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Effects of suberoylanilide hydroxamic acid and trichostatin A on induction of cytochrome P450 enzymes and benzo[*a*]pyrene DNA adduct formation in human cells

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Abstract—In this study, we investigated the effects of histone deacetylase (HDAC) inhibitors suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) on the metabolism of polycyclic aromatic hydrocarbons (PAH) in human mammary carcinoma derived MCF-7 cells in culture. Benzo[*a*]pyrene (B[*a*]P) induces cytochrome P450 (CYP) 1A1, CYP1B1 and other xenobiotic metabolizing enzymes. Results from our study indicated a significant increase in CYP activity in comparison to vehicle control in cells treated with SAHA or TSA as measured by ethoxyresorufin-*O*-deethylase assay. However, co-treatment with 1.0 μM SAHA and BP, reduced the mRNA levels of *CYP1B1* relative to B[*a*]P alone. When co-treated with 1.0 μM TSA and BP, a reduction in the mRNA levels of both *CYP1A1* and *CYP1B1* was observed relative to BP alone. We further investigated to ascertain if the differential expression and activity of CYP1A1 and CYP1B1 influenced levels of B[*a*]P DNA adduct formation. MCF-7 cells co-treated with B[*a*]P and SAHA or TSA formed DNA adducts, although no significant differences in levels of DNA binding were revealed. These results suggest that while CYP enzyme activity and gene expression were affected by the HDAC inhibitors SAHA and TSA, they had no apparent influence on B[*a*]P DNA binding.

Polycyclic aromatic hydrocarbons (PAH) result from the combustion of organic matter and are widely dispersed in the environment. The association between exposure to PAH and human cancer has been recognized for centuries, and a large body of research examines this relationship.[1] Elevated exposures to PAH correspond with increased risk of lung, skin, and bladder cancer, and may contribute to breast cancer incidence.[2,3] Benzo[*a*]pyrene (B[*a*]P) is a particularly well-studied carcinogenic PAH that is found in coal tar, tobacco smoke, and other products of combustion. B[*a*]P binds to the aryl hydrocarbon receptor (AhR), inducing cytochrome P450 (CYP)1A1 and other detoxifying enzymes.[4] The metabolites of B[*a*]P formed by CYP1A1 and CYP1B1 can covalently bind to DNA, and are potentially mutagenic.[5] Changes in CYP expression can affect B[*a*]P metabolism, and alter the levels of B[*a*]P metabolites available to bind to DNA. Approximately 10% of the human population exhibits highly inducible CYP1A1 activity, which in combination with variation in other xenobiotic metabolizing enzymes, appears to contribute to cancer susceptibility.[6]

While variation in genetic sequence is an important consideration in cancer risk and treatment, epigenetic activation or repression of genes may also contribute to human cancer. Histone deacetylases (HDAC) and histone acetyl transferases modify histone proteins and contribute to an epigenetic code recognized by proteins involved in regulation of gene expression.[7] HDACs prevent transcription by maintaining higher order closed chromatin structure.[8] A number of compounds reverse

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HDAC activity, and several have entered clinical trials.[9,10]

Trichostatin A (TSA) is a potent, specific, and reversible HDAC inhibitor affecting cell proliferation and differentiation.[11] Suberoylanilide hydroxamic acid (SAHA), a structurally similar compound, shows strong anti-proliferative effects but low toxicity in vivo.[12] The antitumor activity of SAHA has been demonstrated in several animal models, and has shown promise in phase I and phase II clinical trials.[7,13] SAHA has also been tested as a chemopreventive agent. Here we examined the effect of SAHA and TSA on the activity of the B[*a*]P metabolizing enzyme CYP1A1 in MCF-7 cells in culture. The effects of the HDAC inhibitors on B[*a*]P induced CYP1A1 and CYP1B1 expression were also investigated. A change in the response of CYP levels or activity to B[*a*]P treatment would be expected to result in a change in B[*a*]P-DNA binding. DNA adducts were also assayed as a measure of genotoxic change induced by B[*a*]P. In this case the induction of CYPs seems to have increased detoxification of B[*a*]P rather than increased activation.

The human mammary carcinoma-derived MCF-7 cell line was obtained from the Michigan Cancer Foundation (Karmanos Cancer Center, Detroit, MI). The cells were cultured in 75 cm^2 flasks (Corning, Corning, NY) in F/D medium, a 1:1 mixture of F-12 Nutrient Mixture (Gibco-Invitrogen, Carlsbad, CA) and Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-Invitrogen) at 37 °C with 5% CO_2. The medium was supplemented with 10% fetal bovine serum (FBS) (Intergen, Purchase, NY), 15 mM HEPES buffer and antibiotics (200 units/mL penicillin, 200 μg/mL streptomycin, and 25 μg/mL ampicillin). Cell cultures were subcultured at a ratio of 1:4 when the cells covered the entire surface of the flask.

