig. 3.4). However, FOXO3a knockdown reversed HIV-1 replication to the same level as those in the isomer treated group, suggesting that FOXO3a knockdown blocks the antiviral effect of ONC201 (Fig. 3.4).

To further determine if TRAIL also mediates the antiviral effect of ONC201, we used 50 ng/ml or 100 ng/ml of the soluble TRAIL receptors to act as decoys blocking activities of TRAIL on the surface of macrophages. In HIV-1 RTase activity assay, we found that treatment of soluble TRAIL receptors reversed the HIV-1 replication in ONC201-treated cells to the same level as those in the isomer-treated group (Fig. 3.5A). Consistent with the RTase activity data, treatment with soluble TRAIL receptors also significantly increased intracellular HIV-1 p24 levels (Fig. 3.5B), suggesting that the antiviral effect of ONC201 in HIV-1 infected macrophages is dependent on TRAIL expressions.

The antiviral effect of ONC201 in HIV-1 infected macrophages is associated with apoptosis.

In cancer cells, activation of FOXO3a-TRAIL pathway by ONC201 leads to cell apoptosis and death, while in HIV-1 infected MDM, treatment of human

recombinant TRAIL induces cell apoptosis and death. Thus, we hypothesized that ONC201 treatment should also induce cell apoptosis and death in HIV-1 infected macrophages. However, when MDM were first infected with HIV-1 and then treated with ONC201 for 5 days, ONC201 treatment significantly increased the cell viability (Fig. 3.6), possibly due to the significant decrease of HIV-1 replication at higher doses (3 - 30 μM) of ONC201 treatment. In other words, ONC201 could have killed infected cells to protect the uninfected cells from infection.

For better understanding the cytotoxic effect of ONC201 in HIV-1 infected MDM, we pretreated MDM with ONC201 or its isomer for 5 days before 24-h HIV-1 infection. ONC201 and isomer treatments continued during and after the infection. At 3-day post infection, cell viability was determined by the MTS assays. A significant and dose-dependent decrease of cell viability was observed in ONC201-treated groups at the concentrations from 3 to 30 μM compared with either isomer- or DMSO- treated groups (Fig. 3.7), showing that pretreatment of ONC201 reduced the cell viability of HIV-1 infected MDM. Enhanced level of caspase-3 cleavage is one of the indicators for cell apoptosis. Western blots on caspase-3 showed that the cleavage of caspase-3, determined by the ratio of cleaved caspase-3 over pro-caspase-3, was significantly increased in a dose-dependent manner with ONC201 treatment (Fig. 3.8), which is consistent with the results in cell viability assay. Meanwhile, analysis of HIV-1 replication by RTase activity assay showed that the ONC201 pretreatment significantly lowered viral levels in the cell culture with a similar pattern to

caspase-3 cleavage (Fig. 3.9). Together, we found that the antiviral effect of ONC201 in HIV-1 infected macrophages is associated with apoptosis.

The antiviral effect of ONC201 in HIV-1 infected macrophages is associated with autophagy.

In addition to apoptosis, autophagy as another target of FOXO3a-TRAIL pathway also plays a role in restricting HIV-1 infection (135, 137, 142, 143). To test whether ONC201 treatment induces autophagy in macrophages, we treated both uninfected and HIV-1 infected MDM with various doses of ONC201 or its isomer for 5 days. The levels of LC3 I, LC3 II and β-actin were measured by Western blots to assess the status of autophagy. We found that in both uninfected and HIV-1 infected MDM, the ratio of LC3 II to LC3 I as an indicator for autophagy increased in a dose-dependent manner after ONC201 treatment while the isomer treatment did not increase this ratio (Fig. 3.10 and 3.11). This result indicates ONC201 induces autophagy in macrophage.

To further determine the role of autophagy in the antiviral effect of ONC201, PI3K inhibitor 3-MA was used to block autophagy. HIV-1 infected MDM were treated with either 3-MA, ONC201, or a combination of 3-MA and ONC201 for 5 days. Then the cell lysates were subjected to Western blots for the measurement of p24, LC3 I, LC 3 II and β-actin (Fig. 3.12A). We found that ONC201 treatment increased the LC3II/I ratio while cotreatment with 3-MA reduced the ratio to the baseline, confirming that 3-MA inhibits the autophagy induced by ONC201 (Fig. 3.12B). Meanwhile, ONC201 treatment decreased the

P24 level whereas cotreatment with 3-MA removed this decrease (Fig. 3.12C). Therefore, blocking autophagy by 3-MA reversed the antiviral effect of ONC201.

RANTES, MIP-1α, CD4 and CCR5 are not involved in the antiviral effect of ONC201.

Because downstream targets of ONC201 include TRAIL, which belongs to the TNF superfamily of cytokines, one plausible mechanism for HIV-1 inhibition by ONC201 is through ONC201-induction of cytokines that interfere with the HIV-1 lifecycle. RANTES (CCL5) and MIP-1α (CCL3) are both chemokines that bind to CCR5, which function as a primary coreceptors for HIV-1 entry in MDM cell (147, 148). We tested the RANTES and MIP-1α levels after ONC201 treatment and found that ONC201 did not increase the levels of these chemokines in the supernatants (Fig. 3.13), suggesting that ONC201 is unlikely to inhibit HIV-1 replication through inducing HIV-1 suppressive chemokines in MDM.

To exclude other possible mechanisms that prohibit virus from entering the macrophage, we also used quantitative real-time PCR to measure the levels of HIV-1 coreceptors, CD4 and CCR5, in HIV-1 infected MDM with ONC201 treatment. The expression of both coreceptors did not change after ONC201 treatment (Fig. 3.14). Finally, ONC201 was not able to suppress HIV-1 when directly incubated with HIV-1 virions (Fig. 3.15), suggesting that ONC201 may not directly inactivate virus to protect cells from infection.

3.5 Discussion

After demonstrating that ONC201 inhibits HIV-1 infection of reservoir cells *in vitro* and *in vivo*, we further identified that the antiviral effect of ONC201 is mediated by the FOXO3a/TRAIL pathway, as ONC201 activated FOXO3a and induced TRAIL expression in HIV-1-infected macrophages, and knockdown of FOXO3a or blocking TRAIL abrogated the antiviral activity of ONC201. The antiviral effect of ONC201 is also associated with apoptosis and autophagy in HIV-1 infected MDM, while direct inhibition or blocking viral entry is not the mechanism underlying this effect.

Our studies identify that the antiviral effect of ONC201 in HIV-1 infected macrophage is dependent on FOXO3a and TRAIL expressions. However, the exact molecular interactions between ONC201 and FOXO3a remain to be determined. Besides being a FOXO3a activator, ONC201 has also been proposed as a selective dopamine receptor 2 (DRD2) antagonist (81, 149, 150). DRD2 as one of the D2-class dopamine receptors mediates the dephosphorylation of kinases Akt and ERK (151). However, previous studies have shown ONC201 inactivates both Akt-1 and ERK1/2 to activate FOXO3a and induce the transcription of TRAIL in cancer cell lines (78). We confirmed the dual inactivation of Akt and ERK by ONC201 in HIV-1 infected macrophages. The fact that ONC201 inactivates Akt and ERK is contrary to the expected phosphorylation of these kinases by DRD2 antagonist, suggesting that ONC201 may have multiple targets other than DRD2 and the overall effect is the inactivation of Akt and ERK. We observed that the phosphorylation of FOXO3a

is decreased after ONC201 treatment in both uninfected and HIV-1 infected MDM, indicating ONC201 targets the same signaling pathway in cancer cells. Notably, it takes at least 3 days for FOXO3a to be fully activated by ONC201 (78, 79), which appears slow for direct kinase regulators, suggesting the activation of FOXO3a is a late event following multi-level regulations triggered by ONC201. Meanwhile, ONC201 is also known to “stresses tumor to death” by inducing endoplasmic reticulum (ER) stress-related or integrated stress response (ISR)-related genes, such as ATF4, CHOP, Growth arrest and DNA damage-inducible protein GADD34, and Tribbles homolog 3 (TRIB3) (152). Whether these genes work in tandem with FOXO3a/TRAIL pathway or not warrants future investigations.

Having demonstrated that the antiviral effect of ONC201 relies on the activation of FOXO3a/TRAIL pathway, we sought to elucidate the exact mechanism by which activated TRAIL signaling reduces HIV-1 replication. One potential mechanism is that TRAIL-mediated apoptosis clears the HIV-1 infected reservoir cells. This is supported by previous reports demonstrating that leucine-zipper TRAIL, agonistic anti-TRAIL receptor antibodies, and activated NK cell-derived TRAIL induce apoptosis in MDM and peripheral blood lymphocytes and consequently reduce viral burden in HIV-infected individuals (92, 153, 154). In the HIV-1-infected macrophage culture with post-infection treatment of ONC201, we were not able to pinpoint whether ONC201-mediated HIV suppression is dependent upon the induction of apoptosis. The progress of HIV-1 infection in the culture coincides with not only viral replication but also cell death and

apoptosis. Therefore, if ONC201 inhibits HIV-1 replication by inducing apoptosis in HIV-1-infected MDM, the viral inhibition could also work to negate the effect of HIV-1-induced cell death, resulting in the absence of viability loss in the infected MDM with post-infection treatment of ONC201 as seen in Fig. 3.6. To untangle the possible mixed effects of ONC201 in HIV-1 infected MDM culture, we changed the scheme of ONC201 treatment from post-infection treatment to pretreatment. The short-term HIV-1 infection in this scheme minimizes the interference of HIV-1-induced cell death with ONC201-induced cell death. Pretreating MDM with ONC201 before HIV-1 infection lowered the cell viability of HIV-1 infected MDM culture. An increased cleavage of caspase-3 and a reduced level of HIV-1 infection parallel with this lowered cell viability, showing the cell death induced by ONC201 is associated with cell apoptosis and the latter might be responsible for the antiviral effect of ONC201. ONC201 did not change the cell viability of uninfected MDM but reduce the cell viability of HIV-1 infected MDM after pretreatment, possibly due to the high expression of DR5 on the membrane of HIV-1 infected MDM (62). Nevertheless, an apoptosis-independent mechanism could also seemingly explain the viability of HIV-1-infected MDM upon ONC201 treatment. TRAIL signaling may change the status of immune cells by interfering with the activation of NF-κB, PKB/Akt and MAPKs, which are relevant factors for HIV-1 replication (155). Thus, more specific experiments need to be developed to identify whether apoptosis is the main mechanism for ONC201 and TRAIL effect in HIV-1-infected macrophages.

