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Histone deacetylase inhibitors increase human arylamine *N*-acetyltransferase-1 expression in human tumor cells

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Running title: Regulation of NAT1 by HDAC inhibitors

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ABBREVIATIONS: NAT1, arylamine *N*-acetyltransferase-1; HDAC, histone deacetylase; TSA, trichostatin A; EMSA, electromobility shift assay

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ABSTRACT:

Arylamine *N*-acetyltransferase-1 (NAT1) has been associated with disorders involving folate metabolism, such as spina bifida, as well as numerous human cancers. As a result, the transcriptional and post-transcriptional regulation of NAT1 activity has been extensively studied. However, little work has been reported on the epigenetic control of NAT1 expression. Here, we demonstrate that the histone deacetylase inhibitor trichostatin A (TSA) increases NAT1 activity in human cancer cells by increasing transcription from the proximal promoter NATb. A specific Sp1 binding-site was identified as essential for optimal induction of NAT1 by TSA. However, TSA did not increase the expression of Sp1 in HeLa cells. Instead, TSA increased the acetylation of histones associated with the NATb promoter. This allowed recruitment of Sp1 to the promoter along with acetylated histones. We propose that NAT1 transcription is partially repressed by the local chromatin condensation in the vicinity of NATb and that histone deacetylase inhibition leads to up-regulation of NAT1 expression via a direct change in chromatin conformation.

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Introduction

Arylamine *N*-acetyltransferase-1 (NAT1) is a phase II enzyme that acetylates a range of arylamine and hydrazine substrates (Minchin et al., 1992; Hein et al., 1993). NAT1 is widely distributed in the body and has been associated with disorders involving folate metabolism, such as spina bifida and cleft palate (Jensen et al., 2006; Erickson et al., 2008), as well as susceptibility to various cancers (Hein et al., 2000; Butcher et al., 2002). As a result, the regulation of NAT1 activity has been studied in detail (Butcher et al., 2008). The NAT1 gene consists of 9 exons, encompassing over 52 kb (Boukouvala and Sim, 2005; Butcher et al., 2005) on chromosome 8. Exon 9 contains the entire 870 bp open reading frame (ORF), while various splicing of the first 8 exons make up the 5'-untranslated region (UTR). NAT1 transcription is driven by two alternative promoters (Minchin et al., 2007). The distal NAT1 promoter, NATa, drives the expression of Type I transcripts that begin with exon 1. These transcripts show tissue-specific expression (Barker et al., 2006). A number of putative transcription factor binding-sites were observed within the NATa promoter region (Barker et al., 2006). The proximal or constitutive NAT1 promoter, NATb, drives the expression of Type II transcripts that begin with exon 4. NATb is responsible for the expression of most NAT1 mRNA and transcripts arising from this promoter have been detected in almost all tissues and human cell-lines examined (Butcher et al., 2005; Barker et al., 2006; Husain et al., 2007). Thus, NATb most likely accounts for the ubiquitous expression of NAT1 in human tissues and cell-lines. NATb is also androgen-responsive through an androgen-dependent heat shock factor binding site located 776 base pairs upstream of the transcription start site (Butcher and Minchin; Butcher et al., 2007).

NAT1 is responsible for the acetylation of the folate catabolite *p*-aminobenzoylglutamate (Minchin, 1995; Sim and Ward, 1995). The importance of this metabolite is not well understood,

particularly as mice lacking the ability to acetylate *p*-aminobenzoylglutamate are generally aphenotypic (Cornish et al., 2003; Sugamori et al., 2003). However, NAT1 has been shown to alter the growth kinetics of non-transformed breast cells (Adam et al., 2003) and it also appears to be necessary for cell invasion in the breast cancer cell-line MDA-MB-231 (Tiang et al.). These observations suggest the enzyme may have a role in addition to xenobiotic metabolism. In the gastrointestinal tract, NAT1 activity is relatively constant from the duodenum to the colon but, at the level of the crypts, greater variation has been reported (Hickman et al., 1998). The NAT1 protein is almost absent in the proliferating cells located at the base of the crypt but is highly expressed in the differentiated non-proliferating epithelial cells at the tips of the villus. Thus, NAT1 may be up-regulated as cells enter senescence or as they express a more differentiated phenotype. In the course of our studies into the regulation of NAT1 activity, we observed a marked increase in NAT1 in cells following treatment with the differentiating agents sodium butyrate and trichostatin A (TSA), but not with retinoic acid. Both sodium butyrate and TSA are non-specific histone deacetylase (HDAC) inhibitors. HDACs, along with the histone acetyltransferases, regulate the acetylation status of histones. Histone acetyltransferases add acetyl groups to lysine residues of proteins, while HDACs remove these acetyl groups. Post-translational acetylation of core histones associated with DNA influences chromatin structure and is central to the regulation of transcription (Senese et al., 2007; Smith and Workman, 2009). Interestingly, the major Class I histones are down-regulated during maturation of intestinal cells along the crypt-villus axis (Wilson et al., 2006).

