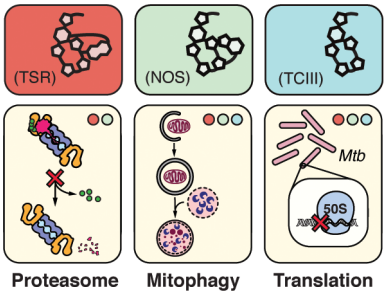
Thiopeptides Induce Proteasome-Independent Activation of Cellular Mitophagy

Kelly E. Bird, Christian Xander, Sebastian Murcia, Alan A. Schmalstig, Xianxi Wang, Michael J. Emanuele, Miriam Braunstein,* and Albert A. Bowers*

ABSTRACT: Thiopeptide antibiotics are emerging clinical candidates that exhibit potent antibacterial activity against a variety of intracellular pathogens, including *Mycobacterium tuberculosis* (*Mtb*). Many thiopeptides directly inhibit bacterial growth by disrupting protein synthesis. However, recent work has shown that one thiopeptide, thiostrepton (TSR), can also induce autophagy in infected macrophages, which has the potential to be exploited for host-directed therapies against intracellular pathogens, such as *Mtb*. To better define the therapeutic potential of this class of antibiotics, we studied the host-directed effects of a suite of natural thiopeptides that spans five structurally diverse thiopeptide classes, as well as several analogs. We discovered that thiopeptides as a class induce selective autophagic removal of mitochondria, known as mitophagy. This activity is independent of other biological activities, such as proteasome inhibition or antibiotic activity. We also find that many thiopeptides exhibit potent activity against intracellular *Mtb* in macrophage infection models. However, the thiopeptide-induced mitophagy occurs outside of pathogen-containing autophagosomes and does not appear to contribute to thiopeptide control of intracellular *Mtb*. These results expand basic understanding of thiopeptide biology and provide key guidance for the development of new thiopeptide antibiotics and host-directed therapeutics.



Mycobacterium tuberculosis (*Mtb*), the bacterium responsible for tuberculosis (TB), and many other intracellular pathogens are quickly becoming resistant to the most common antibiotics. For *Mtb*, the effectiveness of first-line therapy (rifampicin, isoniazid, ethambutol, and pyrazinamide) is waning due to the rise in multi- and extensively drug resistant (MDR; XDR) *Mtb*.[1] Thus, there is a strong need for antibiotics with new mechanisms of action. Thiopeptides may provide such a solution. Thiopeptides are a broad class of structurally complex, macrocyclic, peptide-derived antibiotics, that have been shown to possess activity against mycobacteria.[2−8] This class of promising natural products has been studied for some time but has attracted renewed attention on account of their clinically unique mechanisms of action. Several thiopeptides, including thiostrepton (TSR, **3**), nosiheptide (NOS, **6**), and the family of close homologues known as thiocillins, including micrococcin P1 (MP1, **1**) and thiocillin III (TCIII, **2**), inhibit bacterial protein translation (Figure 1A).[9] Importantly, they bind to bacterial ribosomes in a cleft that is not targeted by other clinically deployed, ribosome targeting antibiotics, such as macrolides, aminoglycosides, and oxazolidinones.[10] Another group of thiopeptides, represented by GE37468 (GE, **4**) target the translation elongation factor, EF-Tu, which shuttles aminoacyl tRNAs into the ribosome.[11,12] EF-Tu represents a novel antibiotic target, and semisynthetic analogs of GE2270A have been advanced to clinical trials.[13−16] Together, these thiopeptides provide potentially attractive starting points for antibiotic development.

Select thiopeptides also have effects on mammalian cancer cells and macrophages.[5,17−26] TSR in particular interacts with several mammalian targets, including the oncogenic transcription factor, Forkhead box M1 (FoxM1), and the 26S proteasome, both of which could potentially be leveraged for anticancer chemotherapy (Figure 1B, pathways 1 and 2).[27,28] Recently, TSR was also shown to induce macroautophagy (hereafter referred to as autophagy) in macrophages.[5,29−31] Autophagy is a bulk intracellular degradation process in which cytoplasmic components are encapsulated in double-membrane bound vesicles (i.e., autophagosomes) and shuttled to lysosomes for degradation (Figure 1B, pathway 3).[32,33] Eukaryotic cells use autophagy for nonspecific processes, such as recycling cytosolic materials for nutrient homeostasis. Additionally, there are also cargo-specific forms of autophagy that target pathogens, damaged organelles (e.g., mitochondria, endoplasmic reticulum), or protein aggregates.[32,34,35] Selective autophagy of pathogens is a host defense against intracellular infections.[35−37] Thus, the autophagy activity of TSR is enticing from an antibiotic development standpoint, and chemical

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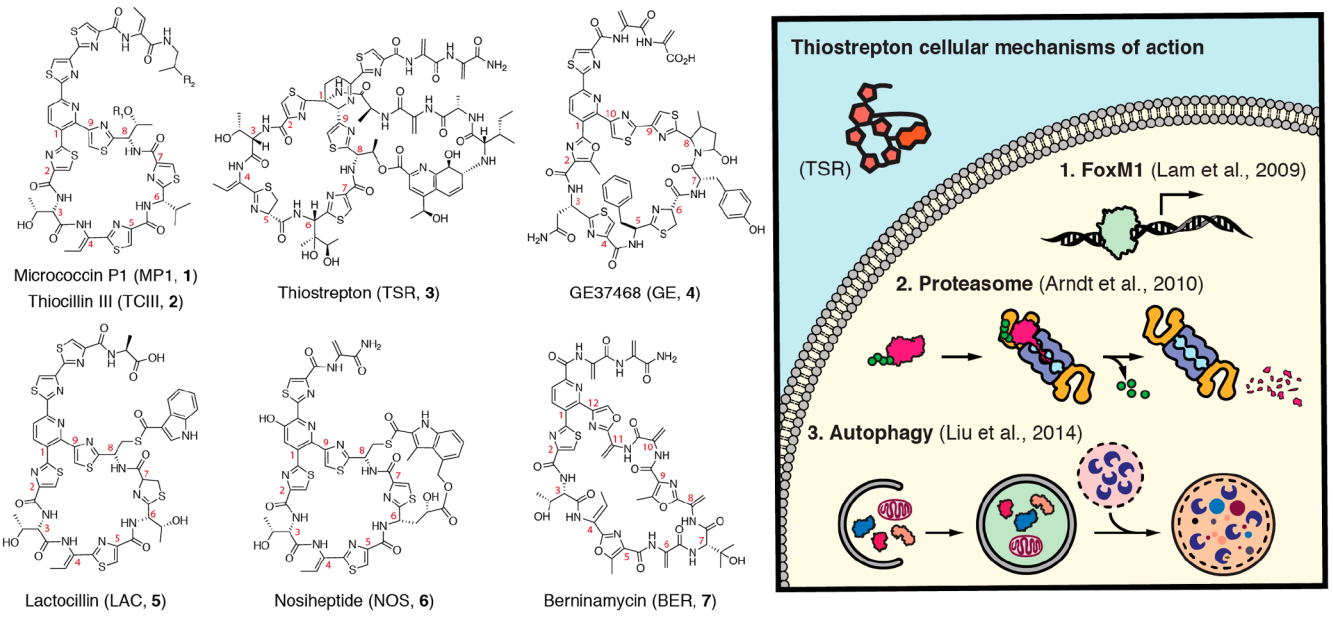