Furthermore, TRAIL is also known to trigger autophagy in human macrophage (143), which selectively degrades the HIV-1 Tat by p62/SQSTM1 (137) and causes preferential cell death of HIV-1-infected macrophages (156). In our study, ONC201 treatment elevated the LC3 II/I ratio in both uninfected and HIV-1 infected MDM and 3-MA treatment reversed the antiviral effect of ONC201, suggesting autophagy might be involved in the antiviral effect of ONC201. Nevertheless, these results need to be interpreted carefully considering that autophagy is a complicated, multi-step process. In the late phase of autophagy, LC3 II is degraded by lysosomal hydrolases considerably and therefore can only serve as a marker for autophagosome in the early phage of autophagy (157). PI3K inhibitor,3-MA, is usually considered to block autophagy in the early phase (158). To further confirm the induction of autophagy by ONC201 and pinpoint its specific role in the antiviral effect of ONC201, more markers and blockers targeting the different phases of autophagy will be needed.

Aside from FOXO3a-TRAIL pathway, other possible mechanisms underlying the antiviral effect of ONC201 were also investigated. ONC201 decreases HIV-1 RTase activities in MDM through FOXO3a and TRAIL but likely not through a direct inhibition of HIV-1 RTase activities since ONC201 is not able to suppress HIV-1 when directly incubated with HIV-1 virions (Fig. 3.15). No change in the expression levels of RANTES, MIP-1α, CD4 and CCR5 were observed after ONC201 treatment, suggesting that ONC201 did not change the

status of HIV-1 receptors on the surface of macrophages to prevent the entry of viruses.

3.6 Figures

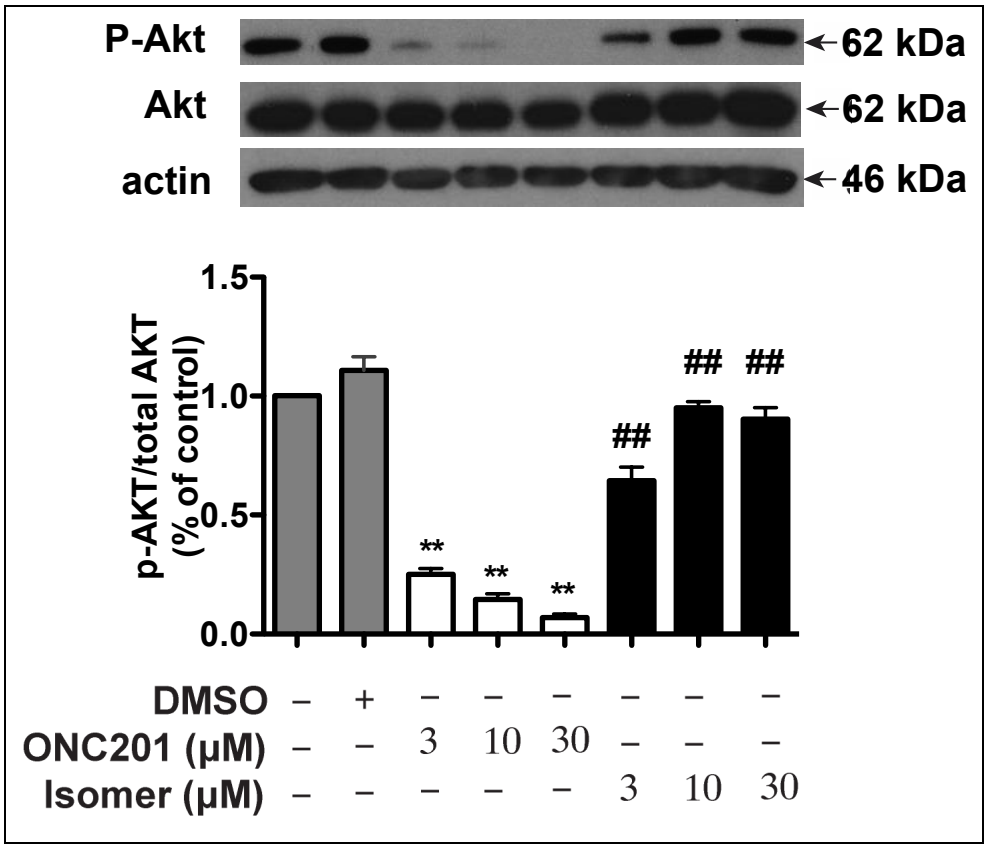


Figure 3.1 ONC201 inactivates Akt in HIV-1 infected macrophages. Human MDM were plated in 24-well plates in triplicate and then infected with HIV-1_{ADA} for 1 day. The cells were incubated with doses of ONC201 or ONC201 isomer ranging from 3 to 30 μM for 5 days. Cells were then harvested and cell lysates were subjected to SDS-PAGE and immunoblotting for the phospho-AKT or total AKT. Actin was used as the loading control. Densitometric quantifications of phospho-AKT in MDM were presented as a ratio to total AKT and normalized as fold changes to the no treatment control. Values represent SEM of biological replicates. ANOVA analysis: ** denotes $p < 0.01$, compared to DMSO group; ## denotes $p < 0.01$, compared to the ONC201 treatment group with the same concentration.

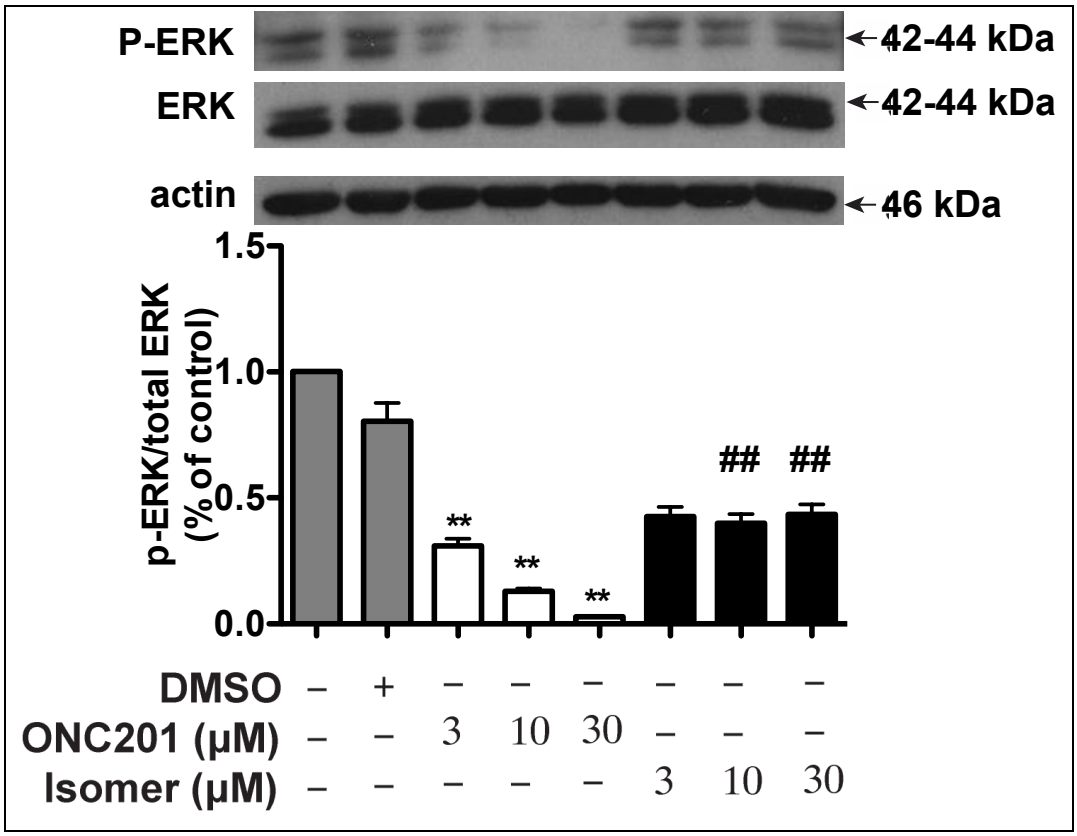
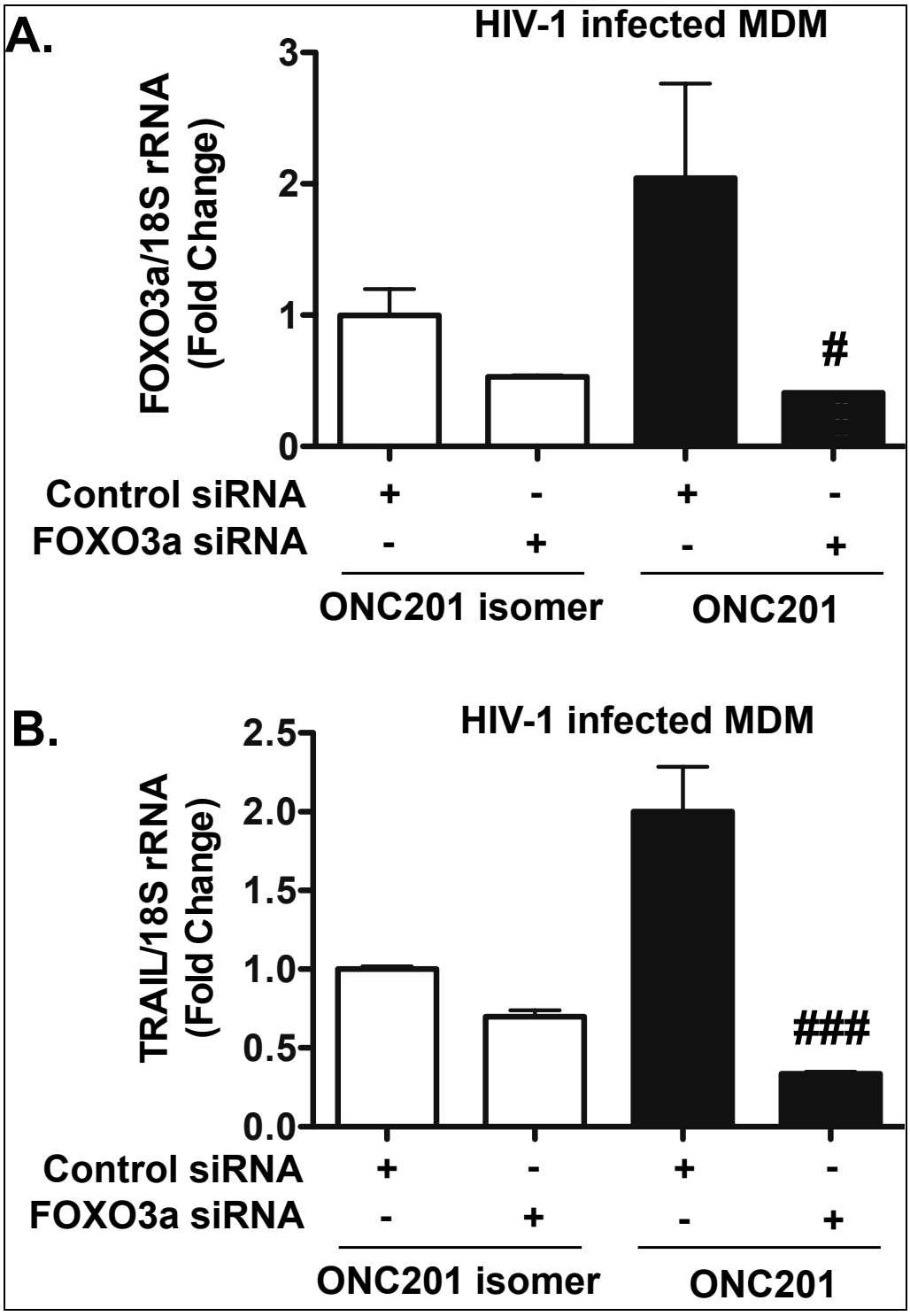


