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The inhibitors of histone deacetylase suberoylanilide hydroxamate and trichostatin A release nitric oxide upon oxidation

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Abstract

Suberoylanilide hydroxamic acid (SAHA, vorinostat, Zolinza®) is the lead compound of a new class of histone deacetylase (HDAC) inhibitors used as anticancer drugs, which have been shown to affect multiple proteins associated with gene expression, cell proliferation and migration. Studies have also demonstrated the essential role of the hydroxamate moiety of SAHA in HDAC inhibition. The ability of SAHA and its structural analog Trichostatin A (TSA) in generating NO upon oxidation was tested directly by spin trapping of NO using Electron Paramagnetic Resonance (EPR) spectroscopy and also indirectly via the determination of nitrite using the Griess assay. H₂O₂/metmyoglobin was used to oxidize SAHA and TSA. These studies demonstrate for the first time, the release of NO from SAHA and its structural analog TSA. We tested the protective effects of SAHA, TSA and valproic acid (VPA) in mammalian Chinese hamster V79 cells exposed to a bolus H₂O₂ for 1 hour and monitoring the clonogenic cell survival. Both SAHA and TSA afforded significant cytoprotection when co-incubated with H₂O₂ whereas VPA was ineffective. These studies provide evidence for the release of NO by hydroxamate containing HDAC inhibitors and their antioxidant effects. Such roles may be an added advantage of this class of HDAC agents which are used for epigenetic therapies in cancer.

Introduction

Suberoylanilide hydroxamic acid (SAHA, Figure 1) is an orally active, inhibitor of histone deacetylase (HDAC), which has been shown to cause cell growth arrest and death [1,2]. The drug is already approved for therapy in patients with primary cutaneous T-cell lymphoma [3] and is currently in multiple clinical studies in patients with hematologic and solid tumor malignancies [4]. While the biological effects of SAHA are associated with inhibition of class I and II zinc containing HDAC, the exact mechanisms of its anticancer effects are still being refined. SAHA has been shown to affect the acetylation of multiple targets, which alters the transcription of genes and the function of proteins that are regulators of proliferation, migration and death of transformed cells [5]. The hydroxamate drug pharmacophoric structure (Fig. 1), which features a cap substructure linked by a hydrocarbon chain to a hydroxamic acid moiety [6] is similar to that of the naturally occurring antifungal antibiotic trichostatin A (TSA, Figure

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1) [7]. X-ray crystallographic studies of a complex of SAHA with a histone deacetylase-like protein showed the mode of binding of the compound in the catalytic domain of the enzyme. While the phenyl group is on the hydrophobic surface of the enzyme, the polymethylene chain extends down a relatively narrow channel to get to the zinc atom at the catalytic site. The hydroxamic acid coordinates the zinc ion through its CO and OH groups resulting in a penta-coordinate Zn^{2+} . Three additional hydrogen bonds exist between the CO, the NH and the OH groups [8,9]. Thus, the presence of the hydroxamate moiety and this interaction seems to be a prerequisite for optimal HDAC inhibitory activity of SAHA and of other hydroxamate based HDAC inhibitors.

Although hydroxamates are employed as therapeutic agents both in cancer and in iron intoxication or overload, it has been suggested that their activity is based not only on their coordination of metals (Zn^{2+} , Fe^{3+}) but also on other factors [10-13]. Previously, hydroxamate containing agents such as hydroxyurea were shown to release nitric oxide upon oxidation [14-17]. Chemical agents which donate NO either spontaneously or enzymatically have shown to afford protection to mammalian cells subjected to oxidative stress. In this study, we show that hydroxamate containing HDAC inhibitors generate NO upon oxidation and also show that these agents provide cytoprotection to mammalian cells subjected to oxidative stress mediated by reactive oxygen species. These attributes of SAHA need to be further evaluated in the context of their use as HDAC inhibitors to identify additional roles which may operate in the treatment of cancer.

