Published in final edited form as:

Bioorg Med Chem Lett. 2014 October 15; 24(20): 4826–4830. doi:10.1016/j.bmcl.2014.08.060.

The Antileishmanial Activity of Isoforms 6- and 8-Selective Histone Deacetylase Inhibitors

Quaovi Sodji^a, Vishal Patil^{a,†}, Surenda Jain^b, James Kornacki^c, Milan Mrksich^c, Babu L. Tekwani^{b,*}, and Adegboyega K. Oyelere^{a,*}

^aSchool of Chemistry and Biochemistry, Parker H. Petit for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, 30332-0400, USA

^bNational Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS, 38677-1848, USA

^cDepartment of Chemistry and Biomedical Engineering, Northwestern University, 2145 Sheridan Rd, Evanston, IL, 60208-3113, USA

Abstract

Histone deacetylase inhibitors (HDACi) pleiotropy is largely due to their non-selective inhibition of various cellular HDAC isoforms. Connecting inhibition of a specific isoform to biological responses and/or phenotypes is essential toward deconvoluting HDACi pleiotropy. The contribution of classes I and II HDACs to the antileishmanial activity of HDACi was investigated using the amastigote and promastigote forms of *L. donovani*. We observed that the antileishmanial activities of HDACi are largely due to the inhibition of HDAC6-like activity. This observation could facilitate the development of HDACi as antileishmanial agents.

Keywords

Histone Deacetylase Inhibitors; Trichostatin A; Tubastatin A; 3-Hydroxypyridin-2-thione; *Leishmania donovani*

Leishmaniasis is a parasitic disease caused by protozoan parasites of the genus *Leishmania*. Human leishmaniasis are present on all continents, but in Antarctica and Australia.¹ Although the majority of leishmanial infections occur in the developing world, leishmaniasis is increasingly common in developed nations as well.^{2,3} Globally, leishmaniasis is endemic in more than eighty countries and affects about 2 million people per year, with approximately 350 million individuals at risk of infection.⁴

© 2014 Elsevier Ltd. All rights reserved.

*Corresponding author. Tel.: +1-404-894-4047; aoyelere@gatech.edu (AKO); Tel.: +1-662-915-7882; btekwani@olemiss.edu (BLT).

†current address: Materials Research Laboratory, University of California, Santa Barbara (USA)

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The limited chemotherapeutic options for the treatment of leishmanial infections are based on amphotericin B, miltefosine, paromomycin, and antimony agents such as sodium stibogluconate and meglumine antimoniate. These are unsatisfactory agents because they require a long course of parenteral injection and many suffer from unacceptable toxicity and poor efficacy.^{5–8} Therefore, there is an urgent need for affordable and safe alternative agents to curtail leishmaniasis.

Leishmania is a member of trypanosomatid protozoa, which are rapidly growing eukaryotes. The genomes of Leishmania, trypanosomatids and many human pathogenic protozoans contain several genes encoding for different histone deacetylase (HDAC) isozymes.^{9–16} In the Leishmania genome, three genes putatively encoding for NAD⁺-dependent HDACs and four genes encoding for zinc-dependent HDACs have been identified. Further analysis of the genes encoding for zinc-dependent HDACs confirmed the presence of major conserved domains and identified these as HDAC1, RpdA/Rpd3 (both Class I), HDAC5 and HDAC4/6 (both class II) homologs (Unpublished results).

HDACs are the primary cellular lysine deacetylases,¹⁷ and their inhibition elicits an array of phenotypes including anti-proliferative activity against nearly all types of transformed cells. Two HDAC inhibitors (HDACi), suberoylanilide hydroxamic acid (SAHA) and FK-228 (romidepsin) (Figure 1), are currently approved by the FDA for the treatment of cutaneous T-Cell lymphoma (CTCL).¹⁷ Interestingly, Leishmania are also susceptible to HDACi,^{18–20} suggesting that HDAC isozymes are essential for their viability and proliferation. However, the identity of the specific Leishmania HDAC isozymes, which confer antileishmanial activity to HDACi is currently unknown. Moreover, the structure activity relationship (SAR) of the antileishmanial activity of HDACi has not been completely worked out.

