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Tropolones As Lead-Like Natural Products: The Development of Potent and Selective Histone Deacetylase Inhibitors

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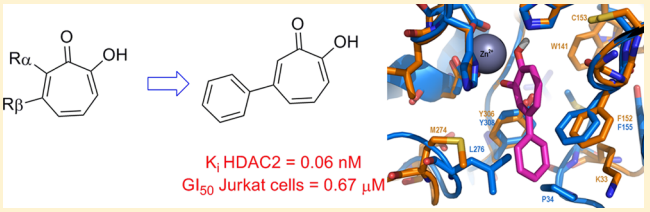
Supporting Information

ABSTRACT: Natural products have long been recognized as a rich source of potent therapeutics but further development is often limited by high structural complexity and high molecular weight. In contrast, at the core of the thujaplicins is a lead-like tropolone scaffold characterized by relatively low molecular weight, ample sites for diversification, and metal-binding functionality poised for targeting a range of metalloenzyme drug targets. Here, we describe the development of this underutilized scaffold for the discovery of tropolone derivatives that function as isozyme-selective inhibitors of the validated anticancer drug target, histone deacetylase (HDAC). Several monosubstituted tropolones display remarkable levels of selectivity for HDAC2 and potently inhibit the growth of T-cell lymphocyte cell lines. The tropolones represent a new chemotype of isozyme-selective HDAC inhibitors.



KEYWORDS: *Tropolone, HDAC, isozyme-selectivity, thujaplicin, metalloenzyme, T-lymphocyte cancer cell lines*

Natural products have long served as a rich source of drugs for a variety of indications ranging from anticancer to antimicrobial to neurological disorders. Typically these natural products are characterized by high molecular weight and potency as well as high levels of structural complexity with limited sites for diversification. In contrast, thujaplicins, members of the tropolone family of natural products, can be regarded as lead-like natural products. β-Thujaplicin (also known as hinokitiol) is characterized by low molecular weight (MW = 164) and a relatively lower level of complexity that allows more extensive structural modification. Thujaplicins are monoterpene natural products isolated from the heartwood of trees in the Cupressaceae family[1] that are associated with antiproliferative activity.[2−4] There have been few attempts to utilize these lead-like compounds in drug discovery, perhaps exacerbated by limited synthetic accessibility to these non-benzenoid aromatics.

The tropolone functionality is uniquely disposed to strongly chelate metal ions, which may be a hallmark of the biological activity of these compounds.[3] Substituted tropolones are a compelling and distinct chemotype for the development of inhibitors of metalloenzyme drug targets. Herein, we describe our efforts to use β-thujaplicin as a lead-like natural product to develop a novel class of inhibitors of histone deacetylase, a validated target in the treatment of cancer.[5,6]

Of the 18 HDAC isoforms, 11 are metalloenzymes that use zinc to remove a terminal acetyl group from lysine residues present in histones and other client proteins. The reversible acetylation and hydrolysis of the ε-acetamide in histones is associated with regulation of gene expression. Interestingly, there are a variety of natural products that inhibit HDACs such as trichostatin A (TSA; Figure 1), romidepsin, and trapoxin.[7]

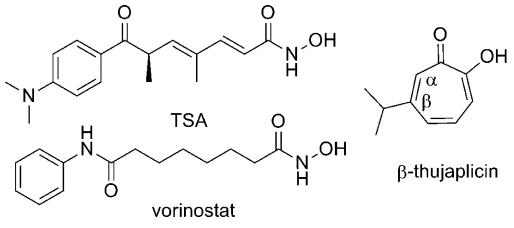


Figure 1. Vorinostat and the natural product, TSA, inhibit HDACs; β-thujaplicin is a lead-like natural product.

Both romidepsin and vorinostat were approved by the FDA for the treatment of cutaneous T-cell lymphoma; the latter possesses a zinc-targeting hydroxamate, similar to TSA.

