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Broad spectrum mixed lineage kinase type 3 inhibition and HIV-1 persistence in macrophages

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Abstract

Mixed lineage kinases (MLKs) are a group of serine-threonine kinases that evolved in part to respond to endogenous and exogenous insults that result in oxidative stress and pro-inflammatory responses from innate immune cells. Human immunodeficiency virus type 1 (HIV-1) thrives in these conditions and is associated with the development of associated neurocognitive disorders (HAND). As part of a drug discovery program to identify new therapeutic strategies for HAND, we created a library of broad spectrum MLK inhibitors with drug-like properties. Serendipitously, the lead compound, URM-099 has proved useful not only in reversing damage to synaptic architecture in models of HAND, but also serves to restore autophagy as a protective response when given in concert with nanoformulated antiretroviral therapy (nanoART) in persistently infected macrophages. These findings are reviewed in the context of MLK3 biology and cellular signaling pathways relevant to new HIV-1 therapies.

Graphical Abstract

Keywords

autophagy; Human immunodeficiency virus type 1; macrophage; mixed lineage kinase; central nervous system

The conceptual underpinnings for understanding the intersection between mixed lineage kinase type 3 (MLK3) function and HIV infection of macrophages are based on the relatively small size of the viral genome and its evolutionary need to rely on mechanisms utilized by the host genome to help perpetuate the viral life cycle. A key feature here is that MLKs respond to exogenous and endogenous cellular stress and the lifecycle of HIV-1 in a cellular reservoir is in part driven by oxidative stress. As with many process of discovery ideas, we were initially searching for therapeutic targets whose direct or indirect engagement by HIV-1 in central nervous system (CNS) reservoirs might be common to both derangement of MLK3-related pathways in infected cells and subcellular elements of bystander end organ target cells (neuronal synapses) damaged during persistent or latent infection. Thus, we were intrigued by an early paper from (Nguyen et al. 2007) that used the X4 tropic strain HIV-IIIb and a high throughput screen consisting of a cDNA library of 15,000 genes to identify novel pro-viral factors that might be involved in enhancement of HIV-IIIb infection. Out of 13 host factors identified in this screen, (Nguyen et al. 2007) chose to focus on the cDNA hit, MLK3 (MAP3K11), based on its already identified roles in pathways known to be hijacked by HIV and its potential for druggability. The irony of capitalizing on data from a model system utilizing an X4 tropic HIV strain that would ultimately result in a drug discovery program to create a small molecule to inhibit toxic pro-inflammatory responses and serendipitously augment efficacy of antiviral therapy nanoformulated to improve cellular uptake in persistently infected mononuclear phagocytes has made us firm believers in paying attention to the old adage that “chance favors a prepared mind.” While the literature is non-existent with respect to demonstration of mechanistic links between R5 HIV strains and MLK3 activation, R5 envelopes do induce mitogen-activated protein kinase (MAPK)-related genes (Arthos et al. 2002; Cicala et al. 2002). Notably, R5 envelopes can modulate 52 MAPK genes, with the majority belonging to the p38 subfamily (Cicala et al. 2006). However, to date there have been no studies that have directly addressed a relationship between HIV tropism and MLK3 activation in brain-resident mononuclear phagocytes.

Regardless of the lack of data directly validating roles between MLK3 and HIV-1 tropism, we speculated that MLK3 activation in response to oxidative and inflammatory stimuli was sufficient to investigate whether it could play a significant role in the pathologic effects of HIV infection of mononuclear phagocytes. Furthermore, we hypothesized that inhibiting activation of MLK3 associated pro-inflammatory pathways could restore homeostasis between innate immune cells and communication between neuronal synapses damaged during persistent HIV-1 infection of the CNS.

Earlier *in vitro* evidence from our laboratories and others demonstrated that non-selective MLK3 inhibition could be both anti-inflammatory and neuroprotective against HIV-1 gene products. Specifically, HIV-1 Tat and gp120 have been shown to induce autophosphorylation of MLK3 (Sui et al. 2006a). HIV Tat- and gp120-induced neuronal apoptosis appears to be dependent on MLK3, as both genetic ablation using over-expression of a dominant negative form of MLK3 in neurons or non-selective small molecule inhibition of MLK3 is neuroprotective *in vitro* (Bodner et al. 2002; Sui et al. 2006a). Finally, Tat- and gp120-induced MLK3 activation in monocytes results in increased production of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF α) (Sui et al. 2006a). In conclusion, these studies indicate that the HIV proteins gp120 and Tat can be directly neurotoxic by activating MLK3 in neurons and can also increase pro-inflammatory cytokine production in mononuclear phagocytes with neurotoxic effects. This increase in MLK3 activity may therefore be a major contributor to neuropathologic mechanisms that are relevant to HIV-1 associated neurocognitive disorders (HAND).