The antiprotozoal activity of HDACi has been suggested to result from the inhibition of protozoan's proteins similar to the class I family HDACs. This suggestion has been supported in the case of *Plasmodium falciparum*, the causative organism of the most prevalent form of human malaria, with the identification and characterization of PfHDAC-1 which has homology with the class I family of HDACs from human, chicken, frog and *Saccharomyces cerevisiae*.^{13,20,21} Additionally, the antimalarial activities of most HDACi tracked very well with their anti- PfHDAC-1 activity.^{18,19,22,23} However, the correlation between class I family HDAC inhibition and antileishmanial activity is a bit tenuous. The pan-HDAC inhibitor, SAHA, is potently active against *P. falciparum* but more than 70-fold less active against *L. donovani*, the causative organism of visceral leishmaniasis.^{18–20} Moreover, several HDACi have inverse relation between HDAC1 inhibition and antileishmanial activity.²⁰ The disparities in the sensitivity of protozoans such as *P. falciparum* and *L. donovani* to HDACi may be due to differences in the key amino acid residues within the active sites of their class I HDAC isozymes. Alternatively, HDAC subclasses other than class I may be essential for the survival and proliferation, particularly for *Leishmania* parasites and may be the potential target for action of antileishmanial HDACi.

We have previously identified a new class of non-hydroxamate HDACi derived from 3-hydroxypyridine-2-thione (3HPT).²⁴ We observed that aryl- and diaryl-analogs of these 3HPT-derived HDACi have selective inhibitory activity against HDAC6 or HDAC8 but are

otherwise inactive against HDAC1. We envisioned that these 3HPT-derived compounds could constitute useful molecular probes for parsing out the contribution of inhibition of classes I and II HDACs to the antileishmanial activity of HDACi. Herein, we showed that despite their inactivity against HDAC1, these 3HPT-HDACi potently inhibit the viability of the amastigote and promastigote forms of *L. donovani*. Additionally, we provided evidence which suggested that the antileishmanial activity of these and other HDACi could be largely due to inhibition of HDAC6-like activity.

We initially evaluated the *in vitro* antileishmanial activities of the 3HPT-derived HDACi and their corresponding 3-hydroxypyridin-2-one (3HP) analogs against the amastigote and promastigote stages of *L. donovani*, the two different stages of Leishmania species. The amastigote is found thriving in the macrophages of the mammalian host while the promastigote starts as the non-infective stage, which replicates within the insect vector and subsequently transforms into infective metacyclic promastigote.¹¹ Viability of the two stages of *L. donovani* was determined using the standard Alamar blue assay, modified to a fluorometric assay.²⁵ We used suberoylanilide hydroxamic acid (SAHA), a standard HDACi as well as Amphotericin B and pentamidine, standard antileishmanial agents, as positive controls. We observed that for each matched pair, the 3HP compounds are relatively weakly cytotoxic to the promastigote form while the 3HPT-HDACi compounds are potently cytotoxic (Table 1). The lead 3HP compound **1a** is inactive at the maximum concentration tested (40 µg/mL) while its 3HPT analog, **1b**, is weakly cytotoxic to the promastigote stage of *L. donovani*. Subsequent modification to **1a** and **1b**, as described in our previous publication,²⁴ afforded matched pairs **2a/2b** through **13a/13b**. Without exception, the 3HPT analogs of each pair (analog “b”) are much more cytotoxic than their 3HP congeners with IC₅₀ ranging from 100 ng/mL to single digit µg/mL. Overall, the potency enhancement of the 3HPT compounds relative to their 3HP analogs range from 11- to >90-folds. The most potent analogs are compounds **2b**, **3b**, **5b** and **7b–10b** (IC₅₀ 100–400ng/mL). The relative inactivity of the 3HP compounds (analog “a”) may be due to their lack of HDAC inhibition activity. Conversely, all the 3HPT compounds, except **5b** and **10b–12b**, inhibit both HDAC6 and HDAC8. Compounds **5b**, **10b** and **12b** inhibit HDCA8 while **11b** inhibits HDAC6. For this set of compounds, we did not see any clear evidence suggesting that the preferential inhibition of HDAC6 or HDAC8 is more deleterious to the viability of the promastigote stage of *L. donovani*. Rather, our result seems to suggest that HDAC1 inhibition may not be necessary for the cytotoxicity of HDACi against the *L. donovani* promastigote stage.

