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The histone deacetylase-6 inhibitor tubacin directly inhibits *de novo* sphingolipid biosynthesis as an off-target effect

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ABSTRACT

Histone deacetylase 6 (HDAC6) controls acetylation of a number of cytosolic proteins, most prominently tubulin. Tubacin is a small molecule inhibitor of HDAC6 selected for its selective inhibition of HDAC6 relative to other histone deacetylases. For this reason it has become a useful pharmacological tool to discern the biological functions of HDAC6 in numerous cellular processes. The interest of this laboratory is in the function and regulation of sphingolipids, a family of lipids based on the sphingosine backbone. Sphingolipid biosynthesis is initiated by the rate limiting enzyme serine palmitoyltransferase (SPT). Sphingolipids have critical and diverse functions in cell survival, apoptosis, intra- and intercellular signaling, and in membrane structure. In the course of examining the role of HDAC6 in the regulation of sphingolipid biosynthesis we observed that tubacin strongly inhibited *de novo* synthesis whereas HDAC6 knockdown very moderately stimulated synthesis. We resolved these seemingly contradictory results by demonstrating that, surprisingly, tubacin is a direct inhibitor of SPT activity in permeabilized cells. Furthermore tubacin inhibits *de novo* sphingolipid synthesis in intact cells at doses commonly used to test HDAC6 function and does so in an HDAC6-independent manner. Niltubacin is a chemical analog of tubacin which lacks tubacin's HDAC6 activity, and so is often used as a control for off-target effects of tubacin. We find that niltubacin is inactive in the inhibition of sphingolipid biosynthesis, and so does not serve to distinguish the inhibitory effects of tubacin on HDAC6 from those on sphingolipid biosynthesis. These results indicate that caution should be used in the use of tubacin to study the role of HDAC6.

1. Introduction

Histone deacetylase 6 (HDAC6), rather than targeting histones as its name implies, has numerous cytosolic, non-histone, substrates. In particular, HDAC6 is responsible for deacetylation of tubulin [1]. Through deacetylation of these substrates HDAC6 has multiple functions in the control of the cytoskeleton and cell migration, heat-shock protein function, and cell signaling, among others (reviewed in [2,3]). Schreiber and colleagues utilized a high throughput screen of a dioxane-based chemical library to identify compounds that selectively inhibit HDAC6, and not other deacetylases, as pharmacological tools for the study of HDAC6 function [4]. The lead compound selected from those studies was termed tubacin, which they carefully characterized with respect to deacetylase specificity and biological effects. Tubacin has subsequently been used by a number of laboratories to explore the biological roles of HDAC6. Our laboratory focuses on the regulation and function of sphingolipids [5–7], a family of signaling and structural lipids with diverse and essential biological roles. In the course of exploring the role of deacetylase activity on regulation of sphingolipid biosynthesis we noted that tubacin inhibited *de novo* ceramide biosynthesis. Although our initial interest was in the role of HDAC6 in regulation of sphingolipid synthesis, our findings, presented below, indicate that the inhibition of sphingolipid synthesis by tubacin is a direct off-target effect of that compound.

2. Materials and methods

2.1. Materials

siRNA oligonucleotides for human HDAC6 (catalog number HS00195869) were from Ambion®. TaqMan® probes for quantitative

Abbreviations: HDAC6, histone deacetylase 6; SPT, serine palmitoyltransferase.

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real-time PCR, Taqman® Gene Expression Master Mix and High Capacity Reverse Transcription Kit were all from Applied Biosystems®. Lipofectamine® RNAiMax transfection reagent and TRIzol® were from Invitrogen (Life Technologies, Grand, Island, NY). L-[^{3}H(G)]-Serine, scintillation vials and scintillation fluid were from Perkin Elmer (Waltham, MA). C16-ceramide standard was from Avanti Polar Lipids (Alabaster, AL). Whatman thin layer chromatography (TLC) plates were from VWR (Radnor, PA). Hela cells were from ATCC (Manassas, VA). Cell culture media and supplies were from VWR. Organic solvents were from Thermo Fisher Scientific (Pittsburgh, PA). Tubacin was from Sigma Aldrich (St. Louis, MO) and niltubacin was from Enzo Life Sciences (Farmington, NY). All other chemicals used were from Sigma Aldrich (St. Louis, MO) unless otherwise indicated.