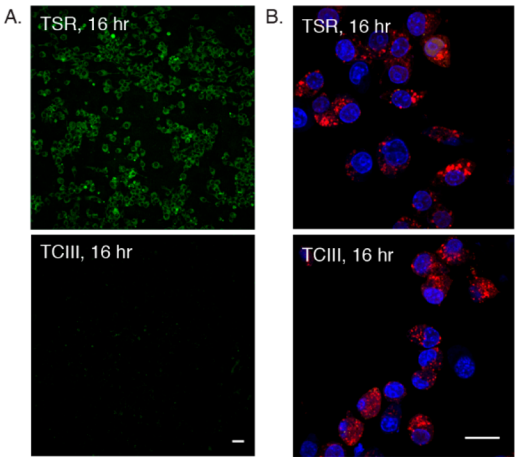
Figure 1. Structures of naturally occurring thiopeptides have similar scaffolds with unique functionalities decorated around the core macrocycle. Micrococcin P1 (MP1, **1**, R1: H, R2: OH), thiocillin III (TCIII, **2**, R1: CH_3, R2: =O), thiostrepton (TSR, **3**), lactocillin (LAC, **5**), and nosiheptide (NOS, **6**) contain 26-membered major macrocycles. GE37468 (GE, **3**) contains a 29-membered macrocycle, and berninamycin contains a 35-membered ring (BER, **7**). Residues on the TCIII scaffold have been highlighted by red numbers; these values correspond to the amino acid sequence of the nascent core peptide. TSR is known to have multiple bioactivities within the cell consisting of (1) decreased FoxM1 expression, (2) inhibition of the proteasome, and (3) induction of autophagy.

inducers of autophagy are actively being investigated as host-directed therapeutics to enhance immune clearance of intracellular pathogens.[36,38] For *Mtb* specifically, a number of compounds that induce autophagy are shown to reduce the intracellular burden of *Mtb*.[36,39−41] A thorough understanding of the structural and mechanistic basis of the host-directed activities of TSR and cognate thiopeptides, as well as their contributions to intracellular bacterial clearance, will benefit further antibiotic development. Moreover, recent work has uncovered numerous thiopeptide biosynthetic gene clusters in human gut-associated microbiota, suggesting that these bacterial metabolites may play additional important roles.[42]

Herein, we investigate the activity of a suite of thiopeptides in macrophages to better understand their host-directed and antibacterial effects. We employ a structurally diverse group of thiopeptides, representative of the five major classes of thiopeptide discovered to date, TSR, NOS, TCIII, GE, and berninamycin (BER, 7), to investigate the activities of this compound set against the known TSR mammalian phenotypes: proteasome inhibition, autophagy induction, and cytotoxicity. The activity profiles of these thiopeptides, as well as a set of semisynthetic and mutasynthetic analogs, demonstrate that not all thiopeptides inhibit the proteasome or exhibit cytotoxic effects. In contrast to these findings, all thiopeptide classes examined in this study induce proteasome-independent activation of autophagy. We further compared the activities of thiopeptides on virulent *Mtb*. We demonstrate potent thiopeptide activity for all antibiotically active compounds on *Mtb* in culture and on *Mtb* in macrophages. Additionally, we reveal that thiopeptide inhibition of intracellular *Mtb* depends on thiopeptide antibacterial activity (i.e., inhibition of bacterial protein translation) and that host-directed effects (i.e., induction of autophagy) play a minimal role. Subsequent biochemical and cellular characterization revealed that the lack of synergy between autophagy and pathogen clearance can be explained by thiopeptides inducing a cargo-specific type of autophagy known as mitophagy (i.e., autophagic elimination of mitochondria), which does not significantly aid in intracellular *Mtb* clearance. Cumulatively, this structure−activity profile of thiopeptides will inform future, rational development of unnatural thiopeptide antibiotics and autophagy as a host-directed strategy for treating intracellular bacteria.

CELLULAR ACTIVITY OF CANONICAL THIOPEPTIDE FAMILY MEMBERS

We first examined the effects of thiopeptides on proteasome activity, cytotoxicity, and autophagy in murine macrophages (RAW 264.7 cells). A previously reported GFP-based reporter assay was used to measure proteasomal activity (Figure S.2.1).[43] This assay relies on a ubiquitin-GFP construct (Ub-R-GFP), which is rapidly degraded by proteasomes in cells; proteasome inhibition then induces accumulation of fluorescence (Figures 2A, S4). Cells containing this reporter were treated with our suite of thiopeptides, as well as the known proteasome inhibitor, MG-132, in a dose-dependent manner (Figure S5). After 16 h, GFP levels were quantified and used to calculate EC_{50} values (Figure 2C). Our results confirm that TSR inhibits proteasomes in cells at micromolar concentrations and identifies NOS and GE as two new classes of thiopeptides that inhibit proteasomes, as indicated by strong accumulation of Ub-R-GFP (Figure 2A). Of these active thiopeptides, GE proved the most potent with an EC_{50} of 0.19 μM, NOS at 0.38 μM, and TSR at 1.45 μM. In contrast, TCIII and BER exhibited no detectable activity in this assay. As many proteasome inhibitors are also well-known to induce pronounced cytotoxicity, we also measured cytotoxicity with a standard cell viability assay, CellTiter Glo. Of the thiopeptides tested, only TSR displayed cytotoxicity, which



C.