We then used non-selective inhibition of MLK3 with the first generation MLK3 inhibitor CEP-1347 in the SCID mouse model of HIV-1 encephalitis (HIVE) (Eggert et al. 2010). CEP-1347 is a semisynthetic small molecule with anti-apoptotic biologic effects that is an analog of the indolocarbazole, K252a. CEP-1347 was originally developed for use in neurodegenerative conditions, most notably Parkinson's disease (PD).

Unfortunately, its use in a Phase II trial for PD was curtailed because of failure to meet primary endpoints for efficacy (PRECEPT, 2007) (Neurology 2007). Further analyses suggested that its unfavorable pharmacokinetic (PK) and metabolic profile were likely the underlying causes of futility in the PRECEPT trial (Goodfellow et al. 2013), but in no way diminished the importance of one of its primary targets, MLK, in mediating neurodegeneration for a number of neurologic diseases.

We used the lessons learned from PRECEPT trial to avoid pharmacologic confounds we felt contributed to the failure of CEP-1347. These included its high molecular weight, poor PK and metabolic profiles, as well as its profile for kinase inhibition (in particular, CAMK2A, CAMK2D, BRSK2 and AMPK 2), raising the specter of mutagenic effects during chronic administration (Goodfellow et al. 2013). By strictly adhering to standard principles of medicinal chemistry for small molecule drug design (i.e. Lipinski's Rule of Five) (Lipinski et al. 2001), we created a library of small molecules based on the 7-azaindole group (aka: 1H-Pyrrolo[2,3-b]pyridine) that allowed for rapid optimization of structure-activity relationships (SAR) to create our 2nd generation broad spectrum MLK3 inhibitor, URM-099. Since MLK3 is expressed in both innate immune cells and in target cells such

as CNS neurons, we used two highly simplified biologic models of HIV-1 associated neurocognitive disorders (HAND) that depend on exposure to the neurotoxic HIV-1 protein Tat and not direct infection of human macrophages with HIV-1 to evaluate the potential for therapeutic efficacy of broad spectrum inhibition of MLK3 using URM-099: (1) in vitro models of mononuclear phagocyte activation and neuronal process simplification and cell death in microfluidic chambers; and (2) an in vivo model of Tat injection into the cortex of irradiation chimeric mice with fluorescent reporters for mononuclear phagocytes and neurons to determine whether MLK3 inhibition could be both anti-inflammatory and neuroprotective. Thus, we were able to demonstrate that URM-099 could reduce inflammatory cytokine production from microglia exposed to HIV-1 Tat, as well as protected neurites from microglial phagocytosis. In vivo, URM-099 treatment also reduced inflammatory cytokine production and preserved synaptodendritic architecture with decreased microglial phagocytosis of synaptic elements in response to HIV-1 Tat exposure (Marker et al. 2013).

While these results were strongly favorable for consideration of URM-099 or its descendant congeners as adjunctive therapy for HAND, our remaining hurdle was to evaluate any potential adverse biologic outcomes from potent MLK3 inhibition during HIV-1 infection that relate to the viral life cycle and mononuclear phagocyte function, particularly in end organs known to harbor infection. While our simplified models of HAND allowed us to perform real time imaging of changes in synaptodendritic architecture and microglial activation in response to HIV-1 Tat, we needed more sophisticated models such as humanized mice engrafted with CD34 cells that could sustain HIV-1 infection.

At the genetic level, early experiments with MLK3 overexpression increased Tat-dependent gene transcription at the HIV LTR 7-fold over control conditions, and this increase was dependent on the MLK3 protein having an intact kinase region. Furthermore, genetic silencing of MLK3 inhibited viral replication *in vitro* (Nguyen et al. 2007). The mechanism by which MLK3 increased viral replication appears to be mediated through NF- κ B, which can be activated by MLK3 (Hehner et al. 2000) and directly enhance viral transcription at the LTR (Yang et al. 1999; Yang and Klionsky 2009). However, these early experiments focused on the role of MLK3 in the lymphocyte and not the macrophage.

MLK3 is a serine/threonine mitogen associated protein kinase kinase kinase (MAPKKK) (Gallo et al. 1994), which ultimately activates Jun-N-terminal kinase (JNK) (Tibbles et al. 1996). Whether MLK3 also activates the p38 MAPK (Buchsbaum et al. 2002) remains speculative, since experiments with MLK3 deletion argued against the specificity of these findings (Branchio et al. 2005; Gallo and Johnson 2002). MLK3 contains a Src-homology-3 domain (SH3) at the amino terminus, followed by a kinase domain, a leucine zipper domain, and a proline-rich domain (Gallo and Johnson 2002). MLK3 is phosphorylated and activated through homodimerization and autophosphorylation (Leung and Lassam 1998), along with direct phosphorylation from upstream Rho family guanosine triphosphatases (GTPases) (Kant et al. 2011).