2.2. Methods

2.2.1. Measurement of serine palmitoyl transferase activity and de novo ceramide biosynthesis

Measurement of serine palmitoyltransferase (SPT) activity in digitonin-permeabilized cells was performed essentially as previously described [7]. Briefly: Hela cells were plated into collagen-coated 24-well plates at 0.3×10^6 cells/well overnight. Cells were then washed with PBS and permeabilized by treatment with 200 μg/ml digitonin for 3 min. SPT activity was measured in a buffer containing 50 mM HEPES, pH 8.0, 1 mM $MgCl_2$, 1 mM ATP, 20 μM 5′ pyridoxal phosphate, 50 μM palmitoyl CoA, 1 mM serine, and ^{3}H-serine at 10 μCi/ml in a volume of 400 μl. Incubations were performed for 60 min at 37 °C. Following incubation cells were harvested into 400 μl alkaline MeOH (7 g KOH/l). 100 μl $CHCl_3$ was added and extracts were vortexed. Phases were broken by the addition of 500 μl $CHCl_3$, 500 μl alkaline H_2O (100 μl 2 N NH_4-OH/100 ml H_2O), and 100 μl 2 N NH_4OH. The top, aqueous layer was aspirated and the remaining organic layer was washed 2× with 1 ml alkaline H_2O. 350 μl of the organic layer was dried under N_2 in a scintillation vial and incorporated ^{3}H-serine was measured by liquid scintillation counting. Measurement of *de novo* ceramide biosynthesis in intact cells was measured by incorporation of ^{3}H-serine into ceramide during a 1 h incubation as previously described [7]. Briefly: Hela cells were plated as above overnight. Cells were then labeled in serine-depleted media (MEM containing 1% dialyzed FBS and 10 μCi/ml ^{3}H-serine) with 1 ml/well for 60 min. Cells were extracted by a modification of the Bligh–Dyer extraction [7]. 25 nMol C16 ceramide was added to the organic phase and samples were then subjected to thin layer chromatography using a solvent system of chloroform:acetic acid:methanol (90:10:2, v/v). After visualizing the ceramide band by exposure in an iodine tank, the ceramide bands were scraped and incorporated ^{3}H-serine was measured by liquid scintillation counting.

2.3. siRNA depletion of HDAC6

Hela cells were plated at 6×10^4 cells/well in collagen-coated 24-well plates overnight. (5 nM) of siRNA oligonucleotide was combined with Lipofectamine® RNAiMax transfection reagent per the manufacturer's recommendations, added to medium, and cells were incubated overnight before treatment and measurement of *de novo* ceramide biosynthesis as described above. The effect of siRNA treatment on expression of HDAC6 was determined by quantitative, real-time PCR using TaqMan® probes as previously described [7].

3. Results and discussion

In initial experiments we found that siRNA depletion of HDAC6 slightly enhanced ceramide biosynthesis, whereas tubacin treatment strongly inhibited ceramide biosynthesis (data not shown.) To explore this in detail we first examined whether tubacin might be a direct inhibitor of the rate-limiting enzyme in sphingolipid