	Cell Based Proteasome Inhibition EC_{50} (μM) ± SD	Induction of Autophagy EC_{50} (μM) ± SD	Cytotoxicity LC_{50} (μM) ± SD
MG-132	0.18 ± 0.06	0.07 ± 0.12	0.47 ± 0.12
Rapamycin	> 10	0.32 ± 0.02	8.98 ± 0.32
Isoniazid	> 10	> 10	> 10
Chloramphenicol	> 10	> 10	> 10
TSR	1.45 ± 0.10	0.52 ± 0.15	2.10 ± 0.13
NOS	0.38 ± 0.04	0.18 ± 0.26	> 10
TCIII	> 10	0.09 ± 0.06	> 10
GE	0.19 ± 0.07	0.26 ± 0.11	> 10
BER	> 10	0.36 ± 0.13	> 10

Figure 2. Natural thiopeptides were analyzed for three biological activities in RAW 264.7 murine macrophages: (1) proteasome inhibition via Ub-R-GFP reporter assay, (2) induction of autophagy via a GFP-RFP-LC3 fusion protein, and (3) cytotoxicity via Cell Titer Glo. (A) Proteasome inhibition was measured by the increased accumulation of GFP signal. Cells were treated with thiopeptide for 16 h at 5 mM and monitored for GFP signal. Representative images of TSR and TCIII inhibition were collected on the INCell Analyzer 2200. Scale bar: 40 μm. (B) Induction of autophagy was measured by average red puncta, or autophagolysosomes, per cell. Cells were treated with thiopeptide (5 μM) for 16 h and monitored for RFP-puncta and nuclei (DAPI). Representative images are shown for TSR and TCIII. Scale bar: 40 μm. (C) These assays were completed in a high-throughput manner using the INCell Analyzer 2200 to calculate thiopeptide EC50s. Cells were treated in a dose-dependent manner. Proteasome inhibition was measured by average GFP fluorescence per cell. Induction of autophagy was quantified by the number of RFP puncta per cell, and cytotoxicity was measured by ATP depletion. Color coding indicates the potency of the compound, spanning from most potent (red) to least potent (green).

was on par with the cytotoxicity of the positive control, MG-132 (Figures 2C, S6). Neither GE nor NOS, both of which exhibited antiproteasome activity, proved cytotoxic in this assay. Overall, this result indicates that while TSR, GE, and NOS all inhibit proteasomes, they have distinct cytotoxicity profiles that may be due to a unique mechanism of proteasome inhibition. Alternatively, TSR may have additional targets that contribute to its cytotoxicity.

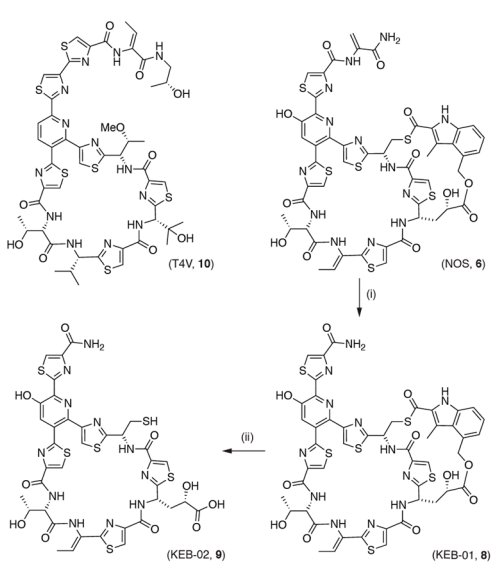
Separately, we employed a commercial reporter assay (RAW-Difluo mLC3) to evaluate the effects of thiopeptides on autophagy (Figure S.2.2).[44,45] mLC3 (microtubule-associated protein 1 light chain 3) localization is a hallmark of autophagosomes, and in the RAW-Difluo mLC3 assay, a mLC3-GFP-RFP fusion protein is used to quantify autophagosomes (Figure 2B). RAW-Difluo mLC3 cells were incubated for 16 h with thiopeptides or rapamycin (RAPA), an mTOR inhibitor and known inducer of autophagy.[46,47] EC_{50}'s were then determined based on the number of RFP-LC3 fluorescent puncta per cell (Figures S7, S8). As expected, based on the work of Zheng et al., TSR potently induced autophagy similarly to the control RAPA (Figure 2.B).[5] Furthermore, we discovered that the proteasome inhibitors NOS and GE also induced autophagy at concentrations comparable to TSR (Figure 2C). The thiopeptides that had no activity against proteasomes, TCIII and BER, also induced autophagy. In particular, TCIII proved the most potent inducer with an EC_{50} of 90 nM (Figure 2B). These data suggest that thiopeptides are broadly capable of inducing autophagy, regardless of perturbations to proteasomal function, a bioactivity characteristic of only a subset of thiopeptides examined in this study.

THIOPEPTIDE DERIVATIVES DISCRIMINATE BETWEEN AUTOPHAGY AND ANTIBIOTIC ACTIVITY

In light of the phenotypic assay results, we sought to build additional structure–activity relationships for a subset of structurally similar thiopeptides: TSR, NOS, and TCIII. These three compounds contain near-sequence identical macrocycles, comprised of four identically spaced sulfur heterocycles at positions 2, 5, 7, and 9 and a 3/4 threonine/dehydrobutyrine pair (Figure 1A). NOS and TSR also exhibit a heavily modified secondary macrocycle, one end of which is appended as an ester to the position 8 residue. With TSR, Arndt and co-workers had previously shown that disruption of this secondary macrocycle via alkaline hydrolysis abrogated proteasome activity.[22] However, this modification leaves a substantial fragment of TSR's secondary ring still attached, which remains a significant departure from the structures of TCIII and NOS and might alter target engagement. In order to build a coherent structure–activity series between the relatively simple TCIII core and the considerably more complex NOS and TSR scaffolds, we focused on chemical derivatization of NOS. More specifically, we sought to transform NOS into a more TCIII-like analog by removing the indolyl side-ring and probing the effect on NOS's antiproteasomal activity. Additionally, we prepared an antibiotically inactive variant of the TCIII scaffold, which contains a T4V mutation, to probe the impact of the antibiotic activity (i.e., translation inhibition) in our cell assays.[48,49]

Analogs of NOS could be prepared by a semisynthetic approach, while the key TCIII analog could be accessed by a previously reported gene replacement strategy (Figure 3). To pare down NOS to a TCIII-like core macrocycle, we began by removing the C-terminal dehydroalanine (Dha), which would largely eliminate the possibility for formation of covalent adducts in cells. This transformation could be carried out by treating NOS with diethylamine in THF for 16 h to give KEB-01 (**8**) in good yield.[50] Subsequent base hydrolysis was used to cleave both ester linkages to the indolyl side-ring, completely removing this group from the scaffold and affording KEB-02 (**9**), a Val6b–OHGlu, Thr8Cys homologue of the core macrocycle of TCIII. Structures of the new analogs were

A.



B.