Glycogen synthase kinase-3 β (GSK-3 β), an important kinase linked to inflammation and the progression of Alzheimer's disease (Jope et al. 2007), also directly phosphorylates and

activates MLK3 in neurons (Mishra et al. 2007). Additionally, pharmacologic and RNAi inhibition of GSK-3 β in response to LPS treatment of microglia blocked MLK3 signaling pathways via disruption of MLK3 dimerization-induced autophosphorylation (Wang et al. 2010). HIV-1 Tat can also activate GSK-3 β in neurons to antagonize the NF κ B survival pathway via modification of RelA subunit at ser468 (Sui et al. 2006b). MLK3 can also negatively regulate its own activity through the interaction of its SH3 domain with its proline-rich region, resulting in steric hindrance of the kinase region (Zhang and Gallo 2001).

MLK3 is expressed at basal levels in peripheral immune cells (Handley et al. 2007), microglia (Wang et al. 2010), and neurons (Maroney et al. 2001), among other cell types. In neurons, MLK3 is upregulated and phosphorylated in response to trophic factor withdrawal, ROS exposure, and UV irradiation, three classical activators of JNK (Lotharius et al. 2005; Xu et al. 2001). This increase in MLK3 activity results in JNK dependent c-Jun up-regulation and activation, which in turn activates pro-apoptotic pathways, leading to apoptosis (Dhanasekaran and Reddy 2008). Additionally, JNK activation is thought to be common to the neuropathogenesis of Alzheimer's disease, PD, and other neurodegenerative disease states (Borsello and Forloni 2007).

As part of our early clinical work with the first generation non-selective MLK3 inhibitor, we used CEP-1347 in conjunction with the protease inhibitor atazanavir/ritonavir (ATV/r) in a three-week controlled open-label, multiple dose study with 20 HIV-1-infected patients (Ma et al. 2013). All patients tolerated CEP-1347 well, with the only reported adverse effects attributable to ATV/r and tenofovir. At study completion (day 21), plasma viral load had decreased from 3.80 ± 3.70 to 2.41 ± 2.03 log copies/mL, a finding that because of small sample size and variability, did not reach statistical significance. The major findings from this pilot study were that CEP-1347 administration significantly increased both the accumulation ratio of ATV by 15% and its half-life from 12.7 to 15.9 hours.

These intriguing findings led us to investigate how URM-099 might interact with ATV/r. Because nanoformulated ATV/r (NanoATV/r) was developed expressly to bypass its very poor pharmacokinetic (PK) profile for penetration into cellular reservoirs for HIV-1 (Gendelman and Gelbard 2014) we reasoned that MLK3 inhibition might further augment desirable PK parameters of NanoATV/r. To model these effects, we used the NSG CD34-engrafted murine model to sustain widespread infection of immune cells (Dash et al. 2012). Unexpectedly, we found that, while URM-099 by itself had at best modest antiviral effects against HIV-1, when given with NanoATV/r, viral reservoirs were eliminated in lymphocyte populations in vivo and strikingly reduced in infected macrophage reservoirs (Zhang et al. 2016). Additionally, endosomal uptake and retention of NanoATV/r were significantly increased in the presence of URM-099 (Zhang et al. 2016).

Macrophages and monocytes play a vital role in HIV infection, serving as persistently-infected viral reservoirs despite cART therapy, and thus contribute to disease progression (Alexaki et al. 2008; Deng and Siliciano 2014). Macrophages harbor diverse viral phenotypes with multiple co-receptor and cell tropism usage during HIV-1 infection (Abbas et al. 2015). Their long lifespan, ability to cross the blood-brain barrier and their capabilities

of mounting an inflammatory response could reactivate HIV replication from latently infected cells; all properties that make them excellent reservoirs for persistent HIV-1 infection (Clayton et al. 2017; Fischer et al. 2014; Zaikos and Collins 2014). These factors highlight the need for an improved method of drug delivery system that can extend drug half-life and successfully eliminate viral dissemination from HIV reservoirs. Having previously demonstrated that nanoformulated ARVs co-administered with URM-099 successfully extended drug half-life, we then investigated the ability of URM-099 to aid in the maintenance of nanoformulated ARV depots in HIV-infected monocyte-derived macrophages (MDMs). URM-099 facilitated a four-fold reduction in virion production and reduced multinucleated giant cell formation when administered to HIV-infected MDMs along with a sub-therapeutic dose of nanoATV.