To further delineate the contribution of HDAC6- and HDAC8-like activities to the viability of the promastigote stage, we investigated the effects of exposure to Tubastatin A²⁶ and PCI-34051,²⁷ prototypical HDAC6 and HDAC8 selective HDACi respectively, on cell viability. Similar to the pan HDACi SAHA, PCI-34051 has only but minimal effect on cell viability. Interestingly, Tubastatin A is much more cytotoxic to the promastigote stage of *L. donovani* than PCI-34051 with IC₅₀ of 4.4µg/mL. This data suggests that the inhibition of the HDAC6-like activity is more deleterious to the viability *L. donovani* promastigote stage. The fact that the apparently HDAC8- selective compounds **5b** and **10b** maintained potent

antileishmanial activity suggests that their cytotoxicity may be due to perturbation of other as yet to be identified intracellular targets.

The axenic amastigote form is generally less responsive to drug treatment including the standard antileishmanial agents, Amphotericin B and pentamidine, and all HDACi investigated. All 3HP compounds are virtually nontoxic to the axenic amastigote except **4a** and **10a**, which are about equipotent to both stages of *L. donovani*. Relative to their effects on the promastigote stage, the 3HPT compounds are between 2- to 115-fold less active against the promastigote stage. Nevertheless, compounds **2b–4b** and **10b–13b** have single digit $\mu\text{g/mL}$ cytotoxic activity. The pan HDACi SAHA and HDAC8-selective HDACi are non-toxic to the amastigote stage at maximum tested concentration of $40\mu\text{g/mL}$. Interestingly, the cytotoxic activity of the HDAC6-selective inhibitor Tubastatin A is only 2-fold lower than that of its effect on the promastigote stage (IC_{50} of $8.8\mu\text{g/mL}$). These data suggest that HDAC6-like activity is essential for the viability of the amastigote stage of *L. donovani* as well. It is worth noting here that a stage-specific response of *Leishmania* spp to HDACi has been previously observed and attributed to overexpression of SIR2, a cytoplasmic NAD^+ -dependent HDAC.²⁸ The weaker response of the amastigote stage to the active HDACi described herein could also be due to compensation from the upregulated SIR2 activity.

To investigate the activity or lack thereof of these 3HPT HDACi against the therapeutically relevant mammalian host stage of *L. donovani*, we tested all compounds *in vitro* in amastigote-macrophage assay. We used a human THP1 macrophage cell line both as the amastigote host cell and as a control for the determination of drug selective toxicity index.²⁹ We observed that all compounds are non-cytotoxic to uninfected THP1 macrophage cells at the maximum tested concentration of $10\mu\text{g/mL}$. However, standard antileishmanial agents, Amphotericin B and pentamidine are potently cytotoxic to the intra-macrophage amastigote while HDAC8-selective PCI-34051 is still inactive (Table 2). The 3HP compound **4a** is moderately active in similar manner to its effect on the promastigote and axenic amastigote stages. Other exceptions in the 3HP series are **3a**, **5a** and **13a** which display moderate to good cytotoxic activities, despite their inactivity against the promastigote and axenic amastigote stages (Supplemental Info Table S1). The target(s) responsible for the moderate activity of these 3HP compounds is unknown at the moment since they are inactive against the HDAC isoforms tested. Except for **6b** and **8b**, which are inactive, all 3HPT HDACi have moderate to strong cytotoxic activities. The potency of compounds **3b**, **4b**, **10b** and HDAC6-selective Tubastatin A was enhanced by 5- to 25-fold relative to their effects on the axenic amastigote (Table 2). Interestingly, SAHA display more than a 50-fold potency enhancement relative to its effect on the axenic amastigotes. The improved potency of these HDACi (comparing axenic amastigote and intramacrophage amastigote stage, Tables 1 and 2) could be due to drug-induced secondary effects on the macrophage. HDACi are known to alter macrophage phenotype and function through perturbation of cytokine production,^{30,31} which might negatively impact the viability of the intra-macrophage amastigote stage. Alternatively, upregulation of reactive oxygen species (ROS) production is another mechanism of HDACi-induced cytotoxicity.³²