Developing an alternative class of HDAC inhibitors based on tropolones may impart greater metabolic stability and isozyme selectivity relative to the hydroxamates. It is well-known that hydroxamates such as vorinostat suffer from relatively short metabolic half-life owing to an easily reduced N−O bond, a hydrolytically labile amide linkage and the formation of glucoronides.[8,9] In contrast, the metal-targeting moiety of the tropolone can be viewed as an extended vinylogous carboxylic acid[8] and would not be subject to reductive or hydrolytic

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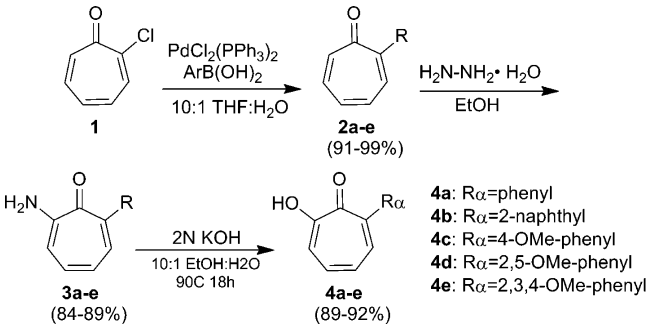
transformations. Additionally, the tropolone scaffold offers several points of modification to access pockets near the metal-binding site that may impart isozyme selectivity, a feature believed to be associated with increased efficacy and lower toxicity.[10] Although some hydroxamate-bearing compounds show some selectivity,[11] most of the compounds in clinical trials are considered to be pan-HDAC inhibitors and exhibit some associated toxicity that may be related to the inhibition of the many functions of the HDAC isozymes.[10] Herein, we describe first-generation tropolones that function as potent inhibitors of the HDAC enzyme and display significant cytotoxicity against T-lymphocyte cancer cell lines.

Structural analysis of HDACs[12,13] revealed hydrophobic pockets near the bound Zn^{2+} ion that would be accessible to lipophilic substituents placed in proximity to the putative metal-binding headgroup. In order to evaluate this design for HDAC inhibitors, an initial series of monosubstituted tropolones was prepared, focusing on placing small lipophilic substituents at the α- or β-positions (Figure 1).

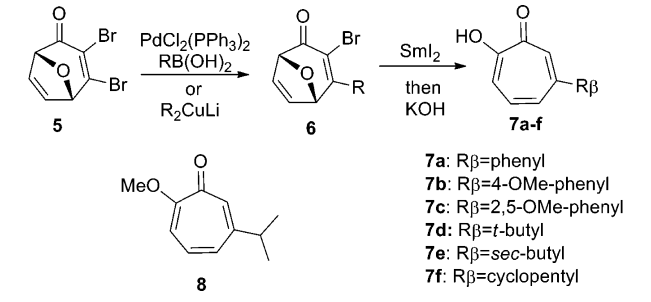
A concise route was developed for α-substituted tropolones from commercially available 2-chlorotropone (**1**). The α-chloro substituent proved very amenable to palladium-catalyzed cross-coupling conditions such that a series of 2-aryl tropones (**2a–e**) were easily prepared by Suzuki reactions. On the basis of a protocol initially reported for the synthesis of α-thujaplicin,[14] the addition of hydrazine led directly to the corresponding 7-aryl 2-amino tropones (**3a–e**). Final hydrolysis of the amino-tropones under basic conditions yielded the α-substituted tropolones (**4a–e**) in good overall yield (Scheme 1).

Scheme 1. Synthesis of α-Tropolones



We have also recently reported a direct and flexible route to β-substituted tropolones from the oxabicyclic dibromoenone **5**[15] (Scheme 2). Substituents can be selectively introduced through cross-coupling or cuprate chemistry to give the bromoenone **6** that is converted in a single step to the β-tropolone by reductive ring-opening with samarium diiodide

Scheme 2. Synthesis of β-Tropolones



followed by basic workup. This route was utilized to generate six analogues (**7a–f**). β-Thujaplicin and the corresponding methylated analogue (**8**, a mixture of positional isomers) were also included for evaluation.