URM-099 can affect JNK phosphorylation which forms an integral part of signalsomes that determine cell fate (Marker et al. 2013), autophagy being one such pathway (Su et al. 2016). HIV is known to inhibit the mechanism of autophagy, which in turn aids viral replication in host cells (Brass et al. 2008). Autophagy, one of the two intracellular degradation pathways, is deployed during cellular stress such as nutrient starvation, hypoxia, viral infection and endoplasmic reticulum (ER) stress (Yang and Klionsky 2009). Although there are three primary types of autophagy (micro-, macro- and chaperone-mediated autophagy) observed in mammalian systems, they all function to sequester and deliver potentially deleterious cargo to the lysosome for degradation. Of the three pathways, macroautophagy is a unique process where the *de novo* synthesis of intracellular vesicles known as autophagosomes enable cargo sequestration. Under nutrient rich conditions, MTORC1 kinase (the mechanistic target of rapamycin complex 1 associates with kinase complexes that are involved in the initiation of autophagy. But cellular stress such as starvation induces the dissociation of MTORC1 allowing dephosphorylation of the sites involved in autophagy (Hosokawa et al. 2009). Further, under normal/nutrient rich conditions, MTORC1 sequesters a transcription factor EB (TFEB), in the cytoplasm. Upon nutrient deprivation, TFEB undergoes nuclear translocation and enhances expression of genes involved in autophagy and lysosomal function (Martina et al. 2012), resulting in the *de novo* synthesis of vesicle membranes called phagophores, which begin to expand and grow spherically through the combined action of the microtubule associated protein 1 light chain 3 β (LC3B) protein and phosphatidylinositol 3-kinase, beclin 1 (BECN1) (Yang and Klionsky 2009). Following the induction of phagophores, they undergo a poorly understood maturation process that enables the vesicles to close off around the cargo (forming autophagosomes) and then enable the fusion of these vesicles to lysosomes.

Several mechanisms have been proposed for this process, including the components of soluble N-ethylmaleimide-sensitive fusion attachment protein receptors (SNARE) such as VAM7 and VAM9, which could play a part in the fusion of autophagosomes with lysosomes (Fader et al. 2009). This highly dynamic intracellular process controls inflammation through the removal of inflammasome activators (such as damaged or surplus organelles) and by inhibiting both activation of pro-IL-1 and the secretion of IL-1 (Harris et al. 2011; Nakahira et al. 2011).

Distilling these very complex signaling pathways into a logical framework that fit with what we were observing in our experimental paradigms, we reasoned that URM-099 was acting via kinase signaling pathways in HIV-infected macrophages exposed to nanoART. We observed that URM-099, when co-administered with therapeutic levels of nanoATV, could reverse the inhibitory effect of HIV-1 on TFEB nuclear translocation in an MTORC1-dependent manner. In line with our hypothesis, we observed that TFEB translocation to the nucleus up-regulated the expression of autophagy markers such as LC3B and BECN1 while maintaining the expression of sequestosome 1 (SQSTM1) (the degradation of which marks the end of autophagy) in HIV-infected MDMs. Because previous reports have demonstrated that HIV-1 Nef protein plays a vital role in the inhibition of autophagy by sequestering TFEB in the cytoplasm (Campbell et al. 2015), we speculated that reversal of HIV-1 Nef's effects on cytoplasmic TFEB sequestration might be responsible for URM-099's ability to restore autophagy in HIV-1 infected macrophages exposed to nanoformulated antiretroviral therapies. Interestingly, Nef has been shown to dysregulate autophagy in astrocytes and can be released in extracellular vesicles from astrocytes to mediate neurotoxicity (Sami Saribas et al. 2017; Sardo et al. 2015). These findings have important implications for the pathogenesis of HAND and may be additional targets for the therapeutic effects of URM-099 in the CNS.

Our encouraging results prompted us to test the effect of stimulating autophagy in vivo in our humanized CD34-engrafted, HIV-1 infected murine model (Dash et al. 2012) using a different class of cART, i.e. integrase inhibitors. The HIV-infected humanized NOD/SCID IL-2R γ c-null (NSG) mice were treated with a single intra muscular dose of nanoformulated dolutegravir (DTG), (aka, nanoDTG) with or without URM-099. A 52-fold increase in the plasma level of the drug was observed in mice also receiving URM-099 (Gnanadhas et al. 2017). Also, an increase in *Tfeb* gene expression was observed in spleen and liver as well as increased expression of autophagy markers such as Map1lc3b, Becn1 and Sqstm1. Taken together, these results demonstrated that URM-099's ability to restore autophagy in vivo is associated with increased levels of nanoDTG, suggesting that it may be possible to ultimately combine URM-099 with nanoformulated antiretrovirals to achieve sustained suppression of persistently infected cellular reservoirs of HIV-1.

