International Journal of Infectious Diseases 69 (2018) 78–84

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Evaluation of the efficacy of valproic acid and suberoylanilide hydroxamic acid (vorinostat) in enhancing the effects of first-line tuberculosis drugs against intracellular *Mycobacterium tuberculosis*

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ARTICLE INFO

Article history:
Received 4 March 2017
Received in revised form 21 February 2018
Accepted 22 February 2018
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Mycobacterium tuberculosis
Adjunct host-directed therapy
Tuberculosis
Histone deacetylase inhibitors
Repurposed drugs

ABSTRACT

Background: New tuberculosis (TB) drug treatment regimens are urgently needed. This study evaluated the potential of the histone deacetylase inhibitors (HDIs) valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA) to enhance the effects of first-line anti-TB drugs against intracellular *Mycobacterium tuberculosis*.
Methods: M. tuberculosis H37Rv cultures were exposed to VPA or SAHA over 6 days, in the presence or absence of isoniazid (INH) and rifampicin (RIF). The efficacy of VPA and SAHA against intracellular *M. tuberculosis* with and without INH or RIF was tested by treating infected macrophages. Bactericidal activity was assessed by counting mycobacterial colony-forming units (CFU).
Results: VPA treatment exhibited superior bactericidal activity to SAHA (2-log CFU reduction), while both HDIs moderately improved the activity of RIF against extracellular *M. tuberculosis*. The bactericidal effect of VPA against intracellular *M. tuberculosis* was greater than that of SAHA (1-log CFU reduction) and equalled that of INH (1.5-log CFU reduction). INH/RIF and VPA/SAHA combination treatment inhibited intracellular *M. tuberculosis* survival in a shorter time span than monotherapy (3 days vs. 6 days).
Conclusions: VPA and SAHA have adjunctive potential to World Health Organization-recommended TB treatment regimens. Clinical evaluation of the two drugs with regard to reducing the treatment duration and improving treatment outcomes in TB is warranted.

Introduction

Tuberculosis (TB) continues to cause 1.5 million deaths every year (WHO, 2015a). Although efforts to bring to the market new chemotherapeutic agents targeting the aetiological agent of TB (*Mycobacterium tuberculosis*) are ongoing, the protracted and expensive drug development process, coupled with a high attrition rate of new drug compounds, has hindered progress in the development of new anti-TB drugs. Efforts towards developing new TB treatment regimens to reduce the duration of treatment and improve treatment outcomes (mortality and long-term pulmonary complications (Manji et al., 2016)), particularly for drug-resistant TB, have focused attention on the repurposing of safe drugs commonly used in medical practice worldwide that are already approved by the United States Food and Drug Administration (FDA) for administration as adjuncts to TB therapy (Zumla et al., 2016). Some noteworthy examples include ibuprofen (Vilaplana et al., 2013), acetylsalicylic acid (Tobin et al., 2012; Byrne et al., 2007), simvastatin (Skerry et al., 2014), metformin (Singhal et al., 2014), and phenylbutyrate (Mily et al., 2015). These drugs may also act in a host-directed fashion (i.e., immune modulation) in concert with standard anti-mycobacterial drugs, thus engaging an additional avenue of ameliorating disease burden.

Various therapeutic agents licensed for non-TB indications appear to be promising against drug-sensitive as well as drug-resistant strains of *M. tuberculosis* (Kaufmann et al., 2014). Clinical testing of the histone deacetylase inhibitor (HDI) phenylbutyrate in patients with pulmonary TB has shown promising preliminary results, augmenting an inhibitory effect against *M. tuberculosis* growth with host macrophage immune responses (Mily et al., 2015; Coussens et al., 2015). HDIs increase the acetylation of lysine

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https://doi.org/10.1016/j.ijid.2018.02.021

residues in histones, leading to chromosome unwinding and gene transcription, thus modifying gene expression (Haery et al., 2015).