	Cell Based Proteasome Inhibition EC50 (μM) ± SD	Induction of Autophagy EC50 (μM) ± SD	Cell Viability LC50 (μM) ± SD	Bacterial Translation Inhibition IC50 (μM) ± SD
TCIII	> 10	0.09 ± 0.06	> 10	0.45 ± 0.11
T4V	> 10	0.11 ± 0.24	> 10	> 100
NOS	0.38 ± 0.04	0.18 ± 0.26	> 10	0.99 ± 0.29
KEB-01	1.95 ± 0.07	0.67 ± 0.46	> 10	0.69 ± 0.13
KEB-02	> 10	0.17 ± 0.07	> 10	> 100

Figure 3. Unnatural thiopeptides probe importance of conserved epitope within nosiheptide structure. (A) Structures of unnatural thiopeptides and methodology for synthetic modifications to the nosiheptide scaffold. Conditions: (i) 20% NEt_2, THF RT, 16 h, 75% yield (II) NaOH (5 equiv), THF/MeOH (3:8), RT 4 h, 68% yield. (B) Unnatural thiopeptide bioactivities were assessed using three reporter assays: (1) proteasome inhibition via Ub-R-GFP reporter assay, (2) induction of autophagy via GFP-RFP-LC3 fusion protein, and (3) cytotoxicity via Cell Titer Glo. These assays were completed in a dose-dependent and high-throughput manner using the INCell Analyzer 2200 to calculate EC_{50}'s. Proteasome inhibition was measured by average GFP fluorescence per cell. Induction of autophagy was quantified by the number of RFP puncta per cell, and cytotoxicity was measured by ATP depletion. Color scale ranges from most potent (red) to least potent (green).

confirmed by both 1D and 2D nuclear magnetic resonance (NMR) spectroscopy.[51−54] Separately, we prepared the antibiotically inactive TCIII variant, T4V (**10**; Figure 3A).[48] Multiple reports demonstrate that replacement of the normally sp^2 alpha-carbon on residue 4 in the TCIII core with an sp^3 homologue demolishes the compound's antibiotic activity, likely by altering the overall conformational rigidity of the ring.[48,49,55] We could access this variant by genetic manipulation of an engineered producer as previously reported.

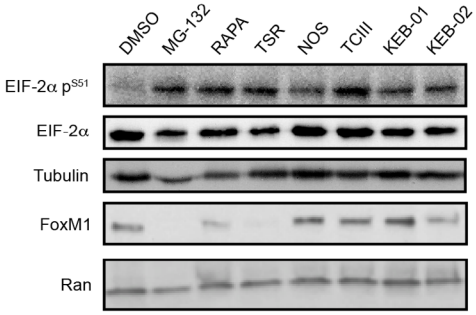
This focused collection of analogs was then tested in the cellular assays for proteasome inhibition, autophagy induction, and cytotoxicity (Figure 3B). Additionally, we tested all new compounds for inhibition of protein translation in an S30 *E. coli* cell lysate assay as an *in vitro* surrogate for antibiotic activity (Figure S11).[56] KEB-01 largely retained all activities of its NOS parent suggesting that the C-terminal Dha was not a key source of this molecule's activity. Meanwhile, removal of the indolyl side-ring of NOS abolished proteasome inhibition, as seen with KEB-02. However, KEB-02 retained the ability to induce autophagy (EC_{50} of 0.17 μM) without cytotoxicity. The autophagy activity of KEB-02 was nearly equipotent to NOS and TCIII, suggesting that the structurally similar macrocycles (Figure S1) may be responsible for the autophagy activity. Unexpectedly, KEB-02 lacked antibiotic activity as determined by the *in vitro* protein translation assay, suggesting that removal of the secondary macrocycle reduces thiopeptide−protein interactions or changes the overall conformation that is required for ribosome binding. Last, the TCIII variant, T4V, behaved similarly to its parent compound (TCIII), displaying no effect in the cellular proteasome assay and no cytotoxicity, but inducing autophagy with an EC_{50} of 0.11 μM. As previously reported, T4V showed no activity in protein translation assays.[48] Cumulatively, the bioactivities of the NOS and TCIII derivatives suggest that antibiotic activity is not essential for the induction of autophagy, as antibiotically inactive thiopeptides induce autophagy, and that the T8 functionalization of the NOS core macrocycle may be important for antiproteasomal activity, as removal of this substituent abolishes this activity.

THIOPEPTIDES BROADLY INDUCE EIF-2α PHOSPHORYLATION, TSR UNIQUELY AFFECTS PROTEASOME, FOXM1

While only a subset of thiopeptides exhibited activity against proteasomes, all thiopeptides within our library, both natural and modified, proved capable of inducing autophagy. Thus, it is possible that other targets previously associated with TSR proteasome inhibition might also be proteasome-independent targets of thiopeptides. In particular, proteasome inhibitors are known to induce endoplasmic reticulum (ER) stress, which can be a signal for autophagy, as part of the unfolded protein response (UPR).[57] TSR has been shown to induce EIF-2α phosphorylation, consistent with a proteasome inhibition-dependent ER-stress response, but this activity could also be related to a broader proteasome-independent mechanism by which thiopeptides induce autophagy.[5,58] TSR has also been shown to inhibit the expression of transcription factor FoxM1. Separate evidence has supported FoxM1 inhibition as either a direct target or a downstream result of proteasome inhibition, but this too could be an effect of a separate thiopeptide mechanism.[17,19−21,23,59,60] Thus, we probed EIF-2α phosphorylation and FoxM1 expression in macrophages treated with thiopeptides to see if these effects were general to thiopeptides, like autophagy, or specific to TSR. Additionally, we used *in vitro* cleavage assays to validate direct proteasome inhibition by thiopeptides and investigate their proteolytic subunit inhibition profiles. For these experiments, we focused on the structurally related subset of TSR, NOS, TCIII, and derivatives.

To probe the impact of thiopeptides on EIF-2α and FoxM1, RAW 264.7 cells were treated with compounds for 16 h, then cell lysates were subjected to Western blot analysis. When we probed ER-stress effects in these cells, we found that all thiopeptides, including TCIII, T4V, and KEB-02, which had no effect on proteasome activity, led to EIF-2α phosphorylation (Figure 4A). Since not all compounds inhibit the proteasome, this result demonstrates that, in many cases, EIF-2α phosphorylation in thiopeptide treated macrophages is not a result of proteasome inhibition. Elsewhere, EIF-2α phosphorylation has been found to be a broad stress signal that can be induced by a number of processes.[61,62] The EIF-2α phosphorylation triggered by thiopeptides may be related to the mechanism of autophagy induction. After probing FoxM1 levels in these cells, we found that only TSR decreased FoxM1

A.



B.