Thus, this study not only suggested a possible autophagy-driven mechanism of how URM-099 might interfere with the viral life cycle in the presence of nanoformulated cART (Figure 1, cartoon), but also highlights the importance of harnessing autophagy in our quest for improved and efficient drug-delivery systems that represent the next generation of cART's ability to improve endosomal trafficking has shown to be key in enhancing the activity of nanoformulated drugs. One possibility that we are actively investigating is that URM-099 inhibits kinase-mediated suppression of transcription factors TFEB and TFE3 in activated macrophages and microglia, allowing these two transcription factors to work together to induce autophagy and lysosomal biogenesis (Pastore et al. 2016).

Having provided extensive correlative evidence that co-administration of URM-099 up-regulates levels nanoformulated antiretroviral medicines as an autophagy inducer (AI), presumably by reversal of a Nef-mediated sequestration of cytoplasmic TFEB (Figure 1, Panel E), the question of whether URM-099's effects could be mimicked by other

pharmacologic agents that induce autophagy became paramount, as well as how URM-099 would compare overall to AIs with respect to overall efficacy. In this study (Thomas et al., 2018), a well-characterized *in vitro* model of MDM ± HIV-1 infection was utilized to compare URM-099 with rapamycin, 2-hydroxy-β-cyclodextrin (HBC), clonidine, metformin and desmethylclomipramine (DMC) on parameters of metabolic health (i.e. mitochondrial dehydrogenase, MTT), autophagy and depot size and retention of endosomal nanoATV. While AIs such as HBC, clonidine and rapamycin exhibited favorable augmentation of parameters that would boost PK parameters of nanoATV, and metformin is currently in a clinical trial in combination with maraviroc to treat hepatic steatosis in people with HIV-1 ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT03129113) Identifier: NCT03129113), the aggregate effects of URM-099 in all these assays demonstrate that it is superior to the other AIs, with the added benefit of being anti-inflammatory and cyto- and neuroprotective (Goodfellow et al., 2013; Marker et al., 2013; Tomita et al., 2017; Ibrahim et al., 2016).

Thus, URM-099, with its broad spectrum of inflammatory kinase inhibitory activity, has the potential to decrease drug dosages with respect to nanoART treatment for HIV-1, which in turn may decrease cART-related toxicity and further augment nanoART antiviral efficacy in persistently infected macrophage reservoirs. Additionally, other diseases where TFEB has been identified as a therapeutic target such as Alzheimer's, Parkinson's and Huntington's may benefit from URM-099 therapy. The observation that all these neurodegenerative diseases, as well as HAND, share the common feature of microglial activation suggests that URM-099's unique broad spectrum of antiinflammatory activity may significantly extend its therapeutic utility for diseases that share macrophage activation as a pathogenetic characteristic.

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BIBLIOGRAPHY

- Abbas W, Tariq M, Iqbal M, Kumar A, Herbein G (2015) Eradication of HIV-1 from the Macrophage Reservoir: An Uncertain Goal? *Viruses* 7:1578–1598 doi:10.3390/v7041578 [PubMed: 25835530]
- Alexaki A, Liu Y, Wigdahl B (2008) Cellular Reservoirs of HIV-1 and their Role in Viral Persistence *Current HIV research* 6:388–400 [PubMed: 18855649]
- Arthos J et al. (2002) The role of the CD4 receptor versus HIV coreceptors in envelope-mediated apoptosis in peripheral blood mononuclear cells *Virology* 292:98–106 doi:10.1006/viro.2001.1266 [PubMed: 11878912]
- Bodner A, Maroney AC, Finn JP, Ghadge G, Roos R, Miller RJ (2002) Mixed lineage kinase 3 mediates gp120IIIB-induced neurotoxicity *Journal of neurochemistry* 82:1424–1434 [PubMed: 12354290]
- Borsello T, Forloni G (2007) JNK signalling: a possible target to prevent neurodegeneration *Current pharmaceutical design* 13:1875–1886 [PubMed: 17584114]