Numerous HDIs are being actively explored for cancer therapy (Haery et al., 2015), among which are valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA/vorinostat). VPA is currently used as an anticonvulsant, and studies are simultaneously testing its clinical efficacy against several types of solid tumour and lymphoma (Haery et al., 2015). SAHA received FDA approval in 2006 for the treatment of cutaneous T-cell lymphoma (CTCL), while further clinical trials are underway to evaluate its adjunctive capacity against a myriad of solid tumours (Haery et al., 2015). Importantly, VPA and SAHA have also shown therapeutic potential against several infections. SAHA can trigger the reactivation of latent HIV-1 infection in human CD4 T cells (Archin et al., 2009), exposing viral progeny to antiretroviral drugs, and is currently under clinical investigation (clinicaltrials.gov identifier: NCT01319383, NCT01365065). SAHA has also shown activity against West Nile virus (WNV) replication and associated disease in experimental mice (Nelson et al., 2015). VPA can also promote latent HIV-1 reactivation (Zeng et al., 2014), and has been shown to induce the disruption of WNV infection of kidney cell lines derived from monkey and hamster, as well as the abrogation of herpes simplex virus 1 (HSV-1) infection of a human oligodendrocyte cell line (Crespillo et al., 2016).

This study evaluated the potential of VPA and SAHA as adjunct therapy for enhancing the effects of the two main first-line anti-TB drugs, namely isoniazid (INH) and rifampicin (RIF), against extracellular as well as intracellular *M. tuberculosis*.

Materials and methods

Reagents and culture media

Middlebrook 7H9 liquid medium supplemented with 10% oleic acid–albumin–dextrose–catalase (OADC), 2% glycerol, and 0.05% Tween 80 (7H9C), as well as Middlebrook 7H11 solid medium supplemented with 10% OADC and 2% glycerol (7H11C), were purchased from the Karolinska University Hospital substrate unit. RPMI Glutamax medium, foetal bovine serum (FBS), and antibiotics (penicillin/streptomycin) for tissue culture were purchased from Life Technologies. Phorbol myristate (PMA), VPA, SAHA, INH, and RIF were purchased from Sigma-Aldrich. SAHA, INH, and RIF stocks were prepared in dimethyl sulfoxide (DMSO), while VPA stocks were prepared in distilled water and stored at −20 °C.

Mycobacterial and THP-1 seed stocks

M. tuberculosis H37Rv was cultured in Middlebrook 7H9 medium supplemented with 10% OADC, 2% glycerol, and 0.05% Tween 80 (7H9C), and grown to mid to late log phase (OD_{600} 0.6–0.8). Mycobacterial cultures were first washed with phosphate buffered saline (PBS) containing 0.05% Tween 80 (PBST80) and resuspended in 7H9C medium supplemented with 16% glycerol stored at −80 °C. THP-1 human monocytes (kindly provided by Dr J. Muvva) were maintained in RPMI Glutamax medium supplemented with 10% FBS and antibiotics (R10+). Cells were washed with R10+ and resuspended in 90% FBS containing 10% DMSO for storage in liquid nitrogen tanks.