Materials and Methods

SAHA was received from Merck & Co., Inc. (Whitehouse Station, NJ, USA). Hydroxyurea (HU), Valproic Acid (VPA, Scheme 1), superoxide dismutase (SOD), and myoglobin (Mb) were purchased from Sigma (St. Louis, MO). TSA and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (carboxy-PTIO) were purchased from Cayman Chemical (Ann Arbor, Michigan). Griess reagents were prepared according to previously published method [18]. H_2O_2 was obtained from Fisher Scientific and its concentration assayed spectrophotometrically using an extinction coefficient of $43.6\text{ M}^{-1}\text{ cm}^{-1}$ at 240 nm.

Griess Assay

SAHA, TSA, HU or VPA were incubated at room temperature (RT) in PBS (pH 7.4) containing $10\text{ }\mu\text{M}$ Mb and 5 mM H_2O_2 with or without SOD (10^3 units/mL). After 0, 10, 20 and 30 min, reaction mixtures were assayed for nitrite using Griess assay [18].

EPR measurements

Solutions containing $25\text{ }\mu\text{M}$ Mb, 10 mM H_2O_2 , $50\text{ }\mu\text{M}$ carboxy-PTIO, and $250\text{ }\mu\text{M}$ SAHA, TSA or VPA were scanned for EPR signals at RT. The reaction mixture was transferred to a gas permeable teflon capillary (Zeus Industries, Orangeburg, SC, USA) having an inner diameter of 0.81 mm, a wall thickness of 0.38 mm and a length of 15 cm. Each capillary was folded twice, inserted into a narrow quartz tube that was open on both edges (2.5 mm inner diameter) and placed within the EPR cavity. EPR spectra were recorded using a Varian E-9 X-band spectrometer with the following instrument settings: Modulation amplitude, 0.5 G; time constant, 0.128 s; field sweep, 25 G/min; modulation frequency, 100 kHz; microwave power, 10 mW.

Cell culture

Chinese hamster V79 lung fibroblasts were cultured in F-12 medium supplemented with 10% (v/v) fetal calf serum and antibiotics. Cells survival was assessed by the clonogenic assay, with

the plating efficiency ranging between 56% and 87%. Stock cultures of exponentially growing cells were trypsinized, rinsed, plated (7×10^5 cells per dish) in 100-cm² petri dishes and incubated for 16 h at 37°C prior to experimental protocols. Cells were exposed to various concentrations of H₂O₂ for 1 h in the absence and presence of SAHA, TSA, HA or VPA. After treatment the cells were washed with PBS, trypsinized, counted, and plated in triplicate for macroscopic colony formation. Each dose determination was plated in triplicate, and experiments were repeated two times. Plates were incubated for 7 days, after which colonies were fixed with methanol/acetic acid, 3:1 (v/v), stained with crystal violet, and counted. Colonies containing >50 cells were scored. Error bars represent the SEM (standard error of the mean) and are shown when larger than the symbol.

Results and Discussion

Indirect evidence

The oxidation of SAHA, TSA, and VPA was induced by their incubation with H₂O₂ and metMb (MbFe^{III}) in PBS (pH 7.4) at room temperature for various periods of time and the yield of nitrite in the reaction mixtures was measured using the Griess assay. Oxidation of SAHA or TSA, but not of VPA resulted in the production of nitrite. The oxidation of hydroxyurea, which was evaluated for comparison purposes, demonstrated similar accumulation of nitrite as previously reported [19-21]. No nitrite accumulation was observed where either metMb, H₂O₂, or any of the drugs was omitted.