	Direct Proteasome Inhibition IC_{50} (μM)		
	Chymotrypsin-Like	Caspase-Like	Trypsin-Like
MG-132	0.01 ± 0.07	0.05 ± 0.02	0.04 ± 0.03
TSR	0.47 ± 0.20	2.56 ± 0.59	0.52 ± 0.20
NOS	2.05 ± 0.50	1.55 ± 0.39	13.00 ± 0.70
TCIII	> 30.00	> 30.00	> 30.00
T4V	> 30.00	> 30.00	> 30.00
KEB-01	1.35 ± 0.40	5.79 ± 0.46	7.47 ± 0.42
KEB-02	> 30.00	> 30.00	> 30.00

Figure 4. Proteasome-related activity of thiopeptides. (A) RAW 264.7 cells were treated with 5 μM of compound for 16 h, and cell lysates were subjected to Western blot analysis. The phosphorylation state of EIF-2α^{Ser51} was observed to assess thiopeptide activated ER stress; β-tubulin was used as a loading control. FoxM1 expression resulting from thiopeptide treatment; Ran was used as a loading control. (B) Thiopeptides were analyzed for direct inhibition of the three proteolytic activities associated with the proteasome: (1) chymotrypsin-like, (2) caspase-like, and (3) trypsin-like using the Proteasome-Glo assay. Purified 20S proteasome was treated with thiopeptides in a dose-dependent manner for 1 h and proteasome activity was assessed to determine IC_{50} values. Color coding indicates the potency of the compound, spanning from most potent (red) to least potent (green).

expression (Figure 4A). While the cytotoxic proteasome inhibitors MG-132 and TSR both showed a significant decrease in FoxM1 protein levels, in line with previous data on cancer cell lines, the proteasome inhibitor NOS and all the other thiopeptides tested had no effect on FoxM1 levels. This result suggests that FoxM1 downregulation is particular to the cytotoxic mechanisms of proteasome inhibition common to TSR and MG-132.

To probe if proteasome inhibition was a direct or indirect effect of thiopeptide treatment, we assessed the activity of reconstituted 20S proteasome *in vitro*. The proteolytic activity of chymotrypsin-, trypsin-, and caspase-like subunits was assessed by cleavage of activity-based luminogenic substrates (Figure S10). In agreement with the phenotypic assays, TSR, NOS, and KEB-01 all directly inhibited the proteasome (Figure 4B). TSR exhibits submicromolar IC_{50} values for chymotrypsin- and trypsin-like activities and is substantially less active against the caspase-like subunits. In contrast, NOS is a weaker and more uniform inhibitor with low micromolar activity against all three active sites; the KEB-01 derivative exhibits a similar profile to NOS. These data suggest that TSR related cytotoxicity is not exclusively related to proteasome inhibition but instead may be a result of interactions with additional biological targets, such as FoxM1.

THIOPEPTIDES DIRECTLY INHIBIT *MTB*

Given the common ability of thiopeptides to induce autophagy, we sought to determine whether this activity could be exploited to promote *Mtb* clearance in macrophages, which had previously been suggested in a study of TSR treatment of *Mycobacterium marinum*.[5] We focused on the series of structurally and mechanistically related thiopeptides, TSR, NOS, TCIII, and derivatives, as there is precedent for these classes of thiopeptides being active against mycobacteria.[2,4−7,36,63] Compounds were first tested for the ability to inhibit *Mtb* H37Rv in culture by the standard resazurin microtiter assay, or REMA (Figure S12).[64] As expected, the natural thiopeptides, TSR, NOS, and TCIII, as well as the semisynthetic derivative, KEB-01, were active against *Mtb in vitro* at IC_{50}'s comparable to kanamycin, a known antibiotic active against *Mtb* (Figure 5A, S13). On the other hand, T4V and KEB-02, which exhibited no inhibition in the *in vitro* assay (Figure 3.B), had no effect on *Mtb* growth with IC_{50} values greater than 10 μM. Notably, this stands as the first report of NOS against *Mtb*, although it has previously been found active against nontuberculosis mycobacteria.[7] On the basis of the result with KEB-02, the side-chain of the NOS scaffold is necessary for antibacterial activity, as it is for inhibition of protein translation.

To specifically assess thiopeptide activity against intracellular *Mtb*, we infected human macrophage-like THP-1 cells with luminescent, *luxBCADE-expressing* H37Rv *Mtb*. Intracellular *Mtb* growth could thus be measured and quantified based on the relative luminescence of this engineered strain (Figure S13). Treatment of infected macrophages with antibiotically active thiopeptides led to potent inhibition of intracellular *Mtb* growth (Figure 5A). TSR, NOS, TCIII, and KEB-01 all exhibited potent inhibitory effects on intracellular *Mtb* growth. Antibiotically inactive thiopeptides, T4V and KEB-02, were significantly less active against intracellular *Mtb* ($p < 0.0001$), which suggests that thiopeptide-induced autophagy on its own is insufficient to inhibit intracellular *Mtb* growth.

To further address the possibility that thiopeptide-induced autophagy might contribute to the activities of TSR, NOS, and TCIII against intracellular *Mtb*, we tested these compounds in the presence of wortmannin (WRT), a potent PI3-kinase (PI3K) inhibitor known to inhibit autophagy.[65,66] We first validated that WRT abrogates thiopeptide-induced autophagy in uninfected THP-1 cells (Figure S14). Subsequently, we evaluated the effect of WRT on thiopeptide activity in *Mtb*-infected macrophages (Figure 5A). Notably, for all three natural thiopeptides, WRT had no significant effect on their anti-*Mtb* activity in macrophages and does not significantly impact thiopeptide inhibition of *Mtb* ($p > 0.05$; Figure S.15). Together with the results of T4V and KEB-02 lacking activity in the intracellular model, it appears as though thiopeptide-induced autophagy does little to promote *Mtb* clearance over the direct activity of these already potent antibiotics.

Although autophagy is a host mechanism used to control intracellular bacteria, *Mtb* has mechanisms to prevent autophagy and fusion with degradative lysosomes.[36,67−69] Therefore, we considered the possibility that the disparity between the ability of thiopeptides to induce autophagy and the minimal contribution of autophagy in clearing intracellular

A.

Compound	*In vitro Mtb* IC_{50}(uM) ± SD	Intracellular *Mtb* IC_{50}(uM) ± SD
Kanamycin (*In vitro*)	1.38 ± 0.36	--
Rifampin (Intracellular)	--	0.08 ± 0.01
TSR	1.44 ± 0.31	2.19 ± 0.78
TSR + WRT	--	2.48 ± 1.04
NOS	1.08 ± 0.37	0.71 ± 0.31
NOS + WRT	--	0.45 ± 0.12
TCIII	2.14 ± 0.91	0.82 ± 0.34
TCIII + WRT	--	1.29 ± 0.05
T4V	> 10	> 10
KEB-01	0.92 ± 0.34	0.73 ± 0.23
KEB-02	> 10	> 10