We showed in this contribution that a set of 3HPT-derived HDACi with HDAC6 and HDAC8 inhibitory activities, but lacking HDAC1 inhibitory activity, are cytotoxic to extracellular and intracellular stages of *L. donovani*. We subsequently parsed out the contribution of inhibition of HDAC6-like and HDAC8-like activities to the overall antileishmanial activities using Tubastatin A and PCI-34051, standard HDAC6 and HDAC8 selective HDACi. We showed that Tubastatin A and 3HPT compounds are cytotoxic to both the extracellular and intracellular stages of *L. donovani* while PCI-34051 is nontoxic. We inferred from our observations in this study that HDAC6-like activity, to a larger extent, is essential for the viability of the amastigote and promastigote stages of *L. donovani*. Targeting protozoan HDAC6 with isoform selective inhibitors may then offer a more focused therapeutic strategy for Leishmaniasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was financially supported by NIH Grant R01CA131217 (A.K.O) and US Department of Defense CDMPR grant No. W81XWH-09-2-0093 (B.L.T. & S.K.J.). Q.H.S. is a recipient of the GAANN predoctoral fellowship from the Georgia Tech Center for Drug Design, Development and Delivery and a dissertation fellowship from the Southern Regional Education Board.

References and notes

- Hide, M.; Bucheton, B.; Kamhawi, S.; Bras-Gonçalves, R.; Sundar, S.; Lemerse, J.; Banuls, A. Understanding Human Leishmaniasis: The Need for an Integrated Approach. In: Tibayrenc, M., editor. Encyclopedia of Infectious Diseases: Modern Methodologies. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2007. p. 87-123.
- Weina PJ, Neafie RC, Wortmann G, Polhemus M, Aronson NE, Strausbaugh LJ. Clin. Infect. Dis. 2004; 39:1674. [PubMed: 15578370]
- Willard RJ, Jeffcoat AM, Benson PM, Walsh DS. J. Am. Acad. Dermatol. 2005; 52:977. [PubMed: 15928615]
- Geneva, Switzerland: World Health Organization; 2001. Tropical Disease Research: Progress 1999–2000: fifteenth programme report/ UNDP/World Bank/WHO Programme for Research and Training in Tropical Diseases; p. 90
- Loiseau PM, Bories C. Curr. Top. Med. Chem. 2006; 6:539. [PubMed: 16719806]
- Mishra J, Saxena A, Singh S. Curr. Med. Chem. 2007; 14:1153. [PubMed: 17456028]
- Verma NK, Dey CS. Diabetologia. 2006; 49:1656. [PubMed: 16752184]
- van Thiel PPAM, Leenstra T, Kager PA, de Vries HJ, van Vugt M, van der Meide WF, Bart A, Zeegelaar JE, van der Sluis A, Schallig HDFH, van Gool T, Faber WR, de Vries PJ. Clin. Infect. Dis. 2010; 50:80. [PubMed: 19951107]
- Ivens AC, Peacock CS, Worthey EA, Murphy L, Aggarwal G, Berriman M, et al. Science. 2005; 309:436. [PubMed: 16020728]
- Vergnes B, Sereno D, Tavares J, Cordeiro-da-Silva A, Vanhille L, Madjidian-Sereno N, Depoix D, Monte-Alegre A, Ouaiissi A. Gene. 2005; 363:85–96. [PubMed: 16236469]
- Ouaiissi A. J. Biomed. Biotechnol. 2007 Article number: 94971.
- Adriano M-A, Vergnes B, Poncet J, Mathieu-Daude F, Silva A, Ouaiissi A, Sereno D. Parasitol Res. 2007; 100:811. [PubMed: 17096142]
- Joshi MB, Lin DT, Chiang PH, Goldman ND, Fujioka H, Aikawa M, Syin C. Mol. Biochem. Parasitol. 1999; 99:11. [PubMed: 10215020]