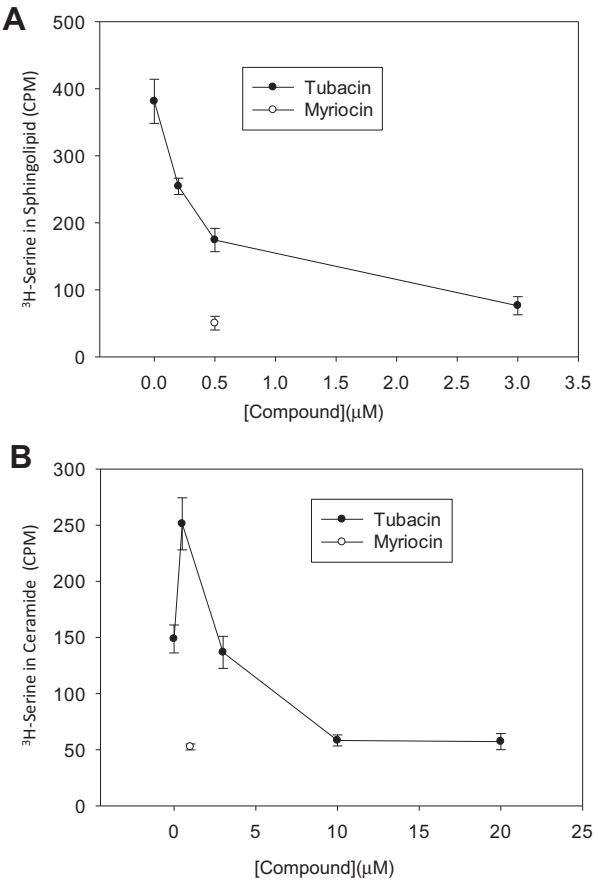

Fig. 1. Tubacin inhibits serine palmitoyltransferase activity in permeabilized cells and *de novo* ceramide biosynthesis in intact cells. (A) Hela cell monolayers were permeabilized and incubated for 60 min in the presence of the indicated concentrations of tubacin or myriocin, co-factors required for serine palmitoyltransferase activity, and ^{3}H-serine to label sphingolipids. Following incubation monolayers were extracted for total sphingolipids and incorporation of ^{3}H-serine was determined by liquid scintillation counting. (B) Hela cell monolayers were incubated for 60 min in the presence of the indicated concentrations of tubacin or myriocin during labeling of sphingolipids with ^{3}H-serine. Following incubation monolayers were extracted and ceramide was isolated by thin layer chromatography. Bands corresponding to ceramide were scraped from the plates and ^{3}H-serine incorporation was determined by liquid scintillation counting. Results for both panels are the means plus and minus standard deviation of quadruplicates. Shown are representative results from at least three independent experiments.

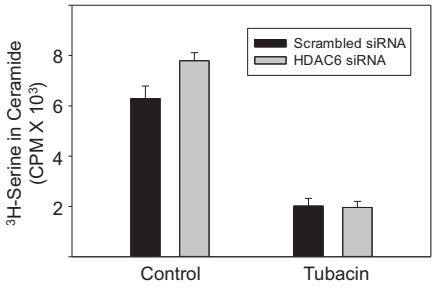

Fig. 2. Tubacin inhibition of *de novo* ceramide biosynthesis is HDAC6 independent. Hela cell monolayers were transfected for 24 h with either control or HDAC6-specific siRNA oligonucleotides. ^{3}H-serine incorporation into ceramide was determined in the presence or absence of 10 μM tubacin as described in the legend to Fig. 1. Results are the means plus and minus standard deviation of 6 replicates. Shown is a representative of two experiments with nearly identical results.