In order to accurately compare inhibition values of different HDAC isozymes, we first performed a thorough Michaelis–Menten analysis of six different HDAC isozymes: 1, 2, 4, 5, 6, and 8 (Supporting Information Table S1). We then evaluated 12 substituted tropolones and show that these compounds function as inhibitors of HDACs and, excitingly, seven (**4b–e**, **7c**,**7d**, and **7f**) show high levels of selectivity (>100-fold) for the inhibition of HDAC2 relative to HDACs 1, 4, 5, 6, and 8 (Table 1). In fact, all of the free tropolones, with the exception

Table 1. Inhibition of HDAC Isozymes[a]

	HDAC isozyme (K_i values in nM)					
compd	1	2	8	4	5	6
TSA	0.87	1.06	69.65	14547	4120	3.02
4a	NA	0.26	1.09	NA	NA	527
4b	NA	0.25	186.30	NA	NA	NA
4c	NA	0.81	83.80	NA	NA	NA
4d	NA	0.42	811.50	NA	NA	NA
4e	NA	0.23	123.65	NA	NA	NA
7a	NA	0.06	1.47	10860	NA	NA
7b	NA	0.12	2.38	8361	NA	NA
7c	NA	0.51	266.30	11204	NA	NA
7d	NA	0.13	12.81	806.13	NA	NA
7e	NA	0.22	2.27	6115	NA	NA
7f	NA	0.04	122.70	990.23	NA	NA
β-tj	NA	15.44	177.95	NA	NA	NA
8	NA	NA	7.87	11,641	NA	NA

[a]For all HDAC isozymes except HDAC4, not active (NA) refers to K_i values >2500 nM, the highest concentration of inhibitor; for HDAC4, NA refers to K_i values >20 000 nM, the highest concentration of inhibitor. β-tj refers to β-thujaplicin.

of β-thujaplicin, were more potent inhibitors of HDAC2 than TSA, which inhibited HDACs 1, 2, and 6 with similar levels of potency. As methylated tropolone **8** shows significantly lower activity than the free tropolones, it is likely that the ionizable α-hydroxy ketone may facilitate strong metal (Zn^{2+}) chelation in the HDAC active site. Overall, both α- and β-substituted tropolones function as very potent inhibitors of HDAC2 with groups larger than the isopropyl group found in β-thujaplicin preferred in the β-position. The selectivity demonstrated by the tropolones is in sharp contrast to the pan-HDAC inhibition exhibited by the hydroxamates TSA and vorinostat.[16]

The selective and potent inhibition of HDAC2 is particularly exciting as the overexpression of this isozyme has been shown to be significant in aggressive forms of some cancers.[17,18] Additionally, specific knockdown of HDAC2 in estrogen receptor-positive cells potentiated tamoxifen-induced apoptosis,[19] while an HDAC2 knockout mouse showed increased synaptic plasticity and memory facilitation.[20] Moreover, it is observed that the tropolones specifically do not inhibit HDAC5; this lack of inhibition is potentially beneficial as deletion of HDAC5 may impair cardiac function.[21]

The addition of HDAC8 inhibition by some of the tropolones (**4a**, **7a**, **7b**, and **7e**) is also noteworthy as TSA and vorinostat have much higher K_i values at 69.65 and 480 nM,[16] respectively. Inhibition of HDAC8 was more sensitive to patterns of substitution with only one α-substituted compound

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(**4a**) showing good inhibition (K_i = 1.09 nM); increasing steric bulk at this position correlated with loss of potency, while β-substitution showed variable effects against HDAC8.

The β-phenyl derivative, **7a**, was docked to the structure of HDAC8[22] (PDB ID 1W22; Figure 2a) using Surflex-Dock. The