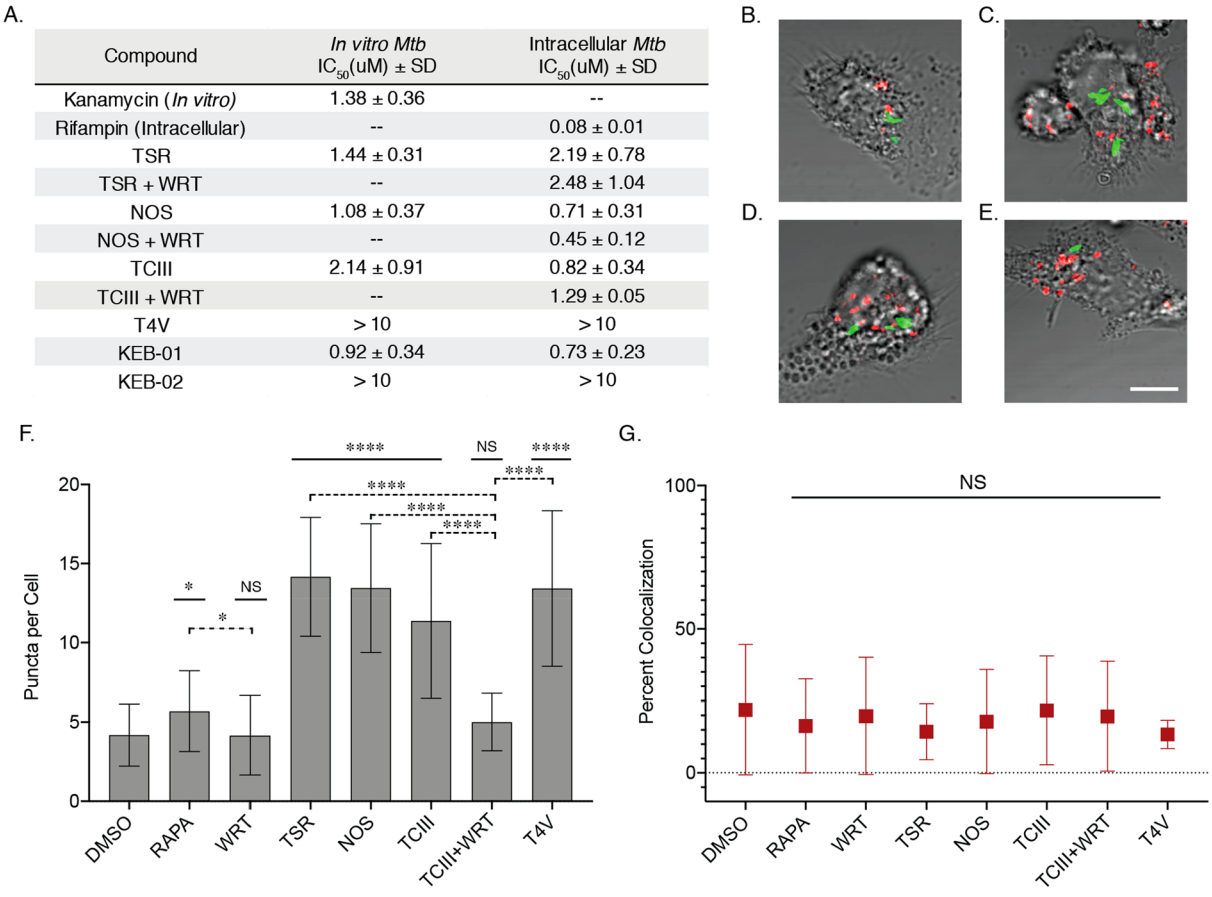


Figure 5. Thiopeptides inhibit the *in vitro* grown *Mtb* in culture and of intracellular *Mtb*. (A) Thiopeptides were measured for their ability to inhibit the growth of *Mtb in vitro* using the REMA assay. *Mtb* was treated with the compound in a dose-dependent manner for 4 days, prior to the addition of resazurin. THP-1 cells were infected with *lux* expressing luminescent *Mtb* a multiplicity of infection (MOI) of 1 and treated with the compound in a dose-dependent manner ± WRT (15 μM) for 7 days. Inhibition of thiopeptide induced autophagy with WRT had no effect on *Mtb* inhibition ($p > 0.05$). Statistics were calculated by repeated measures, one-way ANOVA followed by Tukey's test for posthoc analysis. (B–E) THP-1 cells were differentiated with PMA (30 nM) for 24 h, followed by transfection of RFP-LC3 using JetPrime reagents. RFP-LC3-THP-1 cells were subjected to *Mtb* infection for 1 h with an MOI of 1 followed by thiopeptide treatment for 16 h. (B) DMSO, (C) TSR (2.5 μM), (D) TCIII (10 μM), and (E) T4V (10 μM). Scale bar: 5 μm. (F) *Mtb* infected cells were treated with thiopeptides for 16 h at 10 μM, except for cytotoxic TSR, which was dosed at 2.5 μM to prevent cell death. Infected macrophages were randomly selected ($n = 40$) and quantified for the number of puncta per cell. Error bars: standard deviation (SD). Bold line: Comparisons to DMSO. Dashed line: Comparisons between compounds. Statistics were calculated by repeated measures, one-way ANOVA followed by Tukey's test for posthoc analysis. NS: not significant. ****p value < 0.0001. (G). Percent colocalization represents percent of *Mtb* colocalized to RFP-LC3+ autophagosomes and was measured for 10 FOV containing approximately 15 cells per well. Error bars: standard deviation (SD). NS: not significant.

Mtb might be the result of *Mtb* preventing thiopeptide-induced autophagy. To address this hypothesis, we sought to measure autophagy induction in *Mtb* infected macrophages and assess colocalization of *Mtb* with autophagosomes. To this end, we transfected THP-1 cells with an RFP-LC3 plasmid and infected these macrophages with GFP-expressing H37Rv *Mtb*.[70] With these infected macrophages, fluorescence microscopy could be used to count the number of RFP-$LC3^+$ autophagosomes as a measure of autophagy induced by thiopeptides in the presence of *Mtb*. Additionally, we could track localization of GFP-*Mtb* to RFP-$LC3^+$ autophagosomes relative to controls (Figure 5B–E). All four thiopeptides tested retained their ability to induce autophagy in the presence of *Mtb* infection (Figure 5F). While DMSO and WRT treated cells contained <5 autophagosomes per *Mtb* infected cell, TSR, NOS, TCIII, and T4V significantly increased the number of RFP-LC3 puncta per cell (Figure 5B–F). However, this increase in autophagosome abundance did not result in a concomitant increase in *Mtb* localization to autophagosomes. Across all treatments and controls, 20% of *Mtb* was in autophagosomes (Figure 5G). Significantly, these data demonstrate that while these thiopeptides are still capable of inducing autophagy in *Mtb*-infected cells, thiopeptide-induced autophagy does not enhance the delivery of *Mtb* to autophagosomes.