14. Mukherjee P, Pradhan A, Shah F, Tekwani BL, Avery MA. *Bioorg. Med. Chem.* 2008; 16:5254. [PubMed: 18362073]
15. Andrews KT, Tran TN, Wheatley NC, Fairlie DP. *Curr. Top. Med. Chem.* 2009; 9:292. [PubMed: 19355992]
16. Sriwilaijaroen N, Boonma S, Attasart P, Pothikasikorn J, Panyim S, Noonpakdee W. *Biochem. Biophys. Res. Commun.* 2009; 381:144. [PubMed: 19338767]
17. Gryder BE, Sodji QH, Oyelere AK. *Future Med. Chem.* 2012; 4:505. [PubMed: 22416777]
18. Patil V, Guerrant W, Chen PC, Gryder B, Benicewicz DB, Khan SI, Tekwani BL, Oyelere AK. *Bioorg. Med. Chem.* 2010; 18:415. [PubMed: 19914074]
19. Guerrant W, Mwakwari SC, Chen PC, Khan SI, Tekwani BL, Oyelere AK. *ChemMedChem.* 2010; 5:1232. [PubMed: 20533500]
20. Mai A, Cerbara I, Valente S, Massa S, Walker LA, Tekwani BL. *Antimicrob. Agents Chemother.* 2004; 48:1435. [PubMed: 15047563]
21. Darkin-Rattray SJ, Gurnett AM, Myers RW, Dulski PM, Crumley TM, Allocco JJ, Cannova C, Meinke PT, Colletti SL, Bednarek MA, Singh SB, Goetz MA, Dombrowski AW, Polishook JD, Schmatz DM. *Proc. Nat. Acad. Sci. U.S.A.* 1996; 93:13143.
22. Mwakwari SC, Guerrant W, Patil V, Khan SI, Tekwani BL, Gurard-Levin ZA, Mrksich M, Oyelere AK. *J. Med. Chem.* 2010; 53:6100. [PubMed: 20669972]
23. Patel V, Mazitschek R, Coleman B, Nguyen C, Urgaonkar S, Cortese J, Barker RH, Greenberg E, Tang W, Bradner JE, Schreiber SL, Duraisingh MT, Wirth DF, Clardy J. *J. Med. Chem.* 2009; 52:2185. [PubMed: 19317450]
24. Patil V, Sodji QH, Kornacki JR, Mrksich M, Oyelere AK. *J. Med. Chem.* 2013; 56:3492. [PubMed: 23547652]
25. Mikus J, Steverding D. *Parasitol. Int.* 2000; 48:265. [PubMed: 11227767]
26. Butler KV, Kalin J, Brochier C, Vistoli G, Langley B, Kozikowski AP. *J. Am. Chem. Soc.* 2010; 132:10842. [PubMed: 20614936]
27. Balasubramanian S, Ramos J, Luo W, Sirisawad M, Verner E, Buggy JJ. *Leukemia.* 2008; 22:1026. [PubMed: 18256683]
28. Vergnes B, Vanhille L, Ouaisi A, Sereno D. *Acta Tropica.* 2005; 94:107. [PubMed: 15860278]
29. Jain SK, Sahu R, Walker LA, Tekwani BL. *J. Vis. Exp.* 2012; 70:e4054.
30. Mullican SE, Gaddis CA, Alenghat T, Nair MG, Giacomini PR, Everett LJ, Feng D, Steger DJ, Schug J, Artis D, Lazar MA. *Genes Dev.* 2011; 25:2480. [PubMed: 22156208]
31. Wu C, Li A, Leng Y, Li Y, Kang J. *DNA Cell Biol.* 2012; 31:592. [PubMed: 22054065]
32. Ruefli AA, Ausserlechner MJ, Bernhard D, Sutton VR, Tainton KM, Kofler R, Smyth MJ, Johnstone RW. *Proc. Nat. Acad. Sci. U.S.A.* 2001; 98:10833.

**Trichostatin A
(TSA)**

**suberoylanilide hydroxamic
acid (SAHA)**

PCI-34051

Tubastatin A

FK-228

FIGURE 1.
Representative small molecules HDAC inhibitors.