Figure 3.2 ONC201 inactivates ERK in HIV-1 infected macrophages. Human MDM were plated in 24-well plates in triplicate and then infected with HIV-1_{ADA} for 1 day. The cells were incubated with doses of ONC201 or ONC201 isomer ranging from 3 to 30 μM for 5 days. Cells were then harvested and cell lysates were subjected to SDS-PAGE and immunoblotting for the phospho-ERK or total ERK. Actin was used as the loading control. Densitometric quantifications of phospho-ERK in MDM were presented as a ratio to total ERK and normalized as fold changes to the no treatment control. Values represent SEM of biological replicates. ANOVA analysis: ** denotes $p < 0.01$, compared to DMSO group; ## denotes $p < 0.01$, compared to ONC201 treatment group with the same concentration.

Figure 3.3 Knockdown of FOXO3a reduces the expression of FOXO3a and TRAIL. Human MDM were plated in 24-well plates and then infected with HIV-1_{ADA} for 24 hours before incubation with ONC201 or ONC201 isomer at 30 μM for 5 days. At 2 days post HIV-1 infection, MDM were transfected with either non-targeting control siRNA or FOXO3a siRNA. At the experimental end point, FOXO3a and TRAIL mRNA were detected in total cellular RNA through real time RT-PCR. Data were normalized to 18S rRNA and presented as fold change compared to control siRNA group with isomer treatment. Values represent SEM of biological replicates. ANOVA analysis: ## denotes $p < 0.01$, compared with the control siRNA group with ONC201 treatment; ### denotes $p < 0.001$, compared with the control siRNA group with ONC201 treatment.


A.
HIV-1 infected MDM
FOXO3a/18S rRNA (Fold Change)
3
2
1
0
#
Control siRNA + - + -
FOXO3a siRNA - + - +
ONC201 isomer
ONC201
B.
HIV-1 infected MDM
TRAIL/18S rRNA (Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
###
Control siRNA + - + -
FOXO3a siRNA - + - +
ONC201 isomer
ONC201

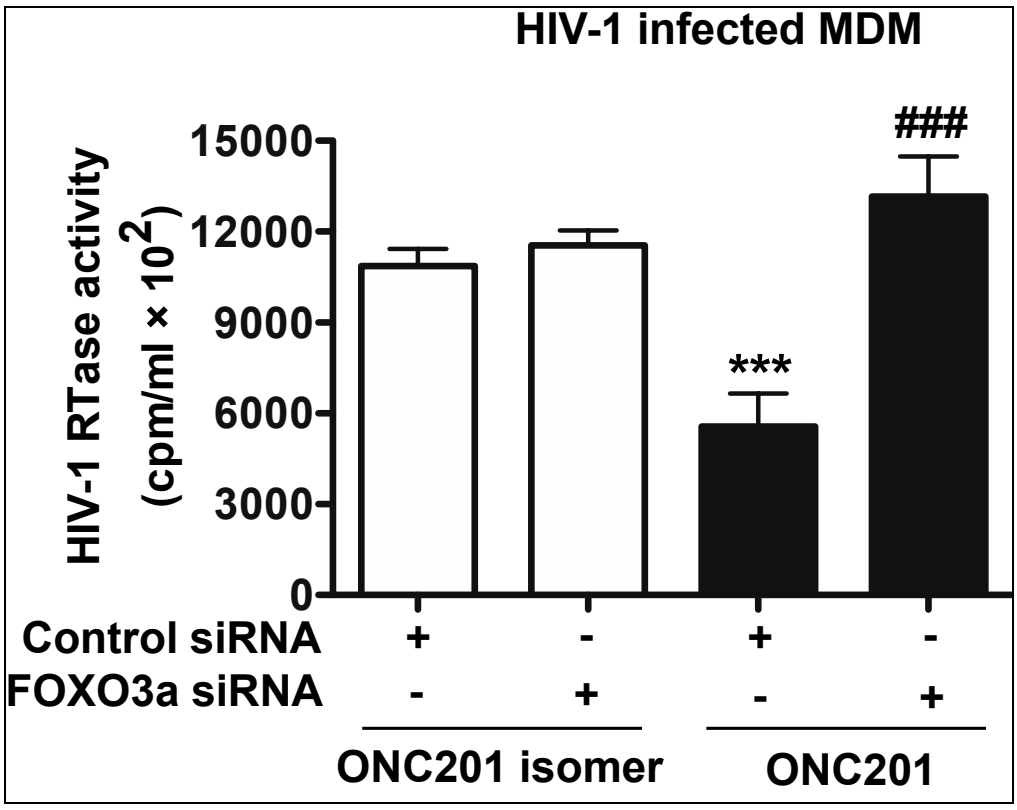
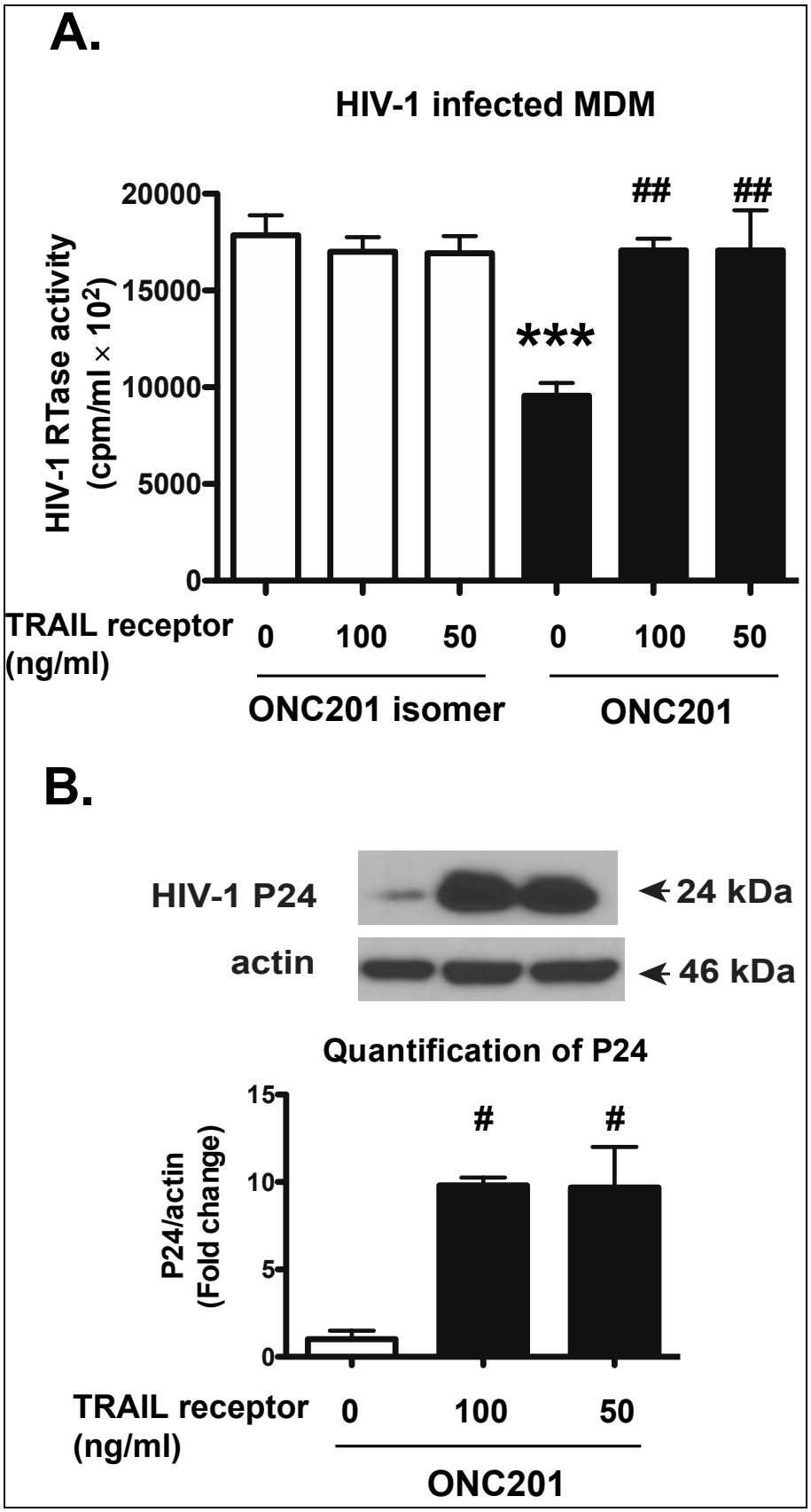


Figure 3.4 Antiviral effect of ONC201 is dependent upon FOXO3a in HIV-1-infected macrophages. Human MDM were plated in 24-well plates and then infected with HIV-1_{ADA} for 24 hours before incubation with ONC201 or ONC201 isomer at 30 μM for 5 days. At 2 days post HIV-1 infection, MDM were transfected with either non-targeting control siRNA or FOXO3a siRNA. HIV-1 infection levels were determined by the RTase activity assay. Values represent SEM of biological replicates. ANOVA analysis: *** denotes $p < 0.001$, compared with the control siRNA group with isomer treatment; ### denotes $p < 0.001$, compared with the control siRNA group with ONC201 treatment.