The cells were treated with 0.01, 0.10, 1.0, 2.0, or 4.0 μM B[*a*]P, SAHA, TSA or an equal volume of dimethylsulfoxide (DMSO) as vehicle control, harvested 12 or 24 h after treatment, washed with phosphate-buffered saline and analyzed immediately, or the cell pellet was stored at −80 °C. The highest concentrations of SAHA and TSA used in these experiments (2 μM) were no more cytotoxic than 1% DMSO, as observed by MTT (ATCC, Manassas, VA) assay (data not shown). B[*a*]P was obtained from Chemsyn Science Laboratories (Lenexa, KS). TSA was obtained from Dr. Roderick Dashwood and Mindy Myzak of the Linus Pauling Institute, Oregon State University, or purchased from Biomol (Plymouth Meeting, PA).

Both microsomes and DNA were isolated from treated cells as previously described.[14,15] Microsomes were used to determine cytochrome P450 activity as measured by ethoxyresorufin O-deethylase (EROD) assay using the protocol from Ciolino and Yeh.[16] EROD activity was derived from measuring the fluorescent resorufin product in a SpectraMax Gemini fluorescent plate reader (Molecular Devices, Sunnyvale, CA) at intervals over 60 min, with an excitation wavelength of 530 nm and emission at 585 nm. While microsomes were used to determine cytochrome P450 (CYP) activity, DNA was used to determine adducts by ^{33}P post-labeling as described previously.[17] Adducts were resolved by elution at 1 mL/min with 0.1 M ammonium phosphate, pH 5.5 (solvent A) and 100% HPLC grade methanol (solvent B). The elution gradient was: 44–60% solvent B over 40 min, 60–80% solvent B over 10 min, elution at 80% solvent B over 10 min, and 80–44% B over 5 min. Peaks were detected by a dry cell on a β-RAM® Model 3 (IN/US Systems, Tampa FL) radioactive detector. The level of DNA binding was calculated based on the labeling efficiency of a [^{3}H]B[*a*]P-7,8-dihydrodiol 9,10-epoxide standard.[18] Four independent sets of the post-labeling reaction were carried out for every sample treated, to determine the total PAH-DNA adduct levels.

Total cellular RNA was isolated from treated cells by using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Relative quantitation of RNA was performed by RT-PCR using Taq-Man™ technology (Applied Biosystems, Foster City CA). The cDNA was synthesized from total RNA using Advantage RT-PCR kit (Clontech, San Jose, CA). The cDNA was diluted 1:10 and 2 μL cDNA was used as a template to perform real-time PCR in a 50 μL reaction mixture containing 2X Taq-Man Universal PCR Master Mix and 20X Assays-on-Demand Gene Expression Primers and Probes for both *CYP1A1* (Hs00153120_m1/X02612), *CYP1B1* (Hs00164383_m1/U03688) and house keeping gene *GAPDH* (Hs99999905_m1/NM002046) (Applied Biosystems/GenBank). This two-step PCR reaction was performed on ABI 7700 Sequence Detection System. Each sample was assayed in duplicate and the Cycle Threshold (C_T) values were normalized to the housekeeping gene (*GAPDH*) and the fold change was calculated using $2_T^{-\Delta\Delta C}$ method.[19]

The data for all the experiments were analyzed with the MIXED procedure (ANOVA and linear mixed models) in SAS version 8.2 (SAS Institute, Cary, NC). Residuals were examined and when EROD was the response, the data were log transformed to achieve homogeneity of variance and approximate normality of residuals. Models for EROD and adduct data had random blocking factors (sets and/or plates and/or post-labelings) and random factors for variation between experimental units (blocks-by-treatment). Models for CYP1A1 and CYP1B1 had a random factor for variation between experimental units (cell preparations within treatments). The statistical models (whenever no data were missing) were equivalent to analyzing the averages over replicate measurements (e.g., wells) on each experimental unit. Multiple comparison procedures used were Dunnett's pairwise comparisons to a control (e.g., DMSO or B[*a*]P) and Tukey's for analyses with additional pairwise comparisons.