THIOPEPTIDES INDUCE MITOPHAGY

After determining that thiopeptide-induced autophagy was not aiding in *Mtb* inhibition, we considered that thiopeptides might be stimulating an alternative mechanism of autophagy that does not overlap with pathogen clearance. In addition to nonselective removal of bulk cytoplasmic components and pathogens, autophagic machinery may be recruited for several other forms of selective autophagy for degradation of protein aggregates or subcellular organelles, such as mitochondria.[71,72] In order to assess whether such an alternative form of

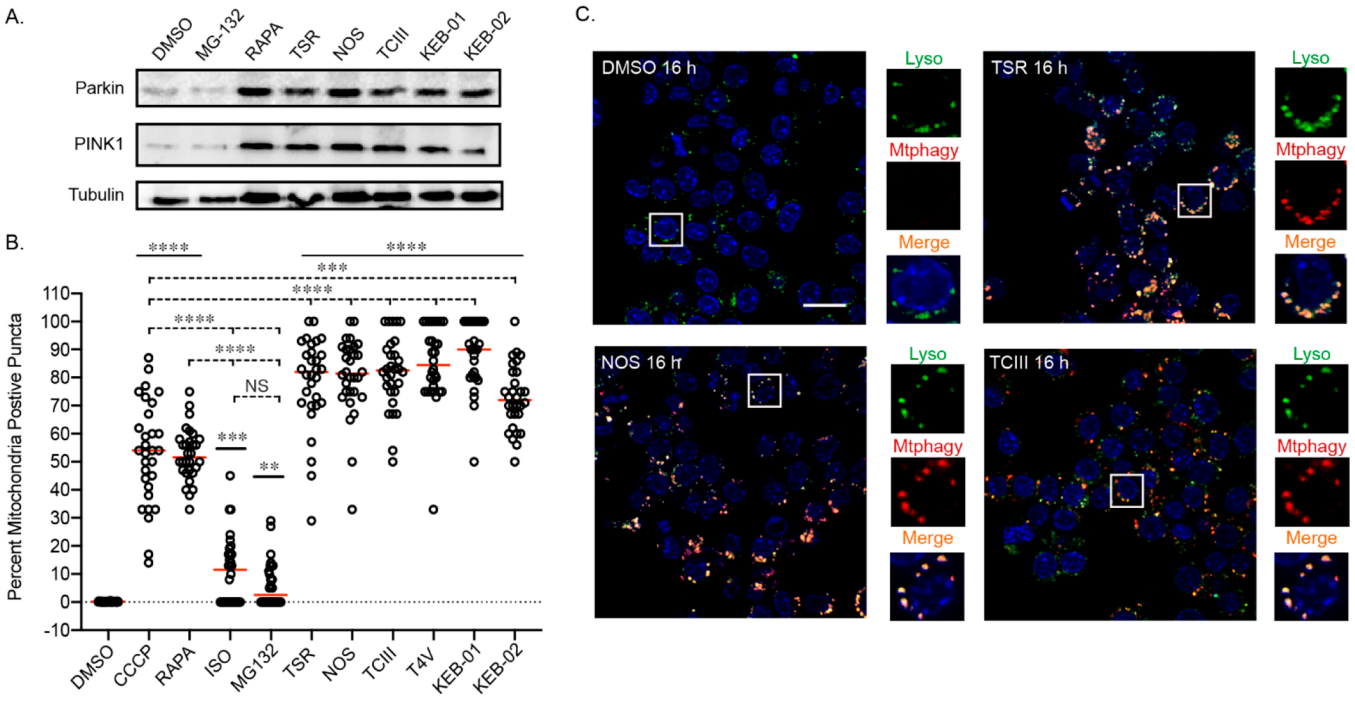


Figure 6. Thiopeptides induce mitophagy. (A) RAW 264.7 cells were treated with 5 μM of compound for 16 h, and cell lysates were subjected to Western blot analysis. Protein levels of Parkin and PINK1 were monitored to probe if a cargo-specific form of autophagy was induced by thiopeptide treatment. β-Tubulin was used as a loading control. (B) RAW 264.7 cells were treated with 5 μM of compound for 16 h. Quantification of mitophagic flux was achieved by calculating the percent mitochondria positive puncta present within lysosomes. A cell profiler pipeline was developed to analyze the number of red (Mtphagy dye) and green puncta (lysotracker) per cell. The ratio of these values was then used to represent the level of mitophagic flux at that specific time point. A total of 30 cells are displayed for each treatment (dots), 10 from each biological replicate. The mean ratio is represented as a red line. Bold line: Comparisons to DMSO. Dashed line: Comparisons between compounds. Statistics were calculated by repeated measures, one-way ANOVA followed by Tukey's test for posthoc analysis. NS: not significant, $^{**}p$ value < 0.01. (C) The induction of a selective form of autophagy, known as mitophagy, was analyzed with the use of Mtphagy dye, an acid-sensitive fluorescent probe that covalently attaches to the mitochondria and fluoresces only when within a lysosome. Four representative photos of DMSO, TSR, NOS, and TCIII (16 h treatment) are shown. One cell is highlighted from each treatment, and the corresponding signals for the LysoDye, MtphagyDye, and respective merged photo are shown to the right. Scale bar: 40 μm.

autophagy was being induced by thiopeptides, we first probed for upregulation of Parkin, an E3 ligase that ubiquitinates unwanted substrates to activate cargo-specific forms of autophagy, such as mitochondria and pathogen clearance.[73,74] We also interrogated the expression of PINK1, a mitochondrial serine/threonine kinase that is stabilized upon mitochondrial dysregulation to initiate organelle degradation via Parkin activation of autophagy.[75] After 16 h of treatment, there was a dramatic increase in Parkin and PINK1 expression levels in all cells treated with thiopeptides (Figure 6A). The control, rapamycin, shows a similar increase in Parkin and PINK1, but the proteasome inhibitor, MG-132, shows little to no increase over basal levels. Our discovery that both Parkin and PINK1 are overexpressed as a result of thiopeptide treatment suggest that these compounds may be inducing mitophagy, a form of cargo-specific autophagy that targets damaged mitochondria for delivery to lysosomes.

To further investigate the possibility of thiopeptide-induced mitophagy, we used the pH-sensitive Mtphagy dye in combination with lysotracker in RAW 264.7 cells (Figure S2.3).[76] Mtphagy dye is an acid-sensitive dye that covalently binds to mitochondria and emits fluorescence upon fusion with the lysosome. The ratio of mitochondria-positive vesicles (Mtphagy dye: red puncta) to lysosomes (lysotracker: green puncta) could be used to monitor the percent of mitochondria positive lysosomes, as an indicator of mitophagy. Values were determined by confocal imaging of cells from three biological replicates (Figures 6C, S17). The basal level of mitophagy was determined by treatment with DMSO (Figure 6B). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and rapamycin, both well-known inducers of mitophagy, were employed as positive controls, while MG-132 and the antibiotic isoniazid were used as negative controls.[77,78] As shown in Figure 6B, both CCCP and RAPA, led to significant increases (p < 0.0001) in mitophagy, whereas isoniazid and MG-132 had little to no effect. All thiopeptide treatments led to significant increases (p < 0.0001) in mitophagy when compared to DMSO. Additionally, our suite of thiopeptides induced mitophagy at levels significantly greater than known mitophagy inducers, CCCP and RAPA. The induction of mitophagy is independent of proteasome inhibition, as shown with TCIII, and is not related to their antibacterial activity as demonstrated with KEB-02 and T4V. These data are significant in demonstrating that thiopeptides of different classes, regardless of their ability to inhibit proteasomes or their antibiotic (antitranslation) activity, induce a highly selective form of autophagy targeted to degrade mitochondria.