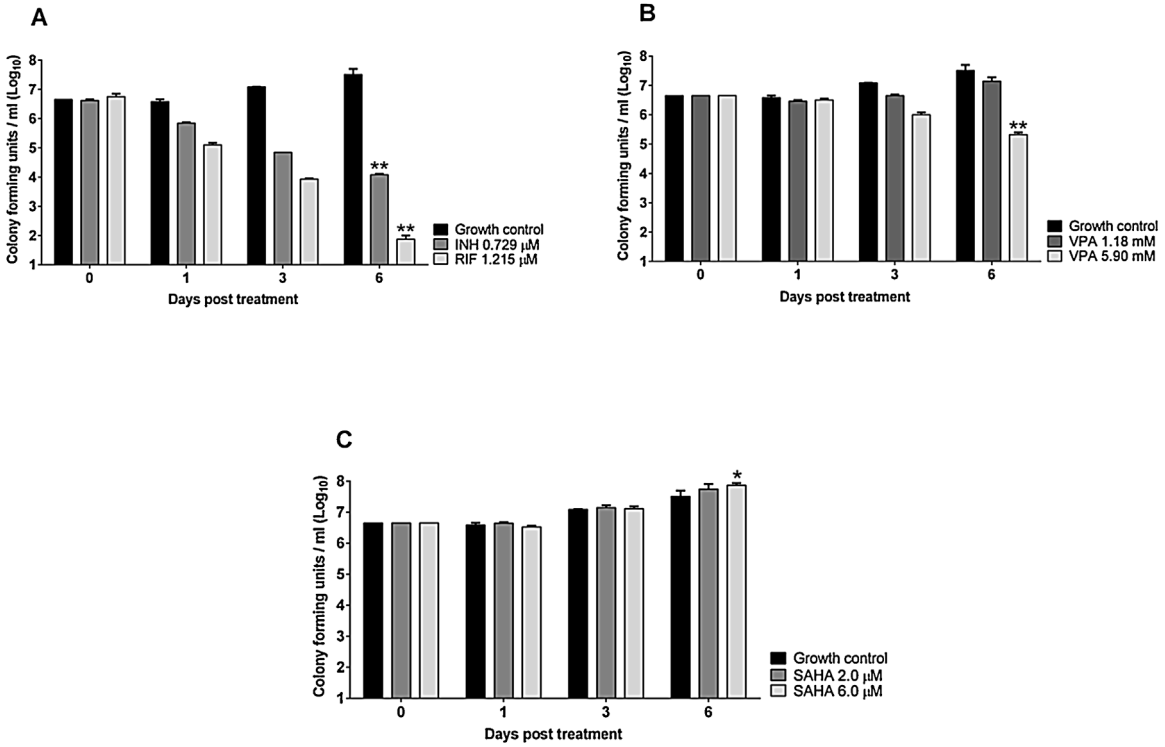

Figure 1. Anti-mycobacterial activity of isoniazid (INH), rifampicin (RIF), valproic acid (VPA), and suberoylanilide hydroxamic acid (SAHA) against *Mycobacterium tuberculosis* in liquid culture when exposed individually. Extracellular *M. tuberculosis* in mycobacterial growth medium were treated with INH or RIF (A), VPA at two different concentrations (1.18 mM and 5.90 mM) (B), or SAHA, also at two different concentrations (2.0 µM and 6.0 µM) (C). Concentrations of INH (0.729 µM) and RIF (1.215 µM) correspond to the World Health Organization-recommended standards (INH 0.1 µg/ml and RIF 1.0 µg/ml). The untreated growth control was used as the reference for bactericidal activity. Mean and SEM of two independent experiments analysed using two-way analysis of variance (ANOVA); $^{*}p < 0.05$, $^{**}p < 0.01$.

Exposure of extracellular M. tuberculosis *to anti-TB drugs and HDIs*

Frozen seed stocks of *M. tuberculosis* were thawed, washed in PBST80 buffer, resuspended in 7H9C medium to the required density, and sonicated (Branson 2510 Ultrasonic Cleaner; Danbury, CT, USA) for 2 × 10 s to disrupt mycobacterial cell clumping. The bacterial culture was then transferred to a 96-well microtitre plate for the drug sensitivity assay. Drugs (INH, RIF, VPA, SAHA) diluted in 7H9C medium were added to the bacterial culture according to the intended final working concentration. No drugs were added to the growth control wells. The bacterial cultures (with and without drugs) were incubated at 37 °C for 6 days. On days 0, 1, 3, and 6 of the experiment, a serial dilution of the bacterial suspension was prepared, plated on 7H11C medium, and incubated at 37 °C for 3–4 weeks for colony-forming unit (CFU) enumeration.

Preparation of THP-1 macrophages, infection with M. tuberculosis*, and drug treatment*

THP-1 human monocytes were differentiated into adherent macrophages in 48-well tissue culture plates with R10+ medium containing 100 ng/ml PMA (overnight incubation at 37 °C with 5% CO_2). The macrophages were subsequently washed with antibiotic-free RPMI medium supplemented with 10% FBS (R10−). For infection, frozen seed stocks of *M. tuberculosis* were thawed, washed in PBST80 buffer, resuspended in R10− medium to the required density, and sonicated (Branson 2510 Ultrasonic Cleaner) for 2 × 10 s to disrupt cell clumping. *M. tuberculosis* suspension was added to wells containing THP-1 macrophages at a multiplicity of infection (MOI) of 5:1, centrifuged at 1500 rpm for 5 min to increase phagocytosis, and incubated at 37 °C with 5% CO_2 for 4 h. The macrophages were then washed with R10− medium and re-incubated at 37 °C with 5% CO_2 following the addition of VPA/SAHA and INH/RIF prepared in R10− medium. Control wells contained drug-free R10− medium only. For *M. tuberculosis* CFU enumeration, infected macrophages were first lysed with PBS + 0.1% Triton X-100, followed by the preparation of serial dilutions in PBST80 buffer for spreading on 7H11C medium. Plates were incubated at 37 °C for 3–4 weeks prior to colony counting.