MCF-7 cells that were treated with SAHA indicated an increase in EROD activity with increasing concentrations (0.2–4.0 μM) of SAHA. At 12 h all concentrations of SAHA had significantly elevated levels of EROD activity compared to DMSO solvent control ($p < 0.04$,

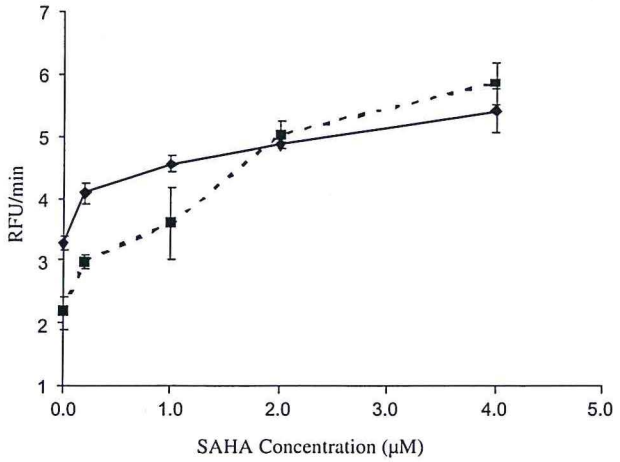

Figure 1. Induction of EROD activity by SAHA: MCF-7 cells in 24-well plates were treated with 4 μM B[*a*]P alone, an equivalent volume of DMSO, or the indicated concentrations of SAHA. After 12 h (◆) or 24 h (■), the plates were assayed for EROD activity. Numbers represent log of relative fluorescent units (RFU)/minute. Data shown is mean ± standard deviation where $n = 2$ plates (after first averaging over the four replicate wells per plate).

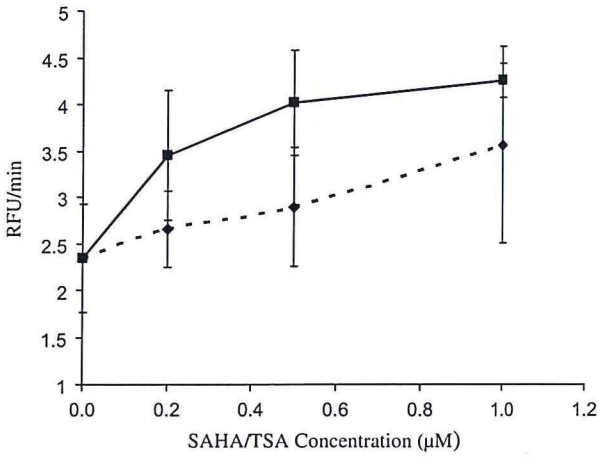

Figure 2. Increase in EROD activity in intact MCF-7 cells after treatment with SAHA and TSA: MCF-7 cells in 24-well plates were treated with 4 μM B[*a*]P, an equivalent volume of DMSO, or the indicated concentrations of SAHA (◆) or TSA (■). After 24 h, the plates were assayed for EROD activity. Numbers are log of RFU/minute. Data shown is mean ± standard deviation for $n = 3$ plates (after averaging over the three replicate wells per plate).

Dunnett adjusted) (Fig. 1). At 24 h, treatment with SAHA (≥1 μM) exhibited significantly elevated levels of EROD activity compared to DMSO ($p < 0.025$ all comparisons, Dunnett adjusted). At the highest dose (4 μM) of SAHA tested, an EROD activity of 5.41 ± 0.34 (12 h) and 5.85 ± 0.33 (24 h) were obtained in comparison to B[*a*]P treatment (4.96 ± 0.21 at 12 h and 5.25 ± 0.14 at 24 h). At 24 h, EROD activity of SAHA (at 2 μM) was most similar to the activity observed with B[*a*]P (at 4 μM), and only the two lowest concentrations of (0.2 and 1 μM) SAHA tested were significantly different from the activity for the B[*a*]P treatment ($p = 0.02$, Dunnett adjusted).

A similar trend was noted when cells treated with SAHA and TSA were compared to B[*a*]P (Fig. 2). Increased EROD activity was observed with increasing concentrations of SAHA and TSA (0.2–1.0 μM, Fig. 2). At all three doses tested, TSA exhibited a significant increase in EROD activity in comparison to DMSO ($p < 0.04$ all comparisons, Dunnett adjusted); TSA (1 μM) and SAHA (1 μM) exhibited activities of 4.26 ± 0.18 and 3.56 ± 1.06 and were comparable to that of B[*a*]P (4 μM) with an activity of 4.44 ± 0.53.