Figure 3.5 Antiviral effect of ONC201 in HIV-1 infected macrophage is dependent on TRAIL expression. HIV-1-infected MDM were incubated with ONC201 or ONC201 isomer at 30 μM, along with 100ng/ml, 50ng/ml soluble TRAIL receptor, or 100ng/ml soluble TNF receptor as control for 5 days. A) HIV-1 infection levels were determined by RTase activity assay. B) Cell lysates were subjected to SDS-PAGE and immunoblotting for HIV-1 p24. Actin was used as the loading control. Densitometric quantifications of p24 in MDM were presented as a ratio to actin and normalized as fold changes to the control group. ANOVA analysis: *** denotes $p < 0.001$, compared with the control group with isomer treatment; ### denotes $p < 0.001$, compared the control group with ONC201 treatment.


A.
HIV-1 infected MDM
HIV-1 RTase activity (cpm/ml × 10²)
20000
15000
10000
5000
0
***
##
##
TRAIL receptor (ng/ml)
0
100
50
0
100
50
ONC201 isomer
ONC201
B.
HIV-1 P24
24 kDa
actin
46 kDa
Quantification of P24
P24/actin (Fold change)
15
10
5
0
#
#
TRAIL receptor (ng/ml)
0
100
50
ONC201

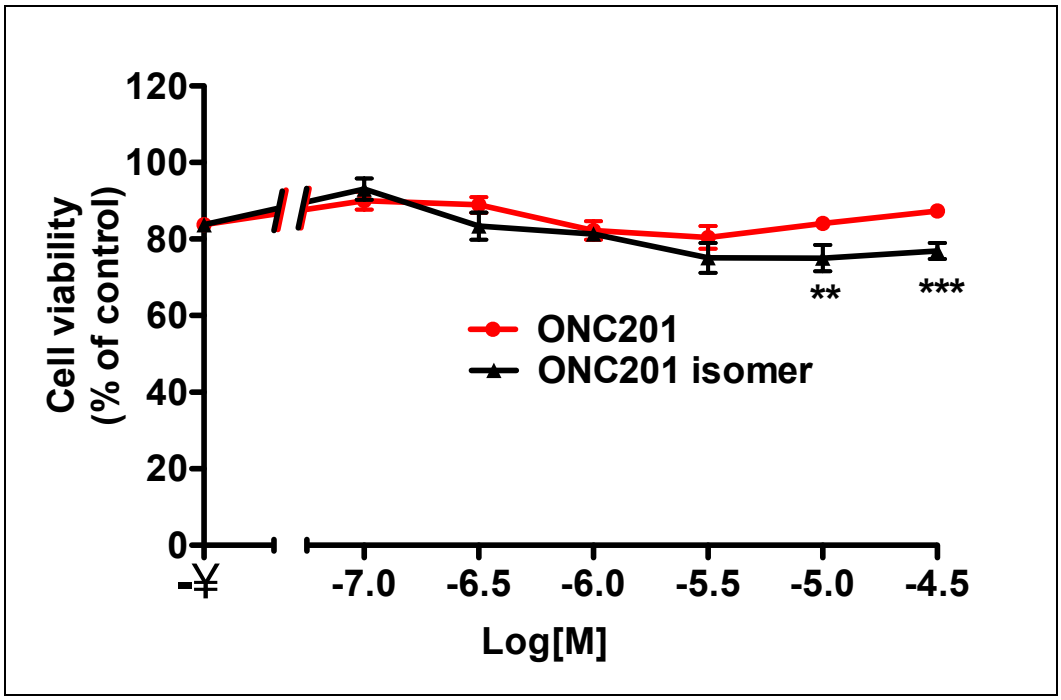


Figure 3.6 Post-infection treatment of ONC201 does not reduce the cell viability of HIV-1 infected macrophage culture. Human MDM were plated in 96-well plates and infected with HIV-1 for 24 hours before incubation with doses of ONC201 or its isomer ranging from 0.03 to 30 μM. At 5-day post ONC201 and isomer treatments, cell viability was determined by the MTS assays. Results shown are from representative experiments performed with three different donors. Data were analyzed by two-way ANOVA: * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$.

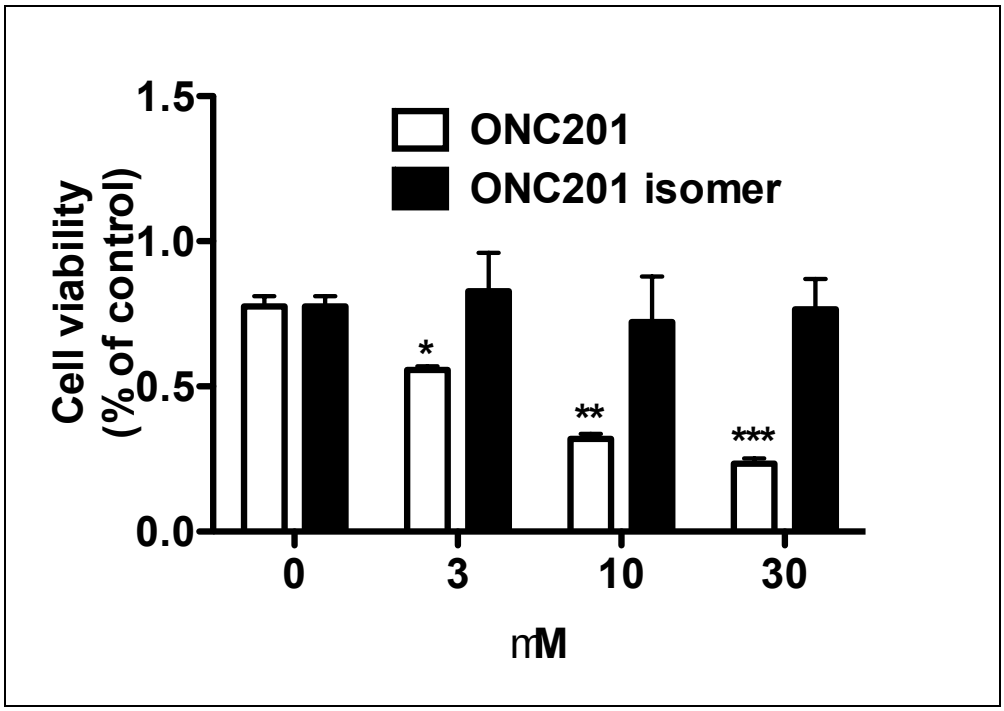
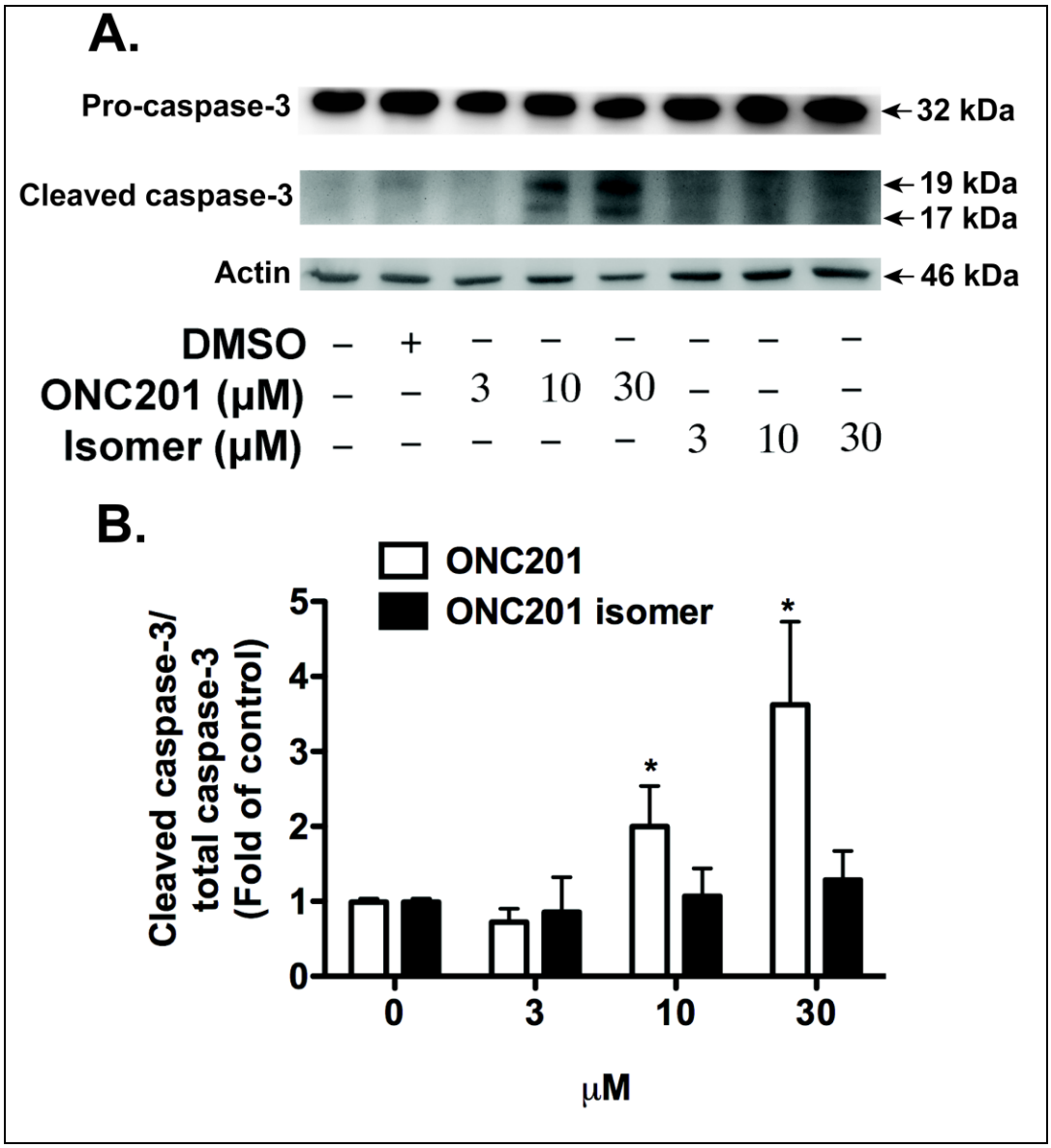