Statistical analysis

The statistical analysis was performed using GraphPad Prism version 6 software. Mean and standard error of the mean (SEM) values were plotted. A *p*-value of 0.05 or lower was considered significant. Each experiment was performed twice.

Results

Efficacy of VPA and SAHA as single agents or in combination with INH/RIF against extracellular M. tuberculosis

The doses of VPA and SAHA used in this study were selected on the basis of previously published data (Archin et al., 2009; Han et al., 2013a), whereas those used for INH (0.1 μg/ml or 0.729 μM) and RIF (1.0 μg/ml or 1.215 μM) are the World Health Organization-endorsed concentrations used in routine clinical diagnostics using the BACTEC MGIT 960 system and Middlebrook media (WHO, 2008). Initial testing was performed to determine whether the HDIs have a direct effect on the viability of *M. tuberculosis* grown in liquid 7H9C culture in the absence and presence of INH or RIF after 1, 3, and 6 days post drug exposure (Figure 1). At 6 days post exposure, INH treatment resulted in a 2.5-log CFU reduction, while treatment with RIF alone afforded a 4.5-log CFU reduction (Figure 1A). VPA treatment alone at 5.9 mM appeared to exert an overall 1.5-log *M. tuberculosis* CFU reduction (Figure 1B). In contrast, VPA at 1.18 mM had only a meagre inhibitory effect on *M. tuberculosis* growth over the same period (Figure 1B). Treatment with SAHA alone at 2.0 μM and 6.0 μM did not seem to affect *M. tuberculosis* growth (Figure 1C).

The combination of INH and VPA (1.18 mM and 5.9 mM) did not enhance the anti-mycobacterial potential of INH (Figure 2A). However, INH + SAHA (2.0 μM and 6.0 μM) had anti-*M. tuberculosis* activity at least as potent as INH alone, although a clear additional effect was not seen (Figure 2B). RIF + VPA (1.18 mM) did not show a much greater anti-mycobacterial effect compared to RIF alone, although in combination with VPA at 5.9 mM, the bactericidal activity of RIF was slightly increased (Figure 2C); the same held true for RIF + SAHA (2.0 μM and 6.0 μM) (Figure 2D). Thus, although VPA had a noticeable bactericidal effect on *M. tuberculosis* growth, neither VPA nor SAHA had an adjunctive effect superior to that of INH or RIF alone on extracellular *M. tuberculosis*.

VPA and SAHA enhance the activity of INH/RIF against intracellular M. tuberculosis

Next, the impact of VPA and SAHA when exposed to intracellular *M. tuberculosis* was assessed. This was done by first infecting THP-1 macrophages with *M. tuberculosis* and then treating them with the drugs. Both HDIs had moderate bactericidal activity at day 3 post treatment, with VPA (5.9 mM) exhibiting an approximate 1.5-log reduction in *M. tuberculosis* load after 6 days of treatment (Figure 3A). Treatment with SAHA (6.0 μM) afforded a modest reduction of *M. tuberculosis* CFUs compared to the growth control (approximately 0.5-log) after 3 days of exposure, but this effect was not maintained up to day 6 (Figure 3A).

To assess whether the HDIs influenced an improvement in the efficacy of anti-TB drugs against intracellular *M. tuberculosis*, the infected macrophages were treated with VPA and SAHA in combination with INH or RIF. As already known, INH alone was very potent at controlling the intracellular bacterial load over 6 days of treatment (1.5-log CFU reduction) (Figure 3B) – similar to the bactericidal effect of VPA. The bactericidal effect of INH was enhanced (2-log CFU reduction) when the infected macrophages were exposed to VPA + INH over the same duration (Figure 3B). When *M. tuberculosis*-infected macrophages were treated with SAHA + INH, the bacterial CFU was reduced by 2.5-log, exceeding the potentiating effect of VPA on INH, which was only 0.5-log (Figure 3B). In general, the INH + VPA/SAHA combinations reduced intracellular *M. tuberculosis* burden most drastically between days 3 and 6 of drug exposure (between 1.0- and 1.5-log) (Figure 3B).