Altered transcription patterns of *CYP1A1* and *CYP1B1* genes in response to either B[*a*]P, SAHA, or TSA are shown in Table 1. *CYP1A1* showed a dose-dependent increase in expression (1.8–25.2-fold) with an increasing dose of B[*a*]P. The results were similar for *CYP1B1* (1.4–4.3 fold) although at a lower magnitude. When the cells were exposed to either SAHA or TSA alone, a dose-dependent change was not observed for either *CYP1A1* or *CYP1B1* (Table 1). Nakajima et al.[20] have shown an increase in *CYP1A1* mRNA in MCF-7 cells after TSA treatment. TSA has also been shown to increase *CYP1A1* mRNA levels in the presence of the AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.[21,22] Activity of the murine AhR promoter has been demonstrated to be increased by TSA,[23] and increased *AhR* and *Arnt* gene expression influence the inducibility of *CYP1A1* by AhR ligands.[24] In our study, SAHA and TSA increased EROD activity in the absence of a known AhR ligand. These results, and those of others[20] suggest that *CYP1A1* as well as AhR may be epigenetically regulated in MCF-7 cells.

Table 1. *CYP1A1* and *CYP1B1* expression in samples treated with B[*a*]P, SAHA, and TSA[a]

Compound	Concentration (μM)	*CYP1A1* (fold change)	*CYP1B1* (fold change)
B[*a*]P	0.01	1.85 ± 0.47	1.36 ± 0.18
	0.10	3.69 ± 8.28	1.70 ± 1.11
	1.0	25.24 ± 4.58	4.35 ± 0.75
SAHA	0.01	0.77 ± 0.43	1.10 ± 0.17
	0.10	2.86 ± 6.09	1.62 ± 0.78
	1.0	0.98 ± 0.68	1.58 ± 0.79
TSA	0.01	0.77 ± 0.43	1.29 ± 0.15
	0.10	0.81 ± 0.15	1.57 ± 0.16
	1.0	3.10 ± 0.88	0.17 ± 0.05

[a] *CYP1A1* and *CYP1B1* gene expression after treatment with varying concentrations of B[*a*]P, SAHA, and TSA relative to DMSO is shown. Results indicate an average fold change compared to DMSO control ± standard deviation where $n = 2$ samples (an average of duplicate measurements).

Additionally, *CYP1A1* and *CYP1B1* expression was measured from samples treated with DMSO alone, B[*a*]P alone, and B[*a*]P with SAHA or TSA (Table 2). In comparison with B[*a*]P-treated samples, expression of *CYP1A1* was significantly reduced after co-treatment of B[*a*]P with TSA ($p = 0.01$, Dunnett adjusted). However, no significant change in *CYP1A1* was observed when the cells were co-treated with B[*a*]P and SAHA

Table 2. *CYP1A1* and *CYP1B1* expression following co-treatment with B[*a*]P and SAHA or TSA[a]

Treatment	Concentration (μM)	*CYP1A1* (fold change)	*CYP1B1* (fold change)
B[*a*]P	1.0	25.55 ± 2.52	4.39 ± 0.55
B[*a*]P + SAHA	1.0	22.92 ± 1.46	*2.40 ± 0.06
B[*a*]P + TSA	1.0	*8.77 ± 3.04	*1.02 ± 0.05

[a] MCF-7 cells were treated with 1.0 μM of B[*a*]P, B[*a*]P plus SAHA or B[*a*]P plus TSA. The cells were harvested after 24 h and *CYP1A1* and *CYP1B1* expression in comparison to cells treated with DMSO was quantified by RT-PCR. Results indicate an average fold change compared to DMSO ± standard deviation for $n = 2$ samples (an average of duplicate measurements). Data with a significant difference in fold change ($p < 0.05$) in comparison to B[*a*]P alone is indicated by an asterisk (*).

($p > 0.50$ Dunnett adjusted). Significant reduction in *CYP1B1* transcript was observed in cells co-treated with either B[*a*]P and TSA, or B[*a*]P and SAHA ($p < 0.02$ both tests, Dunnett adjusted) when compared to the B[*a*]P alone treatment group.