DISCUSSION

Here, we investigated the activity of the major classes of thiopeptide antibiotics against previously identified mammalian targets of TSR. Our results show that all thiopeptides

examined in this study induce autophagy independent of proteasome inhibition and antibiotic activity. Select thiopeptides also exhibit potent activity against intracellular *Mtb* in a macrophage infection model. However, thiopeptide-induced autophagy does not appear to impact intracellular *Mtb* clearance. In fact, autophagy induced by this group of antibiotics does not increase the delivery of *Mtb* to autophagosomes. A closer inspection revealed that all thiopeptides, regardless of antibiotic efficacy, induce mitophagy and thus bypass pathogen delivery to degradative autophagosomes. These results provide key insights into the critical activities of this promising class of antibiotics.

Although thiopeptides possess similar structural features, not all thiopeptides are proteasome inhibitors. Within the 26-membered ring scaffold family of TSR, NOS, and TCIII, only TSR and NOS inhibit proteasomes, and they induce starkly different degrees of cellular cytotoxicity. Given the very similar macrocycles in these three thiopeptides, these results suggest that large modifications to the position 8 residue, as with the indole side ring of NOS or the quinaldic acid macrocycle of TSR, can direct proteasome inhibition in this scaffold family. Absence or removal of these prosthetics, as with TCIII or KEB-02, may either eliminate a key interaction or alter the conformation required for proteasome inhibition. Similarly, substitution at this position could control the pronounced differences in cytotoxicity between TSR and NOS by dictating the divergent proteasome subunit inhibition profiles and/or influencing FoxM1 inhibition activity. Interestingly, a major metabolic effort is required to functionalize this residue in both TSR and NOS and thus to install proteasomal inhibition. It will be of interest to see whether thiopeptides from the human gut microbiome exert similar activity, especially the known gut-associated thiopeptide lactocillin, which displays an indolic acid substituent attached to its position 8 residue (Figure 1).

All thiopeptides within our library induce autophagy. Neither proteasome inhibition nor antibiotic activity is necessary for the autophagy-inducing mechanism of thiopeptides. A previous study suggested that autophagy activation by TSR contributes to its effect on intracellular mycobacteria, however our study does not support the conclusion that thiopeptides are dual mechanism (host and bacteria) therapeutics for *Mtb*.[5] Thiopeptides that inhibited protein translation *in vitro* were effective in limiting the number of *Mtb* in macrophages; however, our results indicate that thiopeptide-induced autophagy does not aid in *Mtb* clearance. This conclusion is supported by our finding that the activity of thiopeptides on *Mtb* in macrophages depends on their antitranslation activity, as antibiotically inactive thiopeptides had negligible effects on *Mtb*. Additionally, blocking autophagy with WRT, a well-known autophagy inhibitor, does not diminish the effects of thiopeptide antibiotics on *Mtb* growth in macrophages. Last, although thiopeptides increase autophagy in *Mtb*-infected macrophages, this does not lead to increased delivery of *Mtb* to autophagosomes. Compared to the previous study of TSR-induced autophagy, we evaluated thiopeptide effects on virulent intracellular *Mtb* as opposed to *M. marinum*, a fish pathogen used as a model for *Mtb*, which may account for the different results. Importantly, our study was not limited to TSR, and we observed the same results with different classes of thiopeptides. Together these results demonstrate that, at least for *Mtb*, thiopeptide-induced autophagy does not synergize with their antibiotic activity. Nevertheless, thiopeptides exhibit potent antibiotic activity against *Mtb*, indicating that they can penetrate both the host cell membrane and the highly impermeable *Mtb* cell wall, auspicious signs for their future use as antibiotics against intracellular pathogens.

Thiopeptide-induced autophagy proved to be a cargo-specific form known as mitophagy, which is specific for the removal of mitochondria, not *Mtb*. Thiopeptide treatment led to significant increases in both PARKIN and PINK1 expression, known markers of mitophagy, as well as significant increases in autophagolysosomes containing mitochondria. Aminoglycosides and macrolides, which are antibiotics that also target bacterial translation, have been found to perturb mitochondrial function and physiology, leading to mitophagy.[79−81] However, in the case of thiopeptides, mitophagy was induced regardless of antibiotic activity, suggesting that inhibition of mitochondrial protein translation was not related to the mechanism of mitophagy induction. Here, mitophagy in particular does not seem to synergize with pathogen clearance, providing a helpful insight for future host-directed therapeutic development: not all forms of autophagy will benefit antimicrobial activity. Compounds that modulate mitophagy are actively being investigated as therapies for a number of neurodegenerative diseases and as adjuvants for several cancer chemotherapies.[78,82−85] Thus, the effect of thiopeptides on mitophagy could be useful for developing these compounds as therapeutics for other diseases. As with proteasome inhibition, it will also be interesting to determine whether microbiome-associated thiopeptides also induce mitophagy and whether this activity is used to interface with host metabolic processes.

Cumulatively, these results demonstrate that thiopeptides, in particular TSR, NOS, and TCIII, exhibit potent activity against *Mtb* in macrophages. In the case of TSR, its specific mechanism of proteasome inhibition, unique among thiopeptides, appears to be a significant liability, as it gives rise to appreciable cytotoxicity in cell culture. In comparison to TSR, TCIII and NOS still exhibit comparable antibiotic activity in macrophages without the attendant toxicity. While the significance of thiopeptides to induce mitophagy is still uncertain, it is clear that this activity does not necessitate proteasome inhibition. Ultimately, TCIII, without the additional rings and side-chain modifications of NOS and TSR, presents a much more synthetically or biochemically tractable molecule with potential for improved solubility, thus making it attractive for further development toward the clinic.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00364.

Full experimental details, procedures, characterization for all compounds, additional figures and tables (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Miriam Braunstein − *Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27599, United States*; Email: miriam_braunstein@med.unc.edu

Albert A. Bowers − *Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy and Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina 27599, United States; Lineberger Comprehensive*

Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States; orcid.org/0000-0001-8214-7484; Email: abower2@email.unc.edu

Authors

Kelly E. Bird − *Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599, United States*

Christian Xander − *Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27599, United States*

Sebastian Murcia − *Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27599, United States*

Alan A. Schmalstig − *Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27599, United States*

Xianxi Wang − *Department of Pharmacology and Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States*

Michael J. Emanuele − *Department of Pharmacology and Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acschembio.0c00364

Notes

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