RIF treatment led to a statistically significant reduction in *M. tuberculosis* load at day 1, which improved steadily over the 6-day period of drug exposure (Figure 3C). RIF + VPA/SAHA also exerted a similar bactericidal effect at day 1 of treatment, although this effect appeared to be better than that of RIF alone at days 3 and 6 of treatment, respectively (Figure 3C). Compared to INH + VPA/SAHA, treatment of infected macrophages with RIF + VPA/SAHA markedly reduced *M. tuberculosis* CFU counts between day 1 and day 3 of exposure, suggesting accelerated anti-mycobacterial activity of these combinations (Figure 3B,C). Furthermore, CFU reduction in the presence of RIF + SAHA was about 1-log greater than that with RIF alone after 3 days of treatment. Although not as effective as RIF + SAHA, RIF + VPA afforded an additional 0.5-log CFU reduction to RIF treatment alone at day 3.

In summary, VPA and SAHA can enhance the potency of RIF and INH against intracellular *M. tuberculosis* in a shorter period.

Discussion

There are several important and novel findings from this study: (1) these data are the first to show that VPA and SAHA can enhance

the activity of the standard first-line anti-TB drugs INH and RIF against intracellular *M. tuberculosis*; (2) VPA on its own possesses some degree of bactericidal activity against extracellular, as well as intracellular *M. tuberculosis*; (3) VPA in combination with INH or RIF further enhances the anti-mycobacterial effect; (4) the combination of RIF and SAHA enhances the reduction of mycobacterial growth in liquid culture; (5) SAHA improves the antibacterial activity of both RIF and INH against intracellular *M. tuberculosis* in macrophages. The data show promise for the potential adjunctive use of VPA and SAHA in combination with INH or RIF for the treatment of drug-susceptible TB and provide the background for further testing in drug-resistant TB.

VPA has already been reported to induce programmed cell death via apoptosis, as well as autophagy in human cancer cells and *M. tuberculosis*-infected macrophages (Schiebler et al., 2015; Fu et al., 2010; Han et al., 2013b). Mechanistically, apoptosis induced by VPA has been linked to its inhibition of eukaryotic sirtuin nicotinamide adenine dinucleotide (NAD)-dependent deacetylases (Sun et al., 2007). Interestingly, the *M. tuberculosis* genome encodes Rv1151c, a sirtuin-like NAD-dependent deacetylase capable of activating acetyl coenzyme A synthetase (ACS) by removing acetyl groups from lysine groups in the latter enzyme (Xu et al., 2011). Deacetylation of ACS is necessary for the synthesis of acetyl coenzyme A, a molecule with significant biological importance in central metabolism in all eukaryotes as well as prokaryotes (Galdieri et al., 2014). It may therefore be hypothesized that pharmacological inhibition of Rv1151c would inhibit ACS deacetylation, inactivate its enzymatic activity, and consequently disrupt bacterial growth, hinting at Rv1151c as a putative *M. tuberculosis* target for VPA. This notion supports the observation in this study of impaired *M. tuberculosis* growth when exposed to VPA alone at 5.9 mM over 6 days, warranting further verification.

Immunologically, VPA is known to starkly inhibit the production of the pro-inflammatory cytokines tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) by THP-1 macrophages and human glioma cells (Ichiyama et al., 2000). VPA has also been linked to alterations of the immune activation status of dendritic cells, marked by reduced antigen presentation as well as their ability to interact with T cells (Frikeche et al., 2012). A balanced upregulation of TNF-α and IL-6 levels in the lung is associated with a protective role in the early stages of TB infection; however, exaggerated concentrations of either cytokine can be detrimental during chronic and active TB disease (O'Garra et al., 2013; Ravimohan et al., 2015). Furthermore, uncontrolled T-cell activation in pulmonary TB is destructive, and only warrants organ damage and ultimately death (Zumla et al., 2015a). Thus, it may be a better option to administer VPA to patients with severe pulmonary TB in the later phase of treatment, which requires dampening and/or modulation of lung immunopathology and fine-tuning of targeted anti-TB immune responses, in conjunction with bacterial killing by anti-mycobacterial drugs.