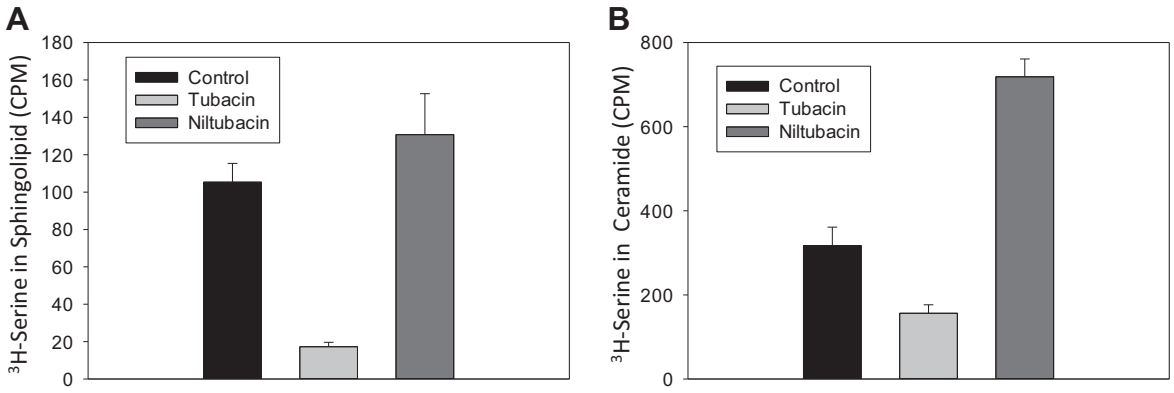

Fig. 3. Niltubacin, an analog of tubacin lacking HDAC6 inhibitory activity, does not inhibit serine palmitoyltransferase in permeabilized cells or *de novo* ceramide generation in intact cells. ^{3}H-serine incorporation into total sphingolipids in permeabilized cells (Panel A) or into ceramide in intact cells (Panel B) was measured in the presence of 3 μM (permeabilized cells) or 10 μM (intact cells) tubacin or niltubacin as described in the legend for Fig. 1 and in Section 2.2. Results are the means plus and minus standard deviation of quadruplicates. Shown is a representative of two experiments with nearly identical results.

biosynthesis, serine palmitoyltransferase (SPT, reviewed in [8]). We utilized a permeabilized cell assay that we have developed as a sensitive, robust, and facile measurement of SPT activity [7]. Tubacin inhibits SPT in permeabilized cells with an IC_{50} of approximately 300 nM (Fig. 1, Panel A). The extent of inhibition under these conditions (81%) is comparable to that of the well-studied SPT inhibitor myriocin (87%) [9,10].

Tubacin inhibited *de novo* synthesis of the bioactive, pro-apoptotic sphingolipid, ceramide in intact cells (Fig. 1, Panel B), as expected from its *in vitro* activity as an SPT inhibitor. The IC_{50} for tubacin (approximately 14 μM) in intact cells is considerably higher than that observed in permeabilized cells. This concentration is, however, well within the range routinely used in cell cultures for the inhibition of HDAC6 (5–20 μM) (e.g.,[11–13]). Interestingly at the lowest dose tested, 0.5 μM, tubacin stimulated *de novo* ceramide biosynthesis. The underlying mechanism is unclear, although, as noted below, we observed a similar phenomenon with the inactive tubacin analog niltubacin.

To determine if the effect of tubacin on sphingolipid synthesis was mediated by tubacin interaction with HDAC6, we depleted cells of HDAC6 by siRNA oligonucleotide transfection (Fig. 2). This treatment depleted HDAC6 mRNA by greater than 84% as determined by real-time PCR (data not shown). HDAC6 depletion by itself slightly, but consistently, elevated *de novo* ceramide generation in Hela cells (Fig. 2, "Control"). As noted above, the enhancement of ceramide generation by HDAC6 depletion stands in sharp contrast to the inhibition of sphingolipid synthesis imparted by tubacin treatment. HDAC6 depletion had no effect on tubacin inhibition of ceramide synthesis (Fig. 2, "Tubacin"). These results demonstrate that the effect of tubacin on sphingolipid biosynthesis is HDAC6-independent.

The above results establish that tubacin has the off-target effect of blunting sphingolipid biosynthesis. Schreiber and colleagues identified a derivative of tubacin, termed niltubacin, which retains the bulk of the chemical structure of tubacin, but lacks HDAC6 inhibitory activity [4]. Niltubacin has been used as a negative control for off-target effects of tubacin. To determine whether niltubacin would be a useful tool to distinguish the on- and off-target effects of tubacin with regard to sphingolipid biosynthesis, we tested the ability of niltubacin to inhibit SPT activity in permeabilized cells and *de novo* ceramide generation in intact cells (Fig. 3). While, as before, tubacin potently inhibited sphingolipid synthesis, niltubacin was inactive in this regard. Interestingly, niltubacin significantly enhanced *de novo* ceramide biosynthesis in intact cells. These data clearly indicate that niltubacin is not useful experimentally to separate the inhibitory effect of tubacin on HDAC6 from the inhibition of sphingolipid biosynthesis.

We have identified inhibition of sphingolipid biosynthesis as a significant off-target, HDAC 6-independent, effect of tubacin. Sphingolipids serve diverse and complex functions in intra- and extracellular signaling (reviewed in [14,15]) and as structural components of cell membranes (reviewed in [16]). Considering the potential biological consequences of altering sphingolipid levels, perturbation of sphingolipid synthesis may complicate the interpretation of tubacin treatment. Tubacin has exquisite isoform specificity for histone deacetylases [4] and so serves a useful purpose as a pharmacological tool for examining HDAC 6 involvement in biological processes. Our results emphasize that use of tubacin should be combined with genetic manipulation of HDAC6 and other controls to ensure that inhibition of HDAC 6, and not inhibition of sphingolipid synthesis, is responsible for observed biological effects.