- Brancho D, Ventura JJ, Jaeschke A, Doran B, Flavell RA, Davis RJ (2005) Role of MLK3 in the regulation of mitogen-activated protein kinase signaling cascades *Molecular and cellular biology* 25:3670–3681 doi:10.1128/MCB.25.9.3670-3681.2005 [PubMed: 15831472]
- Brass AL et al. (2008) Identification of host proteins required for HIV infection through a functional genomic screen *Science (New York, NY)* 319:921–926 doi:10.1126/science.1152725
- Buchsbaum RJ, Connolly BA, Feig LA (2002) Interaction of Rac exchange factors Tiam1 and Ras-GRF1 with a scaffold for the p38 mitogen-activated protein kinase cascade *Molecular and cellular biology* 22:4073–4085 [PubMed: 12024021]
- Campbell GR, Rawat P, Bruckman RS, Spector SA (2015) Human Immunodeficiency Virus Type 1 Nef Inhibits Autophagy through Transcription Factor EB Sequestration *PLoS Pathogens* 11:e1005018 doi:10.1371/journal.ppat.1005018 [PubMed: 26115100]
- Cicala C et al. (2006) R5 and X4 HIV envelopes induce distinct gene expression profiles in primary peripheral blood mononuclear cells *Proceedings of the National Academy of Sciences of the United States of America* 103:3746–3751 doi:10.1073/pnas.0511237103 [PubMed: 16505369]
- Cicala C et al. (2002) HIV envelope induces a cascade of cell signals in non-proliferating target cells that favor virus replication *Proceedings of the National Academy of Sciences of the United States of America* 99:9380–9385 doi:10.1073/pnas.142287999 [PubMed: 12089333]
- Clayton KL, Garcia JV, Clements JE, Walker BD (2017) HIV Infection of Macrophages: Implications for Pathogenesis and Cure *Pathogens & immunity* 2:179–192 doi:10.20411/pai.v2i2.204 [PubMed: 28752134]
- Dash PK et al. (2012) Long-acting NanoART Elicits Potent Antiretroviral and Neuroprotective Responses in HIV-1 Infected Humanized Mice *AIDS (London, England)* 26:2135–2144 doi:10.1097/QAD.0b013e328357f5ad
- Deng K, Siliciano RF (2014) HIV: Early treatment may not be early enough *Nature* 512:35–36 doi:10.1038/nature13647 [PubMed: 25043038]
- Dhanasekaran DN, Reddy EP (2008) JNK signaling in apoptosis *Oncogene* 27:6245–6251 doi:10.1038/onc.2008.301 [PubMed: 18931691]
- Eggert D et al. (2010) Neuroprotective activities of CEP-1347 in models of neuroAIDS *Journal of immunology (Baltimore, Md: 1950)* 184:746–756 doi:10.4049/jimmunol.0902962
- Fader CM, Sanchez DG, Mestre MB, Colombo MI (2009) TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways *Biochimica et biophysica acta* 1793:1901–1916 doi:10.1016/j.bbamcr.2009.09.011 [PubMed: 19781582]
- Fischer T, Wyatt CM, D'Agati VD, Croul S, McCourt L, Morgello S, Rappaport J (2014) Mononuclear phagocyte accumulation in visceral tissue in HIV encephalitis: evidence for increased monocyte/macrophage trafficking and altered differentiation *Curr HIV Res* 12:201–212 [PubMed: 25026899]
- Gallo KA, Johnson GL (2002) Mixed-lineage kinase control of JNK and p38 MAPK pathways *Nature reviews Molecular cell biology* 3:663–672 doi:10.1038/nrm906 [PubMed: 12209126]
- Gallo KA, Mark MR, Scadden DT, Wang Z, Gu Q, Godowski PJ (1994) Identification and characterization of SPRK, a novel src-homology 3 domain-containing proline-rich kinase with serine/threonine kinase activity *The Journal of biological chemistry* 269:15092–15100 [PubMed: 8195146]
- Gendelman HE, Gelbard HA (2014) Adjunctive and Long-Acting Nanoformulated Antiretroviral Therapies for HIV-associated neurocognitive disorders *Current opinion in HIV and AIDS* 9:585–590 doi:10.1097/COH.0000000000000111 [PubMed: 25226025]
- Gnanadhas DP et al. (2017) Autophagy facilitates macrophage depots of sustained-release nanoformulated antiretroviral drugs *The Journal of Clinical Investigation* 127:857–873 doi:10.1172/JCI90025 [PubMed: 28134625]
- Goodfellow VS et al. (2013) Discovery, synthesis, and characterization of an orally bioavailable, brain penetrant inhibitor of mixed lineage kinase 3 *Journal of medicinal chemistry* 56:8032–8048 doi:10.1021/jm401094t [PubMed: 24044867]
- Handley ME, Rasaiyaah J, Chain BM, Katz DR (2007) Mixed lineage kinases (MLKs): a role in dendritic cells, inflammation and immunity? *International journal of experimental pathology* 88:111–126 doi:10.1111/j.1365-2613.2007.00531.x [PubMed: 17408454]