Since treatment with SAHA alone did not directly affect *M. tuberculosis* growth in liquid culture, it was assumed that druggable targets influencing bacterial growth in axenic culture that can be affected by SAHA are likely absent in *M. tuberculosis*. Nevertheless, the meagre anti-mycobacterial effect of SAHA treatment against *M. tuberculosis* growth in infected macrophages hinted at bactericidal activity in a host-dependent fashion. It has been shown previously that SAHA can trigger the generation of reactive oxygen species such as superoxide anion and hydrogen peroxide, which leads to DNA damage and the induction of apoptosis (Petruccelli et al., 2011). SAHA-induced apoptotic cell death has also been observed in acute myeloid leukaemia cell lines (including THP-1 monocytes), which also coincided with reduced cell viability after 2 days of exposure (Silva et al., 2013). Autophagy induction by SAHA involves inhibition of the mammalian target of rapamycin, as observed in human myeloid leukaemia cells as well as HIV-1-infected macrophages (Campbell et al., 2015). More

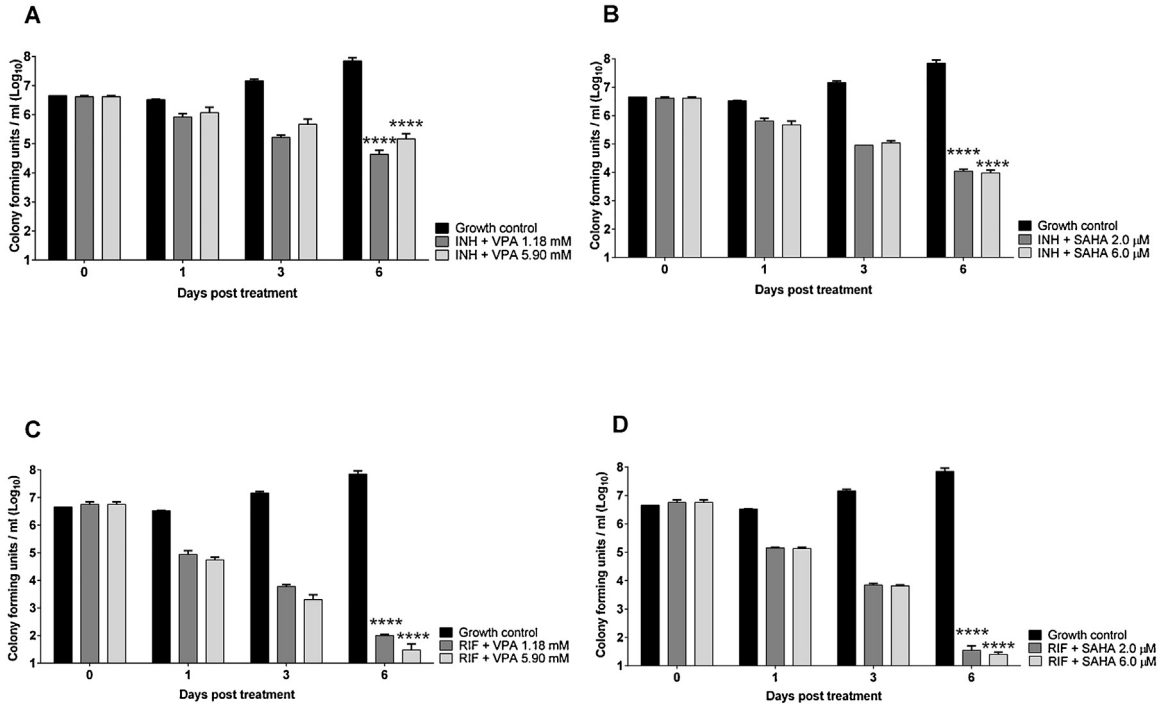

Figure 2. Bactericidal activity of valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA) against *M. tuberculosis* in liquid culture when treated in combination with isoniazid (INH) or rifampicin (RIF). Extracellular *M. tuberculosis* in mycobacterial growth medium were treated with INH or RIF in combination with two different concentrations of VPA (1.18 mM and 5.90 mM) (A, C) or SAHA (2.0 μM and 6.0 μM) (B, D) over a 6-day period. Concentrations of INH (0.729 μM) and RIF (1.215 μM) correspond to the World Health Organization-recommended standards (INH 0.1 μg/ml and RIF 1.0 μg/ml). The untreated growth control was used as the reference for bactericidal activity. Mean and SEM of two independent experiments analysed using two-way analysis of variance (ANOVA); $^{****}p < 0.0001$.