Figure 3.7 Pretreatment of ONC201 reduces the cell viability of HIV-1 infected macrophage culture. Human MDM were plated in 96-well plates and incubated with doses of ONC201 or its isomer ranging from 3 to 30 μM for 5 days before infection with HIV-1 for 24 hours. ONC201 and isomer treatments continued during and after the infection. At 3-day post infection, cell viability was determined by the MTS assays. Results shown are from representative experiments performed with three different donors. Data were analyzed by two-way ANOVA: * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$.

Figure 3.8 Pretreatment of ONC201 induces cleavage of caspase-3 in HIV-1 infected macrophage. Human MDM were plated in 96-well plates and incubated with doses of ONC201 or its isomer ranging from 3 to 30 μM for 5 days before infection with HIV-1 for 24 hours. ONC201 and isomer treatments continued during and after the infection. A) At 3-day post infection, cell lysates were collected and subsequently subjected to SDS-PAGE and immunoblotting for the detection of cleaved caspase-3 and pro-caspase-3. Actin was used as the loading control. B) Densitometric quantifications of cleaved caspase-3 in MDM were presented as a ratio to total caspase-3 and normalized as fold changes to the no treatment control. Values represent SEM of biological replicates. ANOVA analysis: * denotes $p < 0.05$ and ** denotes $p < 0.01$, compared with the DMSO group.


A.
Pro-caspase-3
32 kDa
Cleaved caspase-3
19 kDa
17 kDa
Actin
46 kDa
DMSO – + – – – – – –
ONC201 (μM) – – 3 10 30 – – –
Isomer (μM) – – – – – 3 10 30
B.
ONC201
ONC201 isomer
Cleaved caspase-3/ total caspase-3 (Fold of control)
0
1
2
3
4
5
*
*
0
3
10
30
μM

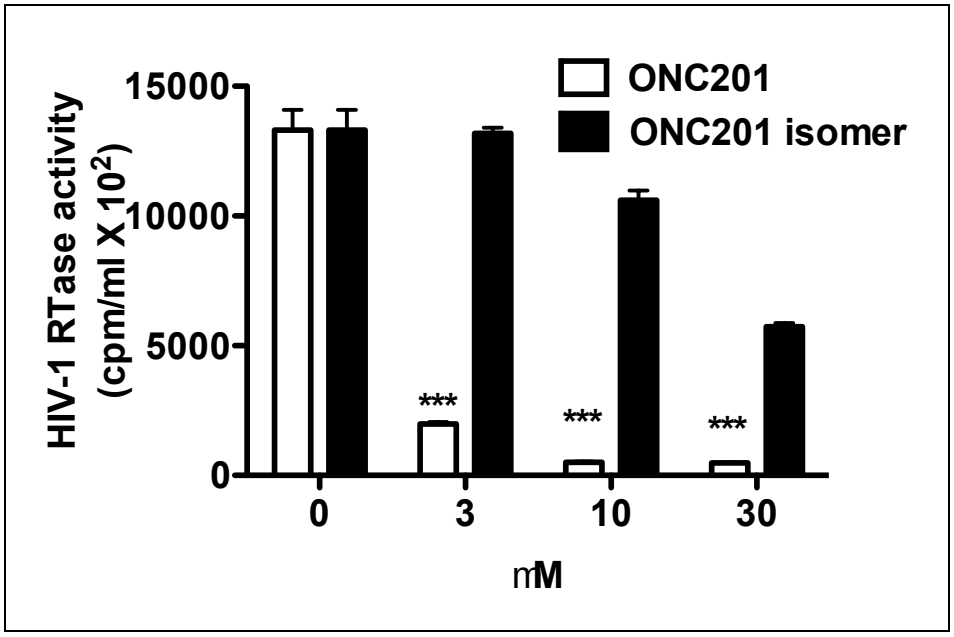


Figure 3.9 Pretreatment of ONC201 on macrophages inhibits HIV-1 infection. Human MDM were plated in 96-well plates and incubated with doses of ONC201 or its isomer ranging from 3 to 30 μM for 5 days before infection with HIV-1 for 24 hours. ONC201 and isomer treatments continued during and after the infection. At 3-day post infection, supernatants were removed and the replication levels of HIV-1 were monitored by RTase activity assay. Results shown are from representative experiments performed with three different donors. Data were analyzed by two-way ANOVA: * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$.

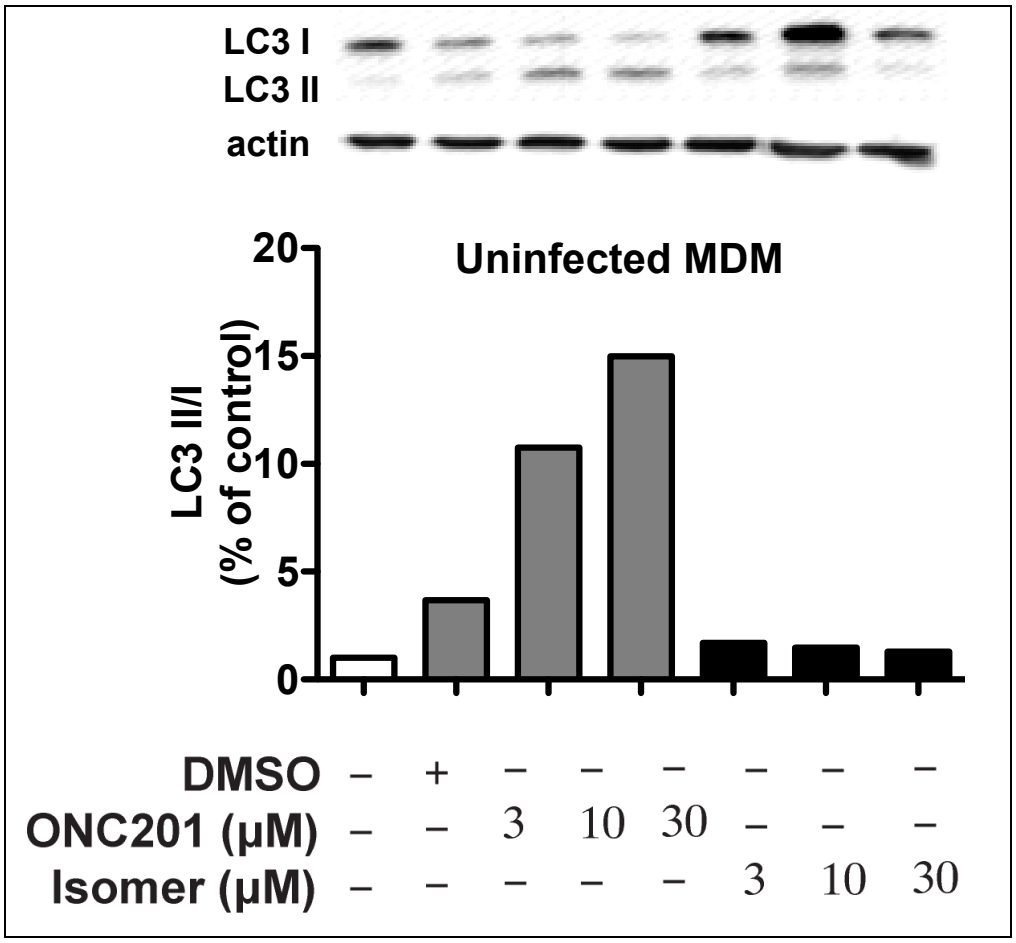


Figure 3.10 ONC201 induces autophagy in uninfected macrophages. Human MDM were plated in 24-well plates and then incubated with ONC201 or ONC201 isomer at 3 to 30 μM for 5 days. Cell lysates were collected and subsequently subjected to SDS-PAGE and immunoblotting for the detection of LC3 I and LC3 II. Actin was used as the loading control. Densitometric quantifications of LC3 II in MDM were presented as a ratio to LC3 I and normalized as fold changes to the no treatment control.