Acknowledgments

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References

[1] Y. Zhang, N. Li, C. Caron, G. Matthias, D. Hess, S. Khochbin, P. Matthias, HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo, EMBO J. 22 (2003) 1168–1179.
[2] Y. Li, D. Shin, S.H. Kwon, Histone deacetylase 6 plays a role as a distinct regulator of diverse cellular processes, FEBS J. 280 (2013) 775–793.
[3] A. Valenzuela-Fernandez, J.R. Cabrero, J.M. Serrador, F. Sanchez-Madrid, HDAC6: a key regulator of cytoskeleton, cell migration and cell–cell interactions, Trends Cell Biol. 18 (2008) 291–297.
[4] S.J. Haggarty, K.M. Koeller, J.C. Wong, C.M. Grozinger, S.L. Schreiber, Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 4389–4394.
[5] S.M. Pitson, R.J. D'Andrea, L. Vandeleur, P.A. Moretti, P. Xia, J.R. Gamble, M.A. Vadas, B.W. Wattenberg, Human sphingosine kinase: purification, molecular cloning and characterization of the native and recombinant enzymes, Biochem. J. 350 (Pt 2) (2000) 429–441.
[6] D.L. Siow, C.D. Anderson, E.V. Berdyshev, A. Skobeleva, S.M. Pitson, B.W. Wattenberg, Intracellular localization of sphingosine kinase 1 alters access to substrate pools but does not affect the degradative fate of sphingosine-1-phosphate, J. Lipid Res. 51 (2010) 2546–2559.
[7] D.L. Siow, B.W. Wattenberg, Mammalian ORMDL proteins mediate the feedback response in ceramide biosynthesis, J. Biol. Chem. 287 (2012) 40198–40204.
[8] K. Hanada, Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism, Biochim. Biophys. Acta 1632 (2003) 16–30.
[9] J.K. Chen, W.S. Lane, S.L. Schreiber, The identification of myriocin-binding proteins, Chem. Biol. 6 (1999) 221–235.

[10] Y. Miyake, Y. Kozutsumi, S. Nakamura, T. Fujita, T. Kawasaki, Serine palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant, ISP-1/myriocin, Biochem. Biophys. Res. Commun. 211 (1995) 396–403.
[11] S. Saito, J.A. Lasky, W. Guo, H. Nguyen, A. Mai, S. Danchuk, D.E. Sullivan, B. Shan, Pharmacological inhibition of HDAC6 attenuates endothelial barrier dysfunction induced by thrombin, Biochem. Biophys. Res. Commun. 408 (2011) 630–634.
[12] S. Saji, M. Kawakami, S. Hayashi, N. Yoshida, M. Hirose, S. Horiguchi, A. Itoh, N. Funata, S.L. Schreiber, M. Yoshida, M. Toi, Significance of HDAC6 regulation via estrogen signaling for cell motility and prognosis in estrogen receptor-positive breast cancer, Oncogene 24 (2005) 4531–4539.
[13] A.D. Tran, T.P. Marmo, A.A. Salam, S. Che, E. Finkelstein, R. Kabarriti, H.S. Xenias, R. Mazitschek, C. Hubbert, Y. Kawaguchi, M.P. Sheetz, T.P. Yao, J.C. Bulinski, HDAC6 deacetylation of tubulin modulates dynamics of cellular adhesions, J. Cell Sci. 120 (2007) 1469–1479.
[14] Y.A. Hannun, L.M. Obeid, The ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind, J. Biol. Chem. 277 (2002) 25847–25850.
[15] M. Maceyka, K.B. Harikumar, S. Milstien, S. Spiegel, Sphingosine-1-phosphate signaling and its role in disease, Trends Cell Biol. 22 (2012) 50–60.
[16] S. Hoetzl, H. Sprong, G. van Meer, The way we view cellular (glyco)sphingolipids, J. Neurochem. 103 (Suppl. 1) (2007) 3–13.