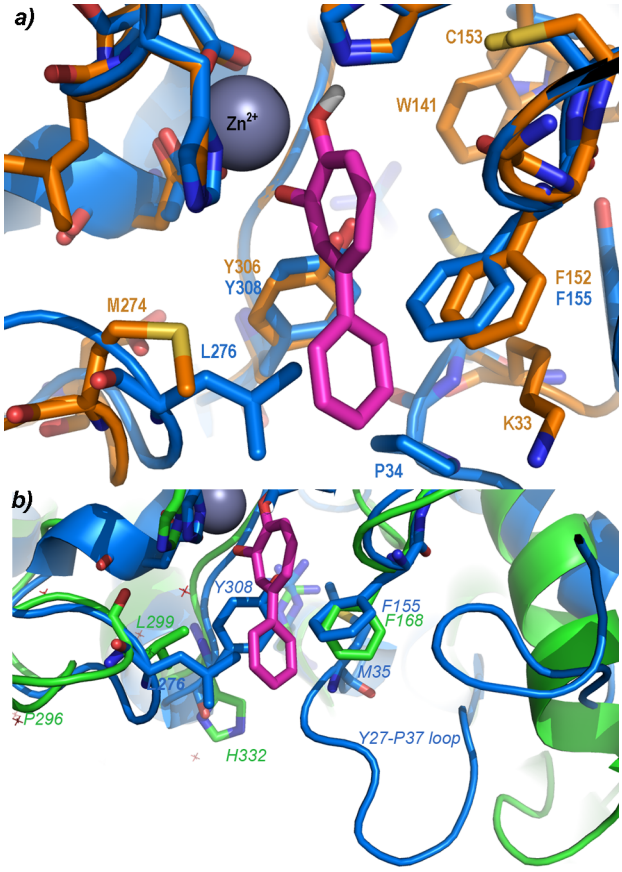

Figure 2. Docked complexes of (a) compound **7a** bound to HDAC2 (blue) and HDAC8 (orange) and (b) compound **7a** bound to HDAC2 (blue) and HDAC4 (green).

docked complex suggests that the bulky, hydrophobic β-substituent preferentially occupies the pocket formed by residues Phe 152, Tyr 306, Met 274, and Lys 33. In fact, a potential hydrogen bond may be formed between the tropolone carbonyl oxygen and the hydroxyl group of Tyr 306 (2.51 Å). It is noteworthy that this pocket is the same one that houses the alkyl chain of vorinostat. As this pocket is relatively large, there may be fewer interactions between other β-substituted tropolones and residues in the pocket, partially explaining the lower potency of β-thujaplicin and **7f**. Branching at this position (compounds **7d** and **7e**) or larger aryl substituents (**7a** and **7b**) is associated with improved HDAC8 inhibition. While the α-phenyl substituent of compound **4a** appears to be accommodated in the pocket comprising Tyr 306, Met 274, and Phe 152, substituted (**4c**−**e**) or bulker (**4b**) phenyl groups would create destabilizing interactions (Supporting Information Figure S1).

For HDAC2, the pocket defined by Tyr 308, Leu 276, Pro 34, and Phe 155 is analogous to that described above for HDAC8, including the same potential hydrogen bond formed with Tyr 304 (Figure 2a). A key substitution of Leu 276 for Met 274 and the addition of Pro 34 produce additional potential for hydrophobic interactions. As such, more β-substituted derivatives show high potency for HDAC2 than for HDAC8; increasing the size of this group relative to the naturally occurring isopropyl substituent is associated with increased potency. The α-substituted derivatives also performed well as inhibitors of HDAC2 and were selective over HDAC8. As the pocket comprising Tyr 308, Leu 276, and Phe 155 may be hindered for these compounds, it is possible that the alpha-tropolones bind in the pocket comprising Cys 156 and Leu 144 (Supporting Information Figure S2). Others have also exploited this foot pocket of HDAC2.[12]

In order to understand the origins of the observed selectivity, structural analysis was extended to HDAC4[23] (Figure 2b), an enzyme that was not inhibited by any of the tropolones. HDAC4 possesses His 332 at the analogous position as Tyr 308 in HDAC2 and appears to be poorly positioned to form a hydrogen bond to the tropolone oxygens. Additionally, major differences appear in two key active site loops. One loop, Pro296−Leu 299 (HDAC4) projects away from the active site relative to the same loop, Asp 274−Leu 276 (HDAC2; only L276 shown for clarity) that projects toward the inhibitor. The second loop in HDAC2, Tyr 27−Pro 37 (containing Pro 34 that interacts with the tropolone), appears to be missing in HDAC4.

The highly potent derivative, compound **4a**, was chosen to validate the competitive mode of inhibition of HDAC8 by the tropolones (Figure S3, Supporting Information). These experiments indicate that compound **4a** affects the binding of substrate and is a competitive inhibitor. Additionally, the measured K_i value for compound **4a** is 0.53 nM, which correlates well with a calculated K_i derived from the measured K_M and measured IC_{50} value shown in Table 1 (1.09 nM). The competitive nature of the inhibition and control experiments with the methylated tropolone (**8**) provide strong evidence that the tropolones inhibit HDAC activity by targeting the bound metal at the active site.