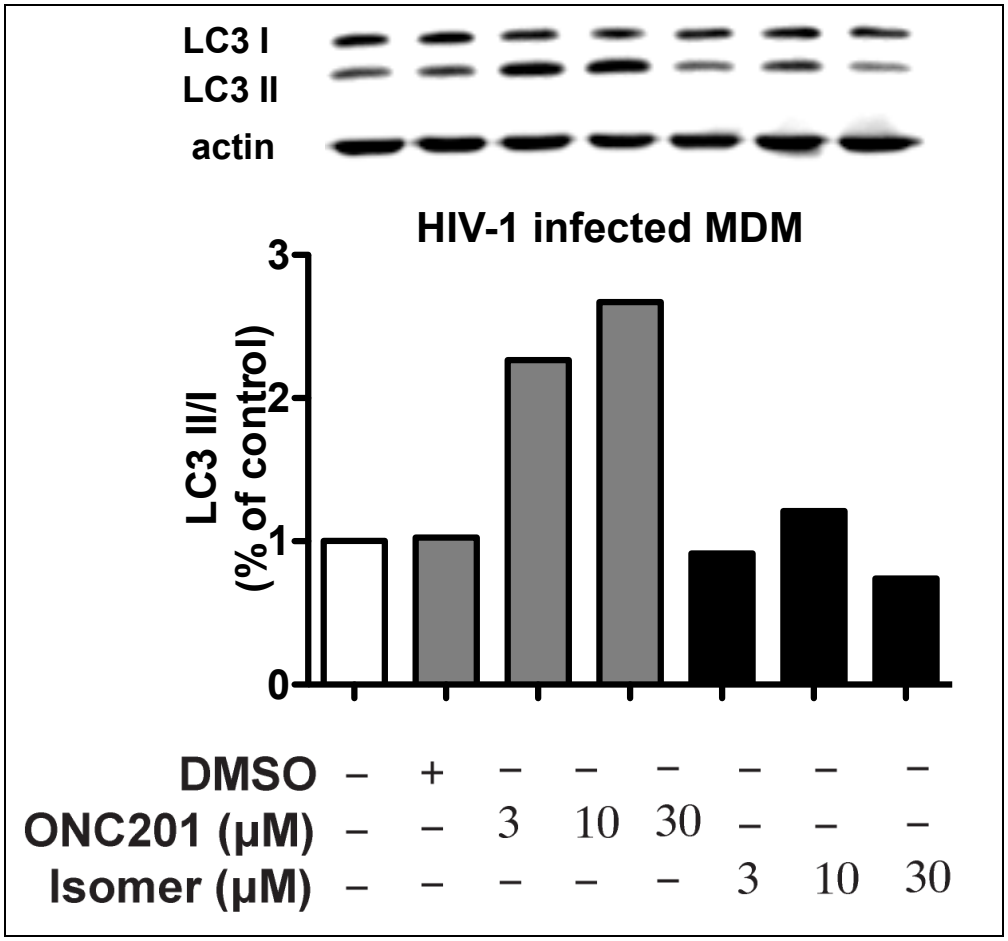
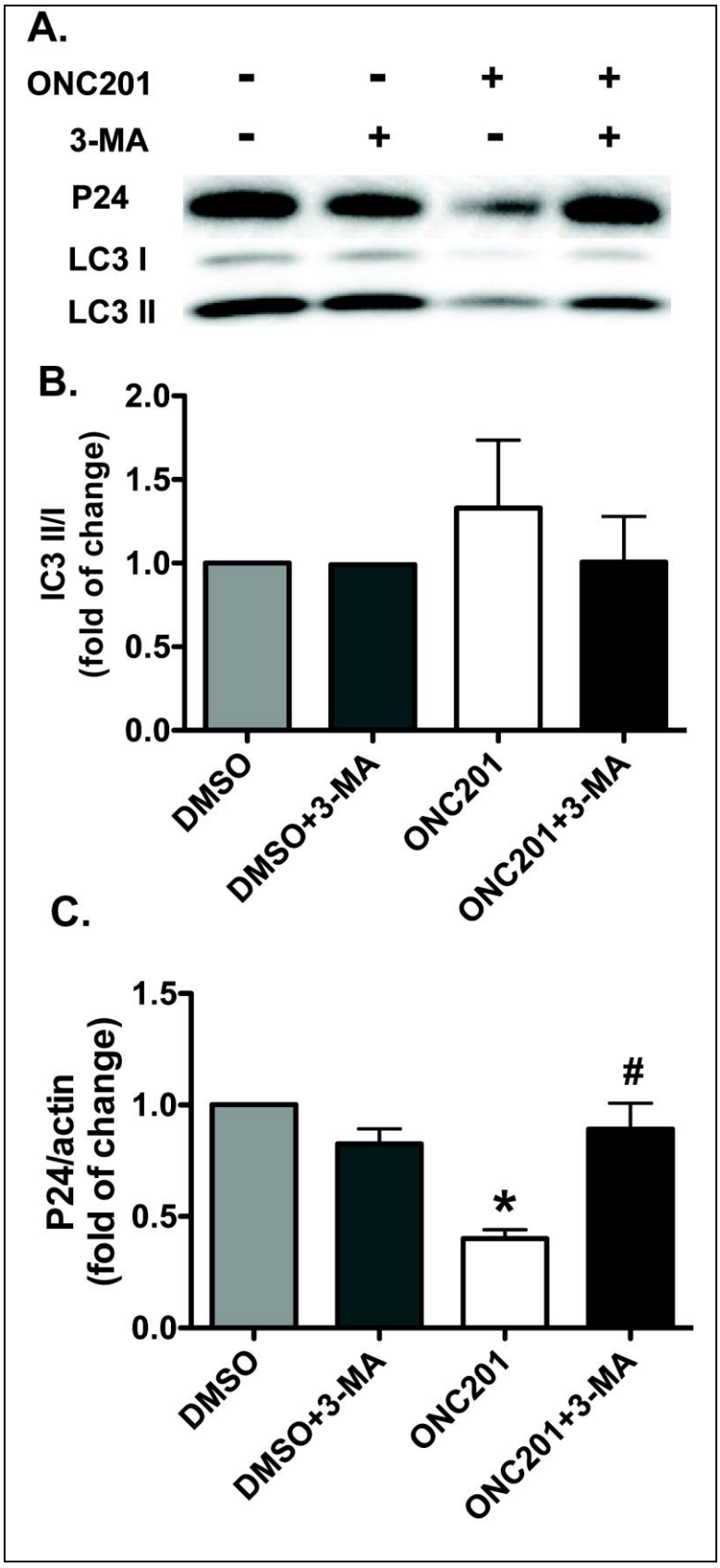


Figure 3.11 ONC201 induces autophagy in HIV-1 infected macrophages. Human MDM were plated in 24-well plates and then infected with HIV-1_{ADA} for 24 hours before incubation with ONC201 or ONC201 isomer at 3 to 30 μM for 5 days. Cell lysates were collected and subsequently subjected to SDS-PAGE and immunoblotting for the detection of LC3 II and LC3 I. Actin was used as the loading control. Densitometric quantifications of LC3 II in MDM were presented as a ratio to LC3 I and normalized as fold changes to the no treatment control.

Figure 3.12 Blocking autophagy by 3-MA reverses the antiviral effect of ONC201. Human MDM were plated in 24-well plates and then infected with HIV-1_{ADA} for 24 hours before incubation with ONC201 (30 μM), 3-MA (100 μM) or a combination of ONC201 and 3-MA for 5 days. A) Cell lysates were collected and subsequently subjected to SDS-PAGE and immunoblotting for the detection of LC3 II, LC3 I and P24. Actin was used as the loading control. B) Densitometric quantifications of LC3 II in MDM were presented as a ratio to LC3 I and normalized as fold changes to the DMSO control. C) Densitometric quantifications of P24 in MDM were presented as a ratio to actin and normalized as fold changes to the control group. ANOVA analysis: * denotes $p < 0.05$, compared to DMSO group; # denotes $p < 0.05$, compared to ONC201 treatment group.


A.
ONC201
3-MA
P24
LC3 I
LC3 II
B.
IC3 II/I
(fold of change)
2.0
1.5
1.0
0.5
0.0
DMSO
DMSO+3-MA
ONC201
ONC201+3-MA
C.
P24/actin
(fold of change)
1.5
1.0
0.5
0.0
*
#
DMSO
DMSO+3-MA
ONC201
ONC201+3-MA

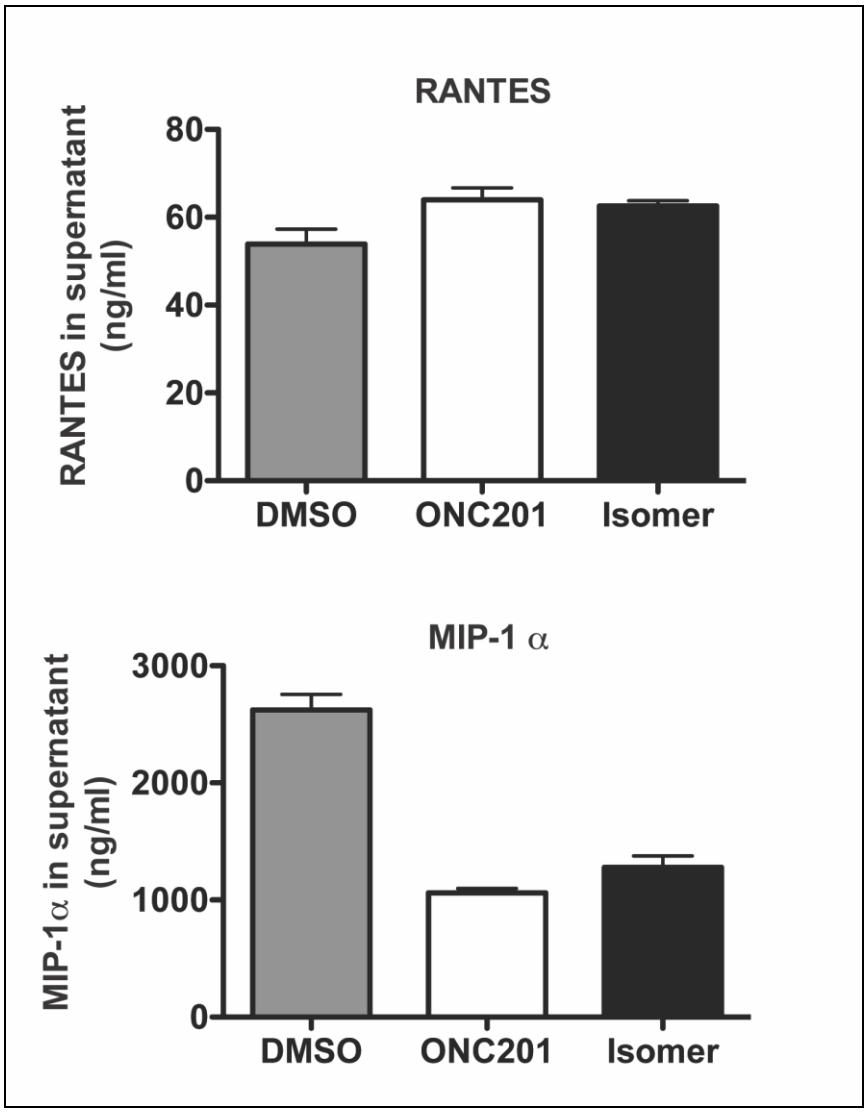


Figure 3.13 ONC201 does not induce the production of RANTES and MIP-1α. Human MDM were plated in 24-well plates in triplicate and then infected with HIV-1_{ADA} for 24 hours before incubation with ONC201 or ONC201 isomer at 30 μM for 5 days. RANTES and MIP-1α concentration (ng/ml) was determined from cell culture supernatants using ELISA and adjusted to intracellular protein concentrations. Values represent SEM of biological replicates. ANOVA analysis: * denotes $p < 0.05$ compared with DMSO group; # denotes $p < 0.05$ compared with ONC201 treatment group.