- Harris J et al. (2011) Autophagy controls IL-1 β secretion by targeting pro-IL-1 β for degradation The Journal of biological chemistry 286:9587–9597 doi:10.1074/jbc.M110.202911 [PubMed: 21228274]
- Hehner SP et al. (2000) Mixed-lineage kinase 3 delivers CD3/CD28-derived signals into the IkappaB kinase complex Mol Cell Biol 20:2556–2568 [PubMed: 10713178]
- Hosokawa N et al. (2009) Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy Molecular biology of the cell 20:1981–1991 doi:10.1091/mbc.E08-12-1248 [PubMed: 19211835]
- Ibrahim SH, et al. 2016 Mixed lineage kinase 3 mediates release of C-X-C motif ligand 10-bearing chemotactic extracellular vesicles from lipotoxic hepatocytes. Hepatology 63:731–44. [PubMed: 26406121]
- Jope RS, Yuskaitis CJ, Beurel E (2007) Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics Neurochemical research 32:577–595 doi:10.1007/s11064-006-9128-5 [PubMed: 16944320]
- Kant S, Swat W, Zhang S, Zhang ZY, Neel BG, Flavell RA, Davis RJ (2011) TNF-stimulated MAP kinase activation mediated by a Rho family GTPase signaling pathway Genes & development 25:2069–2078 doi:10.1101/gad.17224711 [PubMed: 21979919]
- Leung IW, Lassam N (1998) Dimerization via tandem leucine zippers is essential for the activation of the mitogen-activated protein kinase kinase kinase, MLK-3 The Journal of biological chemistry 273:32408–32415 [PubMed: 9829970]
- Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings Advanced drug delivery reviews 46:3–26 [PubMed: 11259830]
- Lotharius J, Falsig J, van Beek J, Payne S, Dringen R, Brundin P, Leist M (2005) Progressive degeneration of human mesencephalic neuron-derived cells triggered by dopamine-dependent oxidative stress is dependent on the mixed-lineage kinase pathway The Journal of neuroscience: the official journal of the Society for Neuroscience 25:6329–6342 doi:10.1523/JNEUROSCI.1746-05.2005 [PubMed: 16000623]
- Ma Q et al. (2013) Pharmacokinetic interactions of CEP-1347 and atazanavir in HIV-infected patients Journal of neurovirology 19:254–260 doi:10.1007/s13365-013-0172-z [PubMed: 23737347]
- Marker DF et al. (2013) The new small-molecule mixed-lineage kinase 3 inhibitor URM-099 is neuroprotective and anti-inflammatory in models of human immunodeficiency virus-associated neurocognitive disorders The Journal of neuroscience: the official journal of the Society for Neuroscience 33:9998–10010 doi:10.1523/jneurosci.0598-13.2013 [PubMed: 23761895]
- Maroney AC et al. (2001) Cep-1347 (KT7515), a semisynthetic inhibitor of the mixed lineage kinase family The Journal of biological chemistry 276:25302–25308 doi:10.1074/jbc.M011601200 [PubMed: 11325962]
- Martina JA, Chen Y, Gucek M, Puertollano R (2012) MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB Autophagy 8:903–914 doi:10.4161/auto.19653 [PubMed: 22576015]
- Mishra R et al. (2007) Glycogen synthase kinase-3 β induces neuronal cell death via direct phosphorylation of mixed lineage kinase 3 The Journal of biological chemistry 282:30393–30405 doi:10.1074/jbc.M705895200 [PubMed: 17711861]
- Nakahira K et al. (2011) Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome Nature immunology 12:222–230 doi:10.1038/ni.1980 [PubMed: 21151103]
- Nguyen DG, Yin H, Zhou Y, Wolff KC, Kuhlen KL, Caldwell JS (2007) Identification of novel therapeutic targets for HIV infection through functional genomic cDNA screening Virology 362:16–25 doi:10.1016/j.virol.2006.11.036 [PubMed: 17257639]
- Pastore N et al. (2016) TFEB and TFE3 cooperate in the regulation of the innate immune response in activated macrophages Autophagy 12:1240–1258 doi:10.1080/15548627.2016.1179405 [PubMed: 27171064]