Here, we report that the HDAC inhibitor TSA up-regulates NAT1 expression through a conserved Sp1 site previously reported to be essential for NAT1 expression from the NATb promoter (Husain et al., 2007). HDAC inhibitors have entered clinical trials for the treatment of a

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number of different cancers. The results from the current study may have important implications in the development of HDAC inhibitors as a class of cancer therapeutics because NAT1 has been shown to positively affect cancer cell growth and invasion (Adam et al., 2003).

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Materials and Methods

Cell culture. Human HeLa, ZR-75-1 and HepG2 cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin. Human MCF-7 cells were cultured in DMEM supplemented with 5% FBS and penicillin/streptomycin (Invitrogen, VIC, Australia). All cell-lines were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were treated with up to 0.5 μM TSA (Sigma) or vehicle (DMSO) for up to 24 hr at 37°C. Actinomycin D (Sigma, MO) was used at a final concentration of 5μg/ml.

NAT1 activity assay. Cells were washed with PBS, resuspended in NAT1 assay buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol) and lysed by sonication. Cell lysates were then immediately assayed for NAT1 activity by HPLC using *p*-aminobenzoic acid as substrate as described previously (Butcher et al., 2000).

Identification of NAT1 transcripts by reverse transcription-PCR (RT-PCR). Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. Approximately 5 μg total RNA was resuspended in RNAse-free water and reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen) and oligo$(dT)_{15}$ primer (Promega, NSW, Australia) according to the manufacturer's instructions. First-strand cDNA from cells grown in the presence or absence of 0.5 μM TSA was amplified by PCR using specific primers designed to detect NATa or NATb transcripts (see Table 1 for primer sequences). PCRs were performed in a final volume of 25 μl containing Thermopol buffer (New England Biolabs, MA), 6.25 pmol of each primer (Geneworks, SA, Australia), 0.25 μM dNTPs, 0.2 U Taq DNA polymerase (New England Biolabs) and 1 μl cDNA. Samples were amplified using the following conditions; initial denaturation at 95°C for 3 min, followed by 36 cycles of denaturation at 95°C for 15 s, annealing

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at 50°C for 20 s and extension at 72°C for 30 s. PCR products were then separated on 2% agarose gels and identified according to size.

Quantitative real-time PCR (qPCR). Expression levels of NATb transcripts were determined using the iCycler system (Bio-Rad, CA). First-strand cDNA was amplified using specific primers for NATb transcripts or β-actin (see Table 1). Reactions contained SensiMix Plus SYBR + Fluorescein (Bioline, NSW, Australia), 6.25 pmol of each primer (Geneworks, SA, Australia) and 1 μl cDNA in a total volume of 25 μl. Samples were amplified using the following conditions; initial denaturation at 95°C for 10 min, followed by 30 (β-actin) or 40 (NATb) cycles of denaturation at 95°C for 15 s, annealing at 50°C for 20 s and extension at 72°C for 60 s (β-actin) or 30 s (NATb). To ensure accurate quantification, qPCR products were assessed by melt curves and also electrophoresed on 2% agarose gels to ensure a single product of the correct size was being amplified. NATb expression levels were normalised to β-actin and the relative fold change in NATb expression calculated using the $^{\Delta\Delta}$CT method (Livak and Schmittgen, 2001).

Transient transfection and luciferase assay. HeLa cells were seeded into 24-well plates at a density of 1.5×10^5 cells/well and allowed to adhere overnight. Cells were transfected with 1 μg of plasmid DNA using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions, and incubated for 4 hr. Cells were then treated with either 0.5 μM TSA (Sigma) or vehicle (DMSO) for 24 hr. Following treatment, cells were washed with PBS, lysed in passive lysis buffer and firefly luciferase activity was measured using a luciferase assay system (Promega) according to the manufacturer's instructions.

Western blot. Cells were washed with PBS and extracted into reducing sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, 0.0025% bromophenol blue). Samples were heated at 95°C for 10 min,

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centrifuged briefly and then 15 µl of each sample was electrophoresed on 12% SDS-polyacrylamide gels at 200 V for 30 min. Proteins were then transferred to nitrocellulose membranes at 350 mA for 1 hr. Following this, membranes were blocked for 1 hr at room temperature with 5% skim milk powder in PBS containing 0.05% Tween-20 (PBST), washed for 3 × 5 min with PBST and immunoblotted for either Sp1 (sc-44221, 1:5000, Santa Cruz Biotechnology, CA) or α-tubulin (DM1A, 1:2000, Merck KGaA, Darmstadt, Germany) at 4°C overnight. Membranes were then washed for 3 × 5 min with PBST and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hr. Finally, membranes were washed 3 × 5 min with PBST and Sp1 and α-tubulin were visualised using enhanced chemiluminescence detection (Bio-Rad). Protein content was estimated from Western blots by densitometry using QuantityOne software, version 4.5 (Bio-Rad).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts from HeLa cells (~ 5×10^6) treated with either 0.5 µM TSA or vehicle were prepared by lysing cells in 0.6 ml ice-cold lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 2 µg/ml leupeptin, 2 µg/ml pepstatin A) by passage through a 27-gauge needle 8-10 times on ice. Crude nuclei were then collected by brief centrifugation and resuspended in 70 µl extraction buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 420 mM KCl, 25% glycerol, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml pepstatin A). After 30 min on ice, 50 µl of storage buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml pepstatin A) was added and extracts were stored as 20 µl aliquots at -80°C until required. Protein concentration was determined by the method of Bradford (Bradford, 1976).