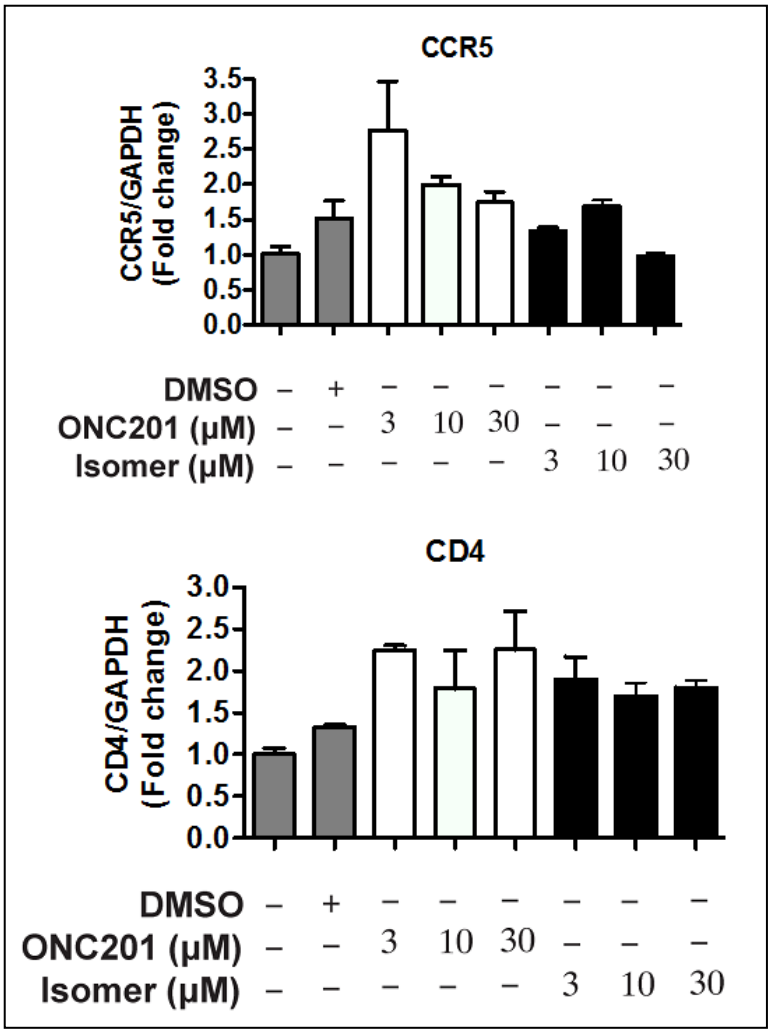


Figure 3.14 ONC201 does not change the expression of CCR5 and CD4 in HIV-1 infected macrophages. Human MDM were plated in 24-well plates in triplicate and then infected with HIV-1_{ADA} for 1 day. The cells were incubated with doses of ONC201 or ONC201 isomer ranging from 3 to 30 μM for 5 days. Cells were then harvested and RNA was isolated. CCR5 and CD4 expression were determined through quantitative real time RT-PCR. Relative CCR5 and CD4 expression levels were determined and standardized with GAPDH internal control. Values represent SEM of biological replicates. ANOVA analysis: ** denotes $p < 0.01$, compared to DMSO group.

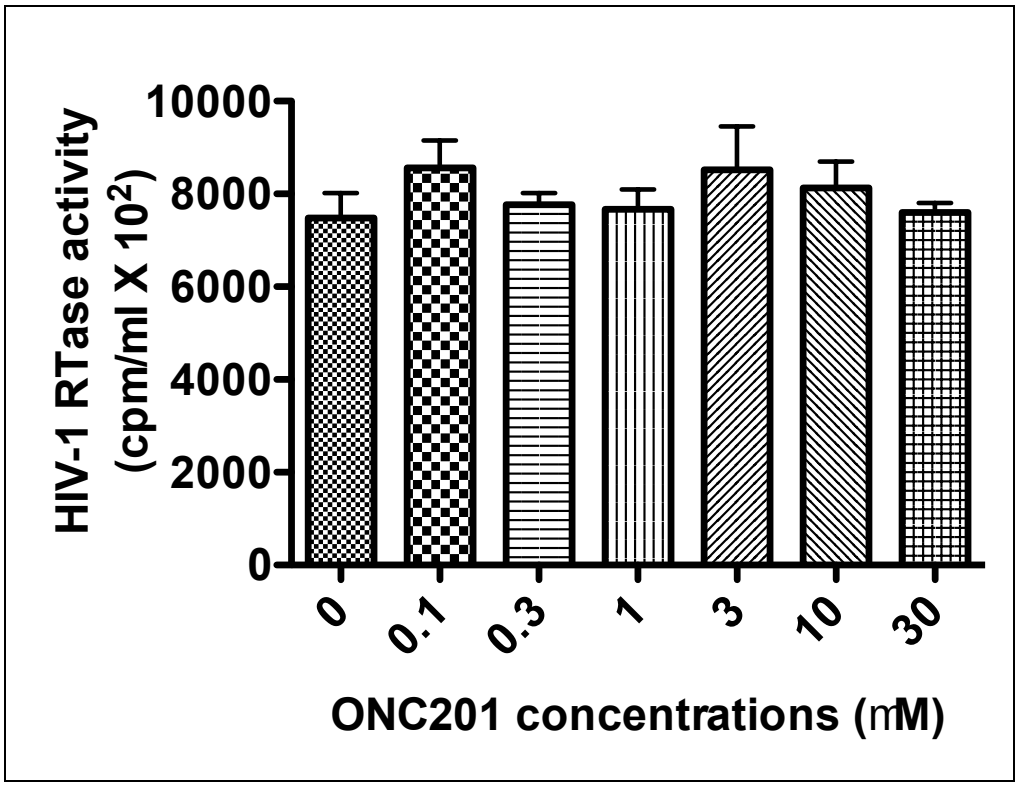


Figure 3.15 ONC201 does not suppress HIV-1 when directly incubated with HIV-1 virions. Equal viral titers (400 $TCID_{50}$/ml) of laboratory-adapted HIV strain ADA were mixed with doses of ONC201 ranging from 0.1 to 30 μM. The reverse transcriptase activity of HIV-1 was monitored by RTase activity assay.

CHAPTER 4

General Discussion and Future Directions

4.1 General discussion

Even in the early phase of infection, HIV enters the CNS and evades ART(159). These viruses incur chronic inflammation and tissue damage that causes the development of complications such as HAND in more than half of infected patients(160). In the post-ART era, the complications are even more prevailing for the long life expectancy of infected patients on ART and poor BBB penetration of ART drugs (161, 162). HIV-1 infected macrophages and microglia, as the reservoir cells in CNS, play a central role in the development of HAND(144). This study targets the long-lived nature of the macrophages and microglia and use the latest understanding of their vulnerabilities to design new therapies for viral eradication. We intended to introduce ONC201, an effective apoptosis-inducing agent in cancer biology, to the HIV field for eradicating infected macrophages and microglia. We observed the antiviral effect of ONC201 on HIV-1 infected macrophages both in vitro and in vivo, and elucidated that the activation of FOXO3a-TRAIL pathway is the main mechanism underlying this antiviral effect.

This study is innovative in three aspects. First, this study makes a conceptual advance in understanding the molecular mechanism of HIV-1 persistence in the CNS. Targeting transcription factors for therapeutic aims is a largely unexplored area. In the current study we uniquely investigated a transcription factor FOXO3a in its crucial contribution to the HIV-1 persistence in the CNS. It provides an important proof-of-concept that endogenous FOXO3a could be harnessed to combat persistent and latent HIV-1 infection. This new

strategy to change the balance of pro- and anti-apoptotic factors in infected cells will stimulate further development of therapeutic strategies to eradicate viral reservoirs. Second, this study takes advantage of the latest drug development on FOXO3a (78). The novel drug has the potential to eliminate HIV-1-infected cells in the CNS and significantly increase the efficacy of ART. Third, this study is innovative in using the technology of HIV-1 encephalitis (HIVE) mouse model. The HIVE mouse model is suitable to study the survival of long-lived HIV-1 infected cells and the effect of TIC10-induced pro-apoptotic genes upregulation. The data from this pilot study on HIVE mouse model are the first evidence of FOXO3a involvement in survival of infected cells in vivo.

The physiological relevance of pharmacological intervention on TRAIL expression is significant. In HIV-1 infected patients, both soluble TRAIL in their serum and surface-bound TRAIL on the membrane of their cells are upregulated (64, 93). However, the elevated level of TRAIL is not sufficient to clear the infected reservoir cells. This could be attributed to the TRAIL resistance conferred from TRAILshort. TRAILshort is a novel splice variant of TRAIL that lacks apoptosis-inducing activity and acts as an antagonist of TRAIL (122, 163). This antagonism is further strengthened by its preference of binding to death receptors, TRAIL-R1 and -R2, rather than decoy receptors, TRAIL-R3 and -R4(122). TRAILshort is produced by both HIV-1 infected cells and uninfected bystander cells, and its production is induced by type I IFNs and TLR7, TLR8, and TLR9 agonists(122). Furthermore, it can be wrapped into microvesicles and then brought to neighboring cells, which establishes a TRAIL-resistant

microenvironment(122). However, supraphysiologic levels of TRAIL agonists and TRAIL-inducer ONC201 are still able to preferentially kill these HIV-1 infected cells(62), suggesting this TRAIL-resistant microenvironment could be overcome by higher levels of TRAIL. Development of more potent TRAIL agonists or TRAIL-inducers are warranted.