Kinetics

The accumulation of nitrite upon oxidation of SAHA or TSA increased as the incubation time increased. The rate of the accumulation was linearly dependent on [hemo-protein] but not on the [drug] from 0.25 to 1 mM. Such kinetic behaviour is consistent with the catalytic nature of the metMb/H₂O₂ system. In the absence of an effective reductant, the hemo-protein operates as catalase-mimic [22-25]. Compound I (MbFe^{IV}=O), which is formed upon MbFe^{III} oxidation by H₂O₂, is further reduced by H₂O₂ to yield the parent metMb. In this case H₂O₂ serves both as an oxidant and as a sink of reducing-equivalents. Hence H₂O₂ is progressively depleted via dismutation while the cycling-rate of the hemo-protein controls the reaction rate. The rate of the reaction was reported to depend on [hemo-protein] with a turnover number of 0.15 min⁻¹ per hemo-protein[25]. In the presence of a reductant, such as hydroxamate, which recycles MbFe^{III} through reduction of compound I and/or ferrylMb, the hemo-protein operates as peroxidase-mimic. Fig. 2 displays the results obtained with 10 μM metMb, 5 mM H₂O₂ and 250 μM drug. The rates of nitrite formation (4 - 5 μM min⁻¹) observed for SAHA and TSA were similar and resembled that found for hydroxyurea (data not shown). This implied a turnover-number of *ca.* 0.45 min⁻¹ per hemo-protein as previously reported in the presence of nitroxide[25].

Direct evidence

The EPR observations further substantiate the conclusion that H₂O₂/MbFe^{III} induced oxidation of SAHA and TSA leads to NO release. The exposure of 0.25 mM SAHA to 25 μM metMb and 5 mM H₂O₂ in PBS containing carboxy-PTIO yielded carboxy-PTI (Fig. 3) as previously observed for several hydroxamate derivatives. [19,21,26-32] Similar EPR spectral changes were observed using TSA and HU but not with VPA as expected since it lacks the hydroxamate moiety. The carboxy-PTI signal was not detected when either H₂O₂, metMb or the drug was excluded from the reaction mixture. Figures 3 and 4 show the progressive decay of carboxy-PTIO signal and the corresponding growth of carboxy-PTI signal monitored by EPR upon oxidation of SAHA and TSA, and compare the time-dependent spectral changes with those observed for hydroxyurea.

The effect of SOD

The release of nitrite (Fig. 2), the decay of the carboxy-PTIO signal (Fig. 4) and the concomitant accumulation of the carboxy-PTI signal (Fig. 5) were also repeated in these reaction systems when 10^3 U/ml SOD was included in the reaction mixture. While the presence of SOD during SAHA oxidation markedly increased the rates of nitrite accumulation, carboxy-PTIO decay and carboxy-PTI appearance, it had a smaller effect on the rates in the case of HU, and no effect detectable in the case of TSA. Superoxide radicals are indeed formed in the $\text{H}_2\text{O}_2/\text{metMb}$ reaction system during H_2O_2 oxidation by $\text{MbFe}^{\text{IV}}=\text{O}$ and/or $\text{MbFe}^{\text{IV}}=\text{O}$. Hence, the mechanism by which SOD enhanced nitrite accumulation (Fig. 2) and the decay of carboxy-PTIO (Fig. 3) during SAHA oxidation could be attributed to the dismutation of $\text{HO}_2^{\cdot}/\text{O}_2^{\cdot-}$, thus preventing its reaction with NO to yield peroxynitrite. Yet, this mechanism cannot be reconciled with the failure of SOD to enhance NO release upon TSA oxidation (Figs 1, 3 and 4). Instead, it can be assumed that the mechanism of NO release from SAHA, though not TSA, involves the intermediacy of HNO/NO^- , [33-39] which is oxidized by SOD to NO ($k = 7 \cdot 10 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ [40]). The present results also agree with previous reports indicating that HU oxidation can lead to production of both NO and HNO/NO^- [21].