- Sami Saribas A, Cicalese S, Ahooyi TM, Khalili K, Amini S, Sariyer IK (2017) HIV-1 Nef is released in extracellular vesicles derived from astrocytes: evidence for Nef-mediated neurotoxicity *Cell death & disease* 8:e2542 doi:10.1038/cddis.2016.467 [PubMed: 28079886]
- Sardo L, Iordanskiy S, Klase Z, Kashanchi F (2015) HIV-1 Nef blocks autophagy in human astrocytes *Cell cycle (Georgetown, Tex)* 14:3781–3782 doi:10.1080/15384101.2015.1105700
- Su KH et al. (2016) HSF1 critically attunes proteotoxic stress sensing by mTORC1 to combat stress and promote growth *Nature cell biology* 18:527–539 doi:10.1038/ncb3335 [PubMed: 27043084]
- Sui Z et al. (2006a) Inhibition of mixed lineage kinase 3 prevents HIV-1 Tat-mediated neurotoxicity and monocyte activation *Journal of immunology (Baltimore, Md: 1950)* 177:702–711
- Sui Z et al. (2006b) Human immunodeficiency virus-encoded Tat activates glycogen synthase kinase-3beta to antagonize nuclear factor-kappaB survival pathway in neurons *The European journal of neuroscience* 23:2623–2634 doi:10.1111/j.1460-9568.2006.04813.x [PubMed: 16817865]
- Thomas MB et al. (2018). Modulating cellular autophagy for controlled antiretroviral drug release. *Nanomedicine (Lond)* doi:10.2217/nnm-2018-0224.
- Tibbles LA, Ing YL, Kiefer F, Chan J, Iscove N, Woodgett JR, Lassam NJ (1996) MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6 *EMBO J* 15:7026–7035 [PubMed: 9003778]
- Tomita K, et al. (2017). Mixed-lineage kinase 3 pharmacological inhibition attenuates murine nonalcoholic steatohepatitis. *JCI Insight* doi: 10.1172/jci.insight.94488.
- Wang MJ, Huang HY, Chen WF, Chang HF, Kuo JS (2010) Glycogen synthase kinase-3beta inactivation inhibits tumor necrosis factor-alpha production in microglia by modulating nuclear factor kappaB and MLK3/JNK signaling cascades *J Neuroinflammation* 7:99 doi: 10.1186/1742-2094-7-99 [PubMed: 21194439]
- Xu Z, Maroney AC, Dobrzanski P, Kukekov NV, Greene LA (2001) The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis *Molecular and cellular biology* 21:4713–4724 doi:10.1128/MCB.21.14.4713-4724.2001 [PubMed: 11416147]
- Yang X, Chen Y, Gabuzda D (1999) ERK MAP kinase links cytokine signals to activation of latent HIV-1 infection by stimulating a cooperative interaction of AP-1 and NF-kappaB *The Journal of biological chemistry* 274:27981–27988 [PubMed: 10488148]
- Yang Z, Klionsky DJ (2009) An overview of the molecular mechanism of autophagy *Current topics in microbiology and immunology* 335:1–32 doi:10.1007/978-3-642-00302-8_1 [PubMed: 19802558]
- Zaikos TD, Collins KL (2014) Long-lived reservoirs of HIV-1 *Trends in microbiology* 22:173–175 doi:10.1016/j.tim.2014.02.015 [PubMed: 24641995]
- Zhang G et al. (2016) The Mixed Lineage Kinase-3 Inhibitor URM-099 Improves Therapeutic Outcomes for Long-Acting Antiretroviral Therapy *Nanomedicine: nanotechnology, biology, and medicine* 12:109–122 doi:10.1016/j.nano.2015.09.009
- Zhang H, Gallo KA (2001) Autoinhibition of mixed lineage kinase 3 through its Src homology 3 domain *The Journal of biological chemistry* 276:45598–45603 doi:10.1074/jbc.M107176200 [PubMed: 11590155]

Figure 1:

Neuroprotection, anti-inflammatory and antiretroviral putative roles of the broad spectrum MLK3 inhibitor, URM-099 in humans; (A) Disease state of the brain during HIV-1 infection with neuroinflammation and impaired synaptic communication, (B) cartoon of a brain section with normal microglia with ramified processes (green). After HIV-1 Infection microglia become amoeboid losing their ramified processes (red). During HIV infection viral proteins, other cellular metabolites such as pro-inflammatory cytokines and chemokines disrupt normal neural function and perpetuate brain inflammation. Eventually all these toxic metabolites contribute to synaptodendritic damage. (C) A magnified cartoon depicts the HIV-1 entry process in microglia through interactions between the chemokines CCR5 or CXCR4 and HIV-1 envelope glycoprotein gp120. (D-E) Panel D depicts the effects

of nanoformulated ART alone after endosomal uptake in an HIV-1 infected macrophage. Panel E depicts the synergistic effect of URM-099 and nanoformulated antiretroviral therapy (nanoART) results in neuroprotection and anti-inflammatory effects associated with increased cell viability, as well as retention of nanoART in mononuclear phagocytes associated with enhanced antiretroviral activity. This very complex process occurs in the setting of HIV-1 infection where nuclear translocation and integration of the viral DNA with the host genome occurs, followed by transcription and translation of viral genes. In particular, HIV-1 Nef protein gets assembled into the budding immature virion during the maturation process. Nef augments viral replication by sequestering TFEB in the cytoplasm, which inhibits autophagy by preventing production of autophagosomes and fusion with lysosomes that would normally allow the infected cell to dispose of immature virions. However, when URM-099 is administered with nanoART, it reverses Nef-mediated sequestration of TFEB in cytoplasm via an as yet unknown, but presumably mixed lineage kinase-mediated mechanism, and allows production of autophagosomes with lysosomal biogenesis. This in turn allows virucidal concentrations of nanoART to accumulate in endosomal depots for prolonged periods of time, and effectively shuts down persistent infection in macrophages.