While it was exciting that several of the substituted tropolones were excellent HDAC inhibitors, it was critical to evaluate whether these compounds are indeed drug-like and could translate enzyme inhibition to cellular effects. As it is well-known that HDAC inhibitors have pronounced antiproliferative effects against hematological cancer cell lines,[24] two T-cell lymphocyte lines (HuT-78 and Jurkat) were chosen for analysis. Additionally, a colon cancer cell line (HCT116) and a pancreatic cancer line (BxPC-3) were also selected as representative solid tumor lines. The tropolone derivatives showed significant half maximal growth inhibition (GI_{50}) values in both T-lymphocyte cell lines (Table 2) with analogues **4c**, **4d**, **7a**, and **7e** showing increased activity relative to vorinostat with GI_{50} values below 1 μM against the Jurkat cell line. Although results against HCT-116 showed that the tropolones were less active, two derivatives (**4a** and β-thujaplicin) did show GI_{50} values less than 20 μM. Likewise, four derivatives (**4b**, **4d**, **7a**, and β-thujaplicin) have GI_{50} values less than 20 μM against the BxPC-3 cells. Evaluation of the compounds against a nonmalignant human dermal fibroblast line indicated a lack of general cytotoxicity with most of the derivatives showing no activity at 100 μM. This selectivity index is significantly greater than that exhibited by vorinostat in the same line.

In order to further characterize the drug-likeness of the leads, we measured the metabolic stability of a representative active tropolone toward phase I and II transformations. Incubation of compound **4a** with mouse liver microsomes showed good metabolic stability with a half-life of 93 min, which compares

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Table 2. Inhibition of Cell Growth

	cell line (GI_{50} values in μM)				
compd	Jurkat	HuT-78	HCT-116	BxPC-3	hDF
4a	3.33	7.83	15.24	29.39	96.46
4b	1.15	4.11	32.06	17.1	93.07
4c	0.62	2.87	56.99	35.93	>100
4d	0.76	3.05	46.65	14.1	>100
4e	1.86	4.74	34.98	21	>100
7a	0.67	4.14	53.44	18.5	>100
7b	4.62	8.95	43.67	91.6	>100
7c	5.89	17.09	>100	34.79	>100
7d	4.45	13.11	62.61	43.02	>100
7e	0.59	3.25	26.86	104	>100
7f	6.30	11.36	>100	180	>100
β-tj	1.10	4.99	6.92	19	>100
8	>100	>100	>100	>100	>100
Vorin.	0.90	2.10	2.50	5.56	18.95

favorably with the 28 min half-life found for vorinostat in a similar system.[9] We also studied the potential for the tropolones to be metabolized through phase II conjugation reactions by the addition of UDPGA to the microsomal incubation and determined a half-life of 60 min and observed the formation of the gluconoride by mass spectrum analysis.

To validate that the tropolones modulate the acetylation state of histones in cells, treated Jurkat cells were probed using antibodies to acetylated histone H4K12. Using flow cytometric analysis to quantify histone modulation,[25] two tropolones (β-thujaplicin and aryl tropolone **4d**) were compared to vorinostat and an untreated control. The geometric mean of the fluorescence intensities (GMFI) show that both vorinostat (GMFI = 100) and the tropolone derivatives (GMFI = 45.40 and 42.20) produce increased levels of histone acetylation for H4K12 as compared to control (GMFI = 7.13). Although these experiments do not exclude the possibility that there are additional targets, the data validate the modulation of HDAC enzymes in cells.

In conclusion, we investigated whether thujaplicins could serve as powerful lead-like natural products targeting HDACs as they possess relatively low molecular weight, ample sites of diversification, and a key metal-directing functional group. Structural analysis suggests that the tropolones form a strong complex with the bound zinc ion and project pendant functionality into different hydrophobic pockets that confer isozyme specificity at the active site. In addition to activity at the enzyme level, further evaluation shows that the compounds exhibit significant cytotoxicity in cancer cells and good metabolic stability and modulate histone acetylation levels. These initial investigations into tropolone-based HDAC inhibitors suggest that this new chemotype could give rise to potent and selective inhibitors that may find application in the study of HDAC function or even as versatile leads for new therapeutic development.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, data for enzyme kinetic parameters, additional figures describing protein:ligand interactions, and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HDAC, histone deacetylase; TSA, trichostatin A; hDF, human adult fibroblasts; UPDGA, uridine diphosphosphate glucoronic acid

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