The antioxidant effect of SAHA and TSA

Since agents, which generate NO , have been previously found to afford cytoprotection to mammalian cells subject to oxidative stress [39,41], it was of interest to test the HDAC-inhibitors in a similar setting. When Chinese hamster V79 cells were exposed to bolus quantities of H_2O_2 at various concentrations ranging 0-1000 μM for 1 h, a H_2O_2 dose dependent-cytotoxicity was observed. Fig. 6 shows that co-incubation of cells with either TSA (2.5 μM) or SAHA (2.5 μM) resulted in a significant inhibition of H_2O_2 -induced loss of cell survival. Conversely, VPA under similar experimental conditions had no protective effect. Previously, it was shown that NO protects against cellular damage and cytotoxicity induced by reactive oxygen species [42]. Although this result does not necessarily imply that NO release activity is required for the antioxidative effect, it still suggests that a hydroxamate moiety in HDAC-inhibitors is required both for NO release and for the antioxidative activity. Similar protection of cells against H_2O_2 was also seen upon treatment with HU (data not shown) which is known to release NO upon oxidation [21,29].

Conclusions

The results of the present study demonstrate for the first time the production of NO by oxidation of SAHA, a hydroxamate-containing inhibitor of HDAC, and its structural analog TSA. Although both HDAC-inhibitors, TSA and SAHA release NO upon oxidation, their exact detailed oxidation mechanisms, which involve loss of 3 electrons, are not exactly the same. The NO-releasing quality of SAHA adds to its already known modes of action and might have biological implications.

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Figure 1. Chemical structures of suberoylanilide hydroxamate (SAHA), trichostatin A (TSA) and valproic acid (VPA)

Figure 2. Nitrite accumulation reflecting $\cdot$ NO release upon oxidation of SAHA, TSA but not of VPA Nitrite formation was determined using the Griess assay following exposure of 250 μ M SAHA (squares) or TSA (triangles) to 10 μ M metMb and 5 mM H_2O_2 in PBS (pH 7.4) at RT for various periods of time, in the absence (open symbols) and the presence (solid symbols) of 10^3 U/ml SOD. VPA did not induce nitrite formation (data not shown). Nitrite release was compared to that obtained with hydroxyurea (data not shown). Data points represent mean values of two experiments.

Figure 3. The EPR spectrum of carboxy-PTI progressively accumulates upon the reaction of carboxy-PTIO with SAHA-borne NO

The EPR spectra monitored during the incubation of 250 μM SAHA with 25 μM Mb, 5 mM H_2O_2 , and 50 μM carboxy-PTIO, Top signal: At $t = 0$ displays the 5-line spectrum of carboxy-PTIO alone, Middle and Bottom signals: At $t = 10$ min and $t = 20$ min, show the progressive emergence of carboxy-PTI spectrum superimposed on the decaying carboxy-PTIO signal.

Figure 4. SOD facilitates NO release upon metMb/H₂O₂-induced oxidation of SAHA but not of TSA

The EPR spectra observed during the incubation of 250 μ M of either SAHA (squares), TSA (triangles) or hydroxyurea (circles) with 25 μ M metMb, 5 mM H₂O₂, and 50 μ M carboxy-PTIO in PBS (pH 7.4) in the absence (open symbols) and the presence (solid symbols) of 10³ U/ml SOD were monitored at several points of time and the decay of carboxy-PTIO signal is presented.

Figure 5. SOD facilitates NO release upon metMb/H₂O₂-induced oxidation of SAHA but not of TSA

The EPR spectra observed during the incubation of 250 μ M of either SAHA (squares), TSA (triangles), and hydroxyurea (circles) with 25 μ M Mb, 5 mM H₂O₂, and 50 μ M carboxy-PTIO in PBS (pH 7.4) in the absence (open symbols) and the presence (solid symbols) of 10³ U/ml SOD were monitored at several points of time and the growth of carboxy-PTI signal is presented.

Figure 6. SAHA, TSA, but not VPA, protect V79 cells against H_2O_2 -induced killing
Survival of Chinese hamster cells exposed to various $[H_2O_2]$ for 1 h in the absence (●) and the presence (■) of 2.5 μM of SAHA, TSA, or VPA.