The elevated EROD activity we observed (Fig. 2), indicating increased CYP1A1 activity, could be predicted to lead to an increase in DNA adducts. Alternately, the decrease in inducible CYP expression (Table 2) when cells were co-treated with HDAC inhibitor and B[*a*]P may lead to a decrease in B[*a*]P–DNA binding. To understand the effect of altered CYP activity and levels that we observed in this study, we examined the ability of SAHA and TSA to influence B[*a*]P DNA binding (Table 3). MCF-7 cells were treated with 4 μM B[*a*]P, or a combination of 4 μM B[*a*]P plus 2 μM SAHA or 2 μM TSA. The patterns of the means was consistent with the hypothesized consequence of increased *CYP1A1* activity, in that the mean adduct formation for B[*a*]P plus SAHA and B[*a*]P plus TSA were about 1.5 times higher than the mean response for B[*a*]P alone. Post-labeling can be sensitive enough to measure one adduct in 10^9 nucleotides, and improved reproducibility of this technique is the goal of many researchers.[25] In this work, there was a large variation between replicate measurements so that there was no statistical evidence of treatment effects ($p > 0.20$, ANOVA). The data suggested this was principally due to variation between treatment sets, and the need to push the limits of sensitivity of the post-labeling technique due to the low levels of DNA adducts formed in cells.

Table 3. B[*a*]P DNA binding in MCF-7 cells co-treated with SAHA or TSA[a]

Treatment	Concentration (μM)	Mean B[*a*]P DNA adducts pmol adducts/mg DNA
B[*a*]P	4.0	34.75 ± 18.66
B[*a*]P + SAHA	4.0 and 2.0	52.18 ± 25.45
B[*a*]P + TSA	4.0 and 2.0	54.15 ± 35.28

[a] MCF-7 cells were treated with B[*a*]P alone, or a combination of B[*a*]P plus SAHA or B[*a*]P plus TSA. Results indicate the mean ± standard deviation over $n = 4$ sets (averaging over two replicate measurements). Differences between treatments were not statistically significant ($p > 0.20$ all comparisons, Tukey adjusted).

HDAC inhibitors have been shown to affect proliferation in cells that are estrogen receptor positive such as MCF-7 cells.[26,27] Loss of estrogen receptor alpha (ER α) is associated with aggressive breast cancer.[28] TSA has been shown to induce re-expression of ER α in MCF-7 cells.[29] CYP1A1 and CYP1B1 are essential to estrogen metabolism, and there is evidence of cross talk between ER α and AhR-regulated responses, including CYP1A1 inducibility.[30] The increased CYP1A1 activity on exposure to SAHA that we have observed in this study invites speculation that ER α and CYP1A1 share common epigenetic regulatory mechanisms.

Another possibility that we have considered is that the effects of SAHA and TSA on CYP1A1 and CYP1B1 are mediated through thioredoxin-1. Thioredoxin-1 (Trx-1) has been shown to be important in many human diseases, including cancer. While the induction of CYP1A1 and CYP1B1 increase oxidative stress,[31] Trx-1 acts as a reducing co-factor[32] and an antioxidant.[33] Trx influences the DNA binding activity of AhR/Arnt.[34] SAHA has been shown to down regulate thioredoxin,[35] which in turn regulates the constitutive expression of CYP1B1, and the inducible expression of CYP1A1 and CYP1B1.[34]

HDAC inhibitors represent a diverse class of antineoplastic agents and their effects in this work exemplify the importance of considering epigenetic regulation in carcinogenesis. They are of great interest because of their ability to suppress the growth of tumor cells in vitro, and to reduce the growth of tumors in animal models. SAHA alone has been found to perturb cell cycle proteins,[9,36] down regulate survival signaling pathways,[37] disrupt the cellular redox state,[38] and exhibit anti-inflammatory properties.[39] Our study is the first to show that CYP1A1 activity was increased when MCF-7 cells were treated with SAHA or TSA. We have also shown that SAHA and TSA reduce the expression of *CYP1A1* and *CYP1B1* induced by B[*a*]P (Table 2). CYP1A1 and CYP1B1 are pivotal enzymes in a network of pathways, and their induction via the AhR is accompanied by that of many other enzymes. It is therefore likely that not only CYP1A1, but other detoxifying enzymes are also influenced by HDAC inhibitors. Further research is necessary to understand how epigenetic regulation of gene expression interacts with AhR-mediated induction.

Because SAHA and other HDAC inhibitors are being investigated for their usefulness in preventing and treating cancer, it is critical that this effect be further examined. TSA is biotransformed rapidly by rat hepatocytes, likely involving cytochrome P450s.[40] If HDAC inhibitors used for clinical trials are transformed in the same manner, additional induction of P450s could expedite this metabolism, decreasing the amount of drug available. HDAC inhibitors appear to be promising chemotherapeutic agents, but further investigation of their roles both in cancer biology and carcinogenesis is warranted.

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