Compared with other currently available TRAIL-based drugs, TRAIL as a small molecule shows advantages. Recombinant TRAIL, adenovirus-delivered TRAIL and longer-lived TRAIL receptor agonist antibodies such as lexatumumab and mapatumumab have been tested in clinical trials and showed antitumor effects (112-118, 164). However, synthesis of these macromolecules is costly, their biodistribution is limited and their half-life is relatively short. ONC201 performs better in bioavailability, biodistribution, stability and cost(79). In addition, the most attractive trait of ONC201 is that it can induce endogenous production of TRAIL not only by the target cells but also by the neighboring cells. It means all the uninfected cells in human body are potentially able to produce and secrete soluble TRAIL that may induce apoptosis of HIV-1 infected cells. Cells can also produce surface-bound TRAIL that kills the infected nearby cells through a bystander effect. Thus, inducing endogenous TRAIL production by a small molecule is much more effective than providing exogenous macromolecules. This also suggests that ONC201 may have a better antiviral effect in a mixed glia cell culture or in the in vivo models than in the current MDM-only culture.

In this study, we proposed that ONC201 inhibits HIV-1 infection through TRAIL-induced apoptosis, which belongs to “prime, shock and kill” strategy of managing latently infected cells. This is further supported by the following facts: 1) HIV-1 infection inhibits Akt-1 phosphorylation (66); 2) FOXO3a- and TRAIL-mediated apoptosis in HIV-1 infected macrophages is dependent on the inhibition of Akt-1 phosphorylation (41, 66); 3) ONC201 activates FOXO3a-TRAIL pathway via inhibition of Akt-1 phosphorylation(78). Together, ONC201 may inhibit Akt-1 to induce apoptosis in HIV-1 infected macrophages, which seems to be the mechanism for the antiviral effect of ONC201. However, latest evidence shows that Akt activation is critical for HIV-1 reactivation in latently infected cell line and ART drugs limits this viral reactivation by inhibiting Akt(125). This suggests that ONC201 as an Akt inhibitor may reduce the size of the HIV reservoir through a “block and lock” strategy, which is opposite to our original hypothesis. It is also possible that the antiviral effect of ONC201 on HIV-1 infected reservoir cells is through both “prime, shock and kill” and “block and lock” strategies. If so, this two-fold effect would make ONC201 more appealing for the future clinical application.

4.2 Future directions

The United States is currently experiencing a grave epidemic of drug abuse such as psychostimulants involving cocaine and methamphetamine, particularly among patients infected with HIV (165). Drug abuse and HIV infection frequently coexist due to the association of drug use with engagement of high-risk behaviors (166). More importantly, drug abuse has been correlated with severe HAND and more rapid progression to AIDS in HIV-infected people (167, 168). Nevertheless, how drug abuse enhances HIV-related neuropathologies are not fully understood. Central to the action of drugs of abuse is dopamine, since psychostimulants such as cocaine and methamphetamine all exert addictive and reinforcing effects through elevation of extracellular dopamine levels in the CNS (144). Thus, macrophages and microglia in CNS are exposed to high level of dopamine for the drug abuser. Previous study has shown that exposure to dopamine during infection increased the entry of R5 tropic HIV into macrophages (146), suggesting that dopamine pathway could be a therapeutic target for delaying or preventing the acceleration of HAND and other HIV-related pathologies enhanced by drug abuse.

Dopamine signals primarily through dopamine receptors (DR), members of the G protein coupled receptor superfamily. DR can be divided into two subclasses, the D1-like DR, DRD1 and DRD5, and the D2-like DR, DRD2, DRD3 and DRD4. Human monocyte-derived macrophages express mRNA for all DR subtypes and protein for DRD1, DRD2, DRD3 and DRD4 in the plasma membrane (144, 145). It has been reported that dopamine increases HIV

replication in human macrophages by activation of DRD2 and increasing ERK 1 phosphorylation (144).

Specific DRD2 antagonist has been lacking. ONC201 was newly found to selectively antagonize the DRD2, but not D1-like subfamily of dopamine receptors (149). ONC201 shows superior traits among current DRD2 antagonists. In addition to high selectivity, ONC201 exhibited a very slow association rate for DRD2 relative to antipsychotics, whereas the dissociation rate was similar to atypical antipsychotics that are better tolerated clinically (unpublished). However, no groups have introduced this drug into HIV field as a DRD2 antagonist for treating HIV-related neurological disorders among infected drug abusers. We have demonstrated that ONC201 reduces HIV-1 replication in human macrophages and microglia both in in vitro primary cultures and in vivo murine HIV encephalitis model. However, whether blocking DRD2 by ONC201 could halt HIV-1/drug abuses co-morbidities remains unclear.

We will first determine whether the antiviral effect of ONC201 in macrophages is through antagonizing DRD2. DRD2-selective agonist quinpirole will be used together with ONC201 on HIV-1 infected macrophage to test whether the DRDR agonist could abrogate the antiviral effect of ONC201. Then we will use two different siRNAs for DRD2 to lower the expression of DRD2 in MDM two days before HIV-1 infection, to see whether knockdown of DRD2 would be sufficient to suppress the HIV-1 infection in macrophage. We will also use HIV_{BaL} containing Vpr-b-lactamase and phenol red free macrophage media with CCF2-AM to perform viral entry assay, to determine whether this DRD2-

mediated antiviral effect of ONC201 is through blocking the entry of HIV into cells. In this assay, uninfected cells fluoresce green and infected cells fluoresce blue.

Evaluating the in vivo therapeutic potential of ONC201 is the most significant part of our study and a natural progression of the in vitro studies. We have used a murine HIV encephalitis (HIVE) model to show the antiviral effect of ONC201 on the HIV-1 infection levels in the brain. Nevertheless, the humanized mice model is more suitable to examine multiple parameters of HIV-1 pathogenesis including eradication of infected macrophages and latently infected T cells as viral reservoir. We will further use the CD34 engrafted model of neuroAIDS to examine whether ONC201 affects multiple parameters of HIV-1 pathogenesis, including persistence of infected macrophages and latently infected T cells as viral reservoir. This first proof-of-principle data will usher in an innovative therapy that targets FOXO3a for viral eradication.

Using CD34-engrafted humanized mouse model of neuroAIDS, we will test whether ONC201 and the standard ART can synergistically reduce the CNS viral load and delay viral rebound. Meanwhile, we will identify whether ONC201 is able to suppress viral replication in the HIV-1-infected humanized mice through induction of TRAIL. After 15 weeks of reconstitution by human CD34+ stem cells, humanized NSG mice will be selected for further study based on their repopulation with human CD45+ cell numbers as monitored in peripheral blood. These animals will be infected with the macrophage tropic virus HIV-1_{ADA} by IP injection and randomized into 9 groups, 8 of which are all HIV-1 infected

animal groups. Four of the groups will receive ART, whereas the other groups serve as control. For ART, HIV-infected mice will be treated by daily injection of ART drugs for 3 weeks. For Non-ART group, mice will be daily-administered ONC201, DMSO/IgG vehicle control, TRAIL neutralizing antibody, or ONC201+ TRAIL neutralizing antibody through IP. At the end point of 3 weeks post HIV-1 infection, whole brain tissues will be collected for DNA/RNA isolation and IHC. For the 4 ART groups, mice will be daily-administrated ONC201, DMSO/IgG vehicle control, TRAIL neutralizing antibody, or ONC201+ TRAIL neutralizing antibody through IP along with ART. At 3 weeks post HIV-1 inoculation, all ART will discontinue, facilitating viral rebound. All 4 groups will be followed for another two weeks to the experimental point. Whole brain tissues will be collected for DNA/RNA isolation and IHC. A ninth group of humanized NSG mice will be uninfected and untreated, serving as negative controls (HIV−).

In addition to whole brain tissues, spleen, brain, liver, lymph nodes and plasma will be also collected for drug levels and antiviral activity determination. Specifically, FACS tests and ELISA of peripheral blood will be performed at three different time points after infection to monitor the counts of CD4 + T-cells and plasma viral load in HIV-1 infected mice. Immunohistochemistry or RNA In Situ Hybridization (RNA ISH) on spleen, brain and lymph nodes will be performed by staining for CD3, CD68 and HIV-1p24 or hybridizing HIV-1 RNA to confirm the viral load tests. Pharmacokinetic analyses will be performed in spleen, brain, and liver to measure the drug levels of ONC201 in such sites.

Lastly, we will evaluate the cognitive function of the humanized mice through fear conditioning tests the day before sacrifice.

One limitation of the humanized mouse models is that microglia within the brain parenchyma are not humanized and could not be infected. Therefore, the models could not fully recapitulate HIV-1 infection in these cell types. In humanized mice, only peripheral monocytes and macrophages and CD4+ T lymphocytes migrating across the BBB are infected and that infection is limited to the meninges and perivascular spaces (169, 170). Although the current models are suitable for testing ONC201 in vivo, this limitation reminds us that continued improvement of animal model for HIV-1 infection is imperative.

As the growing epidemics of drug abuse and HIV become even more interconnected, it has become increasingly important to understand the influence of drugs of abuse in concert with HIV on the development of HAND and intervene with this pathogenesis. The proposed studies will provide important proof-of-concept that DRD2 on macrophages and microglia could be a potential target for multiple drugs of abuse. Understanding the effect of ONC201 on HIV-1-infected macrophages and microglia in drug abuse and its underlying mechanism will reveal new insights for delaying or preventing the acceleration of HAND and other HIV-related pathologies enhanced by drug abuse.

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