Table 1

In Vitro HDAC inhibition (nM) and Antileishmanial Activities ($\mu\text{g/mL}$)

Compound	X	Promastigote IC_{50} ($\mu\text{g/mL}$) [*]	Amastigote IC_{50} ($\mu\text{g/mL}$) [*]	HDAC6 IC_{50} (nM) ^d	HDAC8 IC_{50} (nM) ^d
1a, X = O 1b, X = S	1a	>40	>40	NI	NI
	1b	12.7	30.7	681 $\pm$ 110	3675 $\pm$ 1201
2a, X = O 2b, X = S	2a	9.3	>40	NI	NI
	2b	0.1	6.5	457 $\pm$ 27	1272 $\pm$ 200
3a, X = O 3b, X = S	3a	6.8	30.9	NI	NI
	3b	0.1	6.3	847 $\pm$ 188	4283 $\pm$ 1548
4a, X = O 4b, X = S	4a	4.6	5	NI	NI
	4b	0.7	6.1	957 $\pm$ 159	2075 $\pm$ 459
5a, X = O 5b, X = S	5a	9	>40	NI	NI
	5b	0.4	11.2	44% ^f	1701 $\pm$ 717
6a, X = O 6b, X = S	6a	26.7	>40	NI	NI
	6b	0.9	31.7	372 $\pm$ 35	1907 $\pm$ 771
7a, X = O 7b, X = S	7a	6	29	NI	NI
	7b	0.3	31.3	454 $\pm$ 42	800 $\pm$ 304
8a, X = O 8b, X = S	8a	12.6	32.5	NI	NI
	8b	0.3	32.6	812 $\pm$ 286	2496 $\pm$ 1180

Compound		Promastigote IC ₅₀ (μg/mL) [*]	Amastigote IC ₅₀ (μg/mL) [*]	HDAC6 IC ₅₀ (nM) ^d	HDAC8 IC ₅₀ (nM) ^d
9a, X = O 9b, X = S	9a	27.6	31.6	NI	NI
	9b	0.3	34.4	306 ± 69	3105 ± 1649
10a, X = O 10b, X = S	10a	7.5	4	NI	NI
	10b	0.2	7.4	NI	2858 ± 944
11a, X = O 11b, X = S	11a	38.9	>40	NI	NI
	11b	2.3	6.1	2390 ± 458	NI
12a, X = O 12b, X = S	12a	>40	18	NI	NI
	12b	3.2	6.2	41% [†]	1570 ± 1067
13a, X = O 13b, X = S	13a	14.5	>40	NI	NI
	13b	1.3	5.3	1023 ± 99	1868 ± 723
SAHA	-	25.7	>40	144 ± 23	232 ± 19
Tubastatin A	-	4.4	8.8	15 ± 1 ^b	854 ± 40 ^b
PCI-34051	-	30.5	>40	2900 ^c	10 ^c
Pentamidine	-	0.6	8.9	NT	NT
Amphotericin B	-	0.4	1.6	NT	NT

NT = Not tested; NI = No Inhibition at maximum tested concentration of at 10 μM (percentage inhibition < 20%);

^{*} Average ± SD of triplicate observations.

[†] % inhibition of the compounds at 10 μM are given if the IC₅₀ was above 10 μM;

^a Cited from ref. 24, except for the control Tubastatin A and PCI-34051.

^b Cited from ref. 26.

^c Cited from ref. 27.

Table 2Antileishmanial Activities ($\mu\text{g/mL}$) against the Intra-macrophage Amastigote Stage of *L. donovani*.

Compound	Cytotoxicity (THP1) IC_{50} ($\mu\text{g/mL}$) [*]	Macrophage Amastigote IC_{50} ($\mu\text{g/mL}$) [*]
1b	>10	6.1
2b	>10	5.8
3b	>10	0.6
4b	>10	0.9
5b	>10	3.9
6b	>10	>10
7b	>10	3.7
8b	>10	>10
9b	>10	2.1
10b	>10	0.3
11b	>10	2.6
12b	>10	2.7
13b	>10	1.5
SAHA	>10	0.5
Tubastatin A	>10	1.9
PCI-34051	>10	>10
Pentamidine	>10	0.4
Amphotericine B	>10	0.2

^{*} Average $\pm$ SD of triplicate observations.