recently, adjunctive SAHA treatment was shown to potentiate the antiviral activity of an experimental drug against WNV infection in mice, in addition to reducing immunopathology in the brain (Nelson et al., 2015). It is therefore tempting to speculate that the apoptosis- and autophagy-inducing properties of VPA and SAHA would reduce the number of *M. tuberculosis*-infected cells in the host and expose the bacilli to anti-mycobacterial drugs such as INH and RIF – as shown in this study. Therefore, VPA- or SAHA-induced

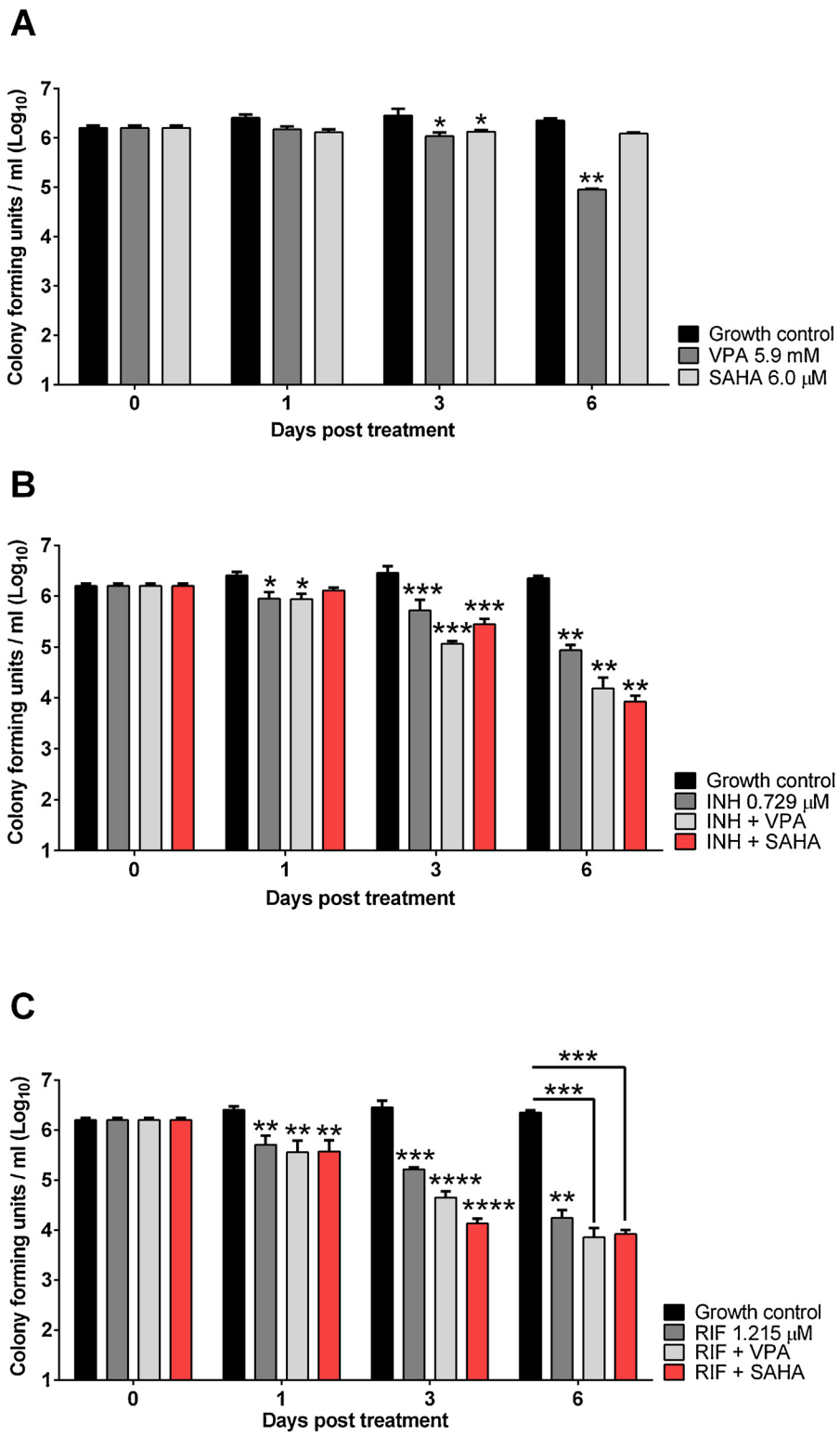

Figure 3. Efficacy of valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA) against intracellular *M. tuberculosis* as single agents or in combination with isoniazid (INH) or rifampicin (RIF). *M. tuberculosis*-infected THP-1 macrophages were exposed to VPA (5.9 mM) or SAHA (6.0 μM) alone (A), or in combination with INH (B) or RIF (C) for 6 days in cell culture medium. Concentrations of INH (0.729 μM) and RIF (1.215 μM) correspond to the World Health Organization-recommended standards (INH 0.1 μg/ml and RIF 1.0 μg/ml). The untreated growth control was used as the reference for bactericidal activity. Mean and SEM of two independent experiments analysed using two-way analysis of variance (ANOVA); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

programmed cell death may enhance the contact of INH and RIF with intracellular *M. tuberculosis*, enabling increased killing of bacteria by the drugs. However, the possibility of VPA and SAHA having a direct impact on the intra-bacterial activation of INH and/or RIF cannot be excluded, and therefore remains to be determined.

Taken together, the data suggest that VPA and SAHA further potentiate the mycobactericidal activity of INH and RIF against intracellular *M. tuberculosis* bacilli. Based on evidence in the literature, this effect could possibly be achieved through a host-directed mechanism of VPA/SAHA, by depriving the bacteria of cellular reservoirs in the host via apoptosis and/or autophagy (Schiebler et al., 2015; Fu et al., 2010; Han et al., 2013b). VPA has been used for over half a century in healthcare systems globally (WHO, 2015b). It is cheap and has an excellent safety profile. SAHA (vorinostat; marketed as Zolinza by Merck) has been included in an access programme by the company for free or discounted distribution to needy patients (Hematology ASo, 2015). Plasma concentrations of VPA between 50 mg/l and 100 mg/l in patients with epilepsy are considered therapeutic (Patsalos et al., 2008), while SAHA reaches up to 658 ± 439 ng/ml in patients with CTCL (Kavanaugh et al., 2010). Thus, the clinically relevant bioavailability of either drug should be reached in patients with TB. However, VPA has been shown to have moderate interactions with INH; the latter increases SAHA bioavailability in plasma (Medscape, 2017), which has to be taken into consideration in the design of clinical trials.

There is growing interest in 'repurposing' clinically approved drugs (based on their host-directed biological activity) for use as adjunctive therapy for improving treatment outcomes in cancer and infectious disease (Wallis and Hafner, 2015; Zumla et al., 2015b; Mahon and Hafner, 2015; Schito et al., 2015; Zumla et al., 2015c). Pertinent to TB, the contribution of host-directed therapies (HDTs) to standard anti-TB drug therapy promises not only improved treatment outcomes in the short term, but also reduced long-term effects suffered by surviving individuals (Manji et al., 2016; Zumla et al., 2015c). An international consortium of 64 clinical partners in five continents was formed in the spring of 2015 to bolster the progress of FDA-endorsed therapeutic agents with promising anti-TB potential to bedside use (Zumla et al., 2015b). Clinical evaluation of these drugs with regard to reducing the duration of treatment and improving treatment outcomes for all forms of TB, as well as the clinical determination of safety and the pharmacological interactions between these repurposed drugs and conventional TB drugs, is now required through randomized clinical trials.

Acknowledgements

We are grateful to Dr J. Rao Muvva (Karolinska Institutet) for providing the primary seed stocks of THP-1 monocytes and to Dr Berit Carow (Karolinska Institutet) for providing the primary seed stocks of *M. tuberculosis* H37Rv.

Funding

This work was supported by the European Union FP7 RID-RTI (grant number 304865 to A.I.Z.), the Swedish Research Council (Vetenskapsrådet; to M.J.M.), Vinnova (to M.J.M.), and the Swedish Heart and Lung Foundation (Hjärt-Lungfonden; to M.J.M.).

Conflict of interest

The authors declare no conflict of interest.

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