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EMSAs were performed as described previously (Butcher and Minchin, 2010). Briefly, 1 × 10^5 d.p.m. of ^{32}P-labeled oligonucleotides probes (see Table 1) and 2 μg of nuclear extracts were incubated at room temperature for 30 min in binding buffer (4% Ficoll 400, 50 mM KCl, 20 mM HEPES, pH 7.9, 2.5 mM DTT, 20 μM $ZnSO_4$, 5 μg poly[d(I-C)]). For competition analysis, a 100-fold molar excess of unlabelled oligonucleotides was used. For supershift assays, 2 μg of Sp1 antibody (sc-44221, Santa Cruz) was added before the 30 min incubation. Samples were then resolved on 5% non-denaturing polyacrylamide gels (120 V for 45 min) at 4°C. Gels were dried at 70°C for 40 min, placed on a phosphorimage screen overnight and then analysed using a BAS-5000 phosphorimager (Berthold, NSW, Australia).

Chromatin immunoprecipitation (ChIP) assays. HeLa cells (~2 × 10^6) treated with either 0.5 μM TSA or vehicle for 6 hr were washed once with PBS and cross-linked in 1.5% formaldehyde (in PBS) for 15 min at room temperature. Cells were washed once in cold PBS, lysed on ice in lysis buffer (10 mM EDTA, 50 mM Tris/HCl, pH 8.0, 1% SDS, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A) and sonicated on ice (3 × 15 s bursts) to fragment the chromatin to an average length of approximately 600 bp. Lysates were then centrifuged at 4°C for 15 min at 14,000 × *g*. A 20 μl aliquot of each supernatant was diluted up to 50 μl in dilution buffer (2 mM EDTA, 150 mM NaCl, 20 mM Tris/HCl, pH 8.0, 1% Triton X-100, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A), and stored for use as positive controls for PCR. The remaining supernatants were diluted 1:10 in dilution buffer and divided into 3 tubes of equal volume. Chromatin-protein complexes were immunoprecipitated with approximately 2 μg of either Sp1 antibody (sc-44221, Santa Cruz), acetyl-histone H4 (Lys8) antibody (2594S, Genesearch, QLD, Australia) or purified goat-IgG overnight at 4°C. After equilibration in dilution buffer, 50 μl of Protein-A sepharose beads (GE Healthcare, NSW, Australia) were added

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to each sample and rotated at 4°C for 2 hr. After extensive washing, chromatin-protein complexes were eluted in 100 µl elution buffer (0.1 M $NaHCO_3$ and 1% SDS) at room temperature for 10 min. Samples, including those used as positive PCR controls, were heated at 65°C overnight to reverse cross-linking. DNA was then purified using MinElute PCR purification spin columns (Qiagen, VIC, Australia). PCR was performed on purified DNA using primers flanking the NATb promoter region containing the Sp1 binding site (186 bp fragment) or GAPDH as a negative control (180 bp fragment) (see Table 1 for primer sequences) Reactions contained Thermopol buffer (New England Biolabs), 6.25 pmol of each primer, 0.25 µM dNTPs, 0.2 U Taq DNA polymerase (New England Biolabs) and 3 µl DNA in a final volume of 25 µl. Samples were amplified using the following conditions; initial denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 57°C (NATb) or 51°C (GAPDH) for 15 s and extension at 72°C for 20 s. PCR products were then separated on 2% agarose gels and identified according to size.

Data analysis and statistics. Data are expressed as mean ± standard error (SE). Statistical comparisons between different treatments were assessed by Student's *t*-tests or one-way analysis of variance (ANOVA) using significance at $p < 0.05$. NATb mRNA half-life was estimated by fitting a linear regression to the data using Prism 4 (GraphPad Software Inc., CA).

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Results

NAT1 activity is induced by TSA in a cell density-dependent manner. TSA significantly increased NAT1 enzyme activity in several cell-lines following treatment for 24 hr (Fig. 1A). The highest increase (3.2-fold) was seen in HeLa cells, a cervical adenocarcinoma. Induction was not dependent on the basal level of NAT1 expression, which was 4.2 ± 0.2, 6.7 ± 0.1, 22.9 ± 0.7 and 38.9 ± 1.6 nmol/min/mg protein for HeLa, HepG2 (liver), MCF-7 (breast) and ZR-75-1 (breast) cells, respectively. The effect of TSA on gene expression has been reported to be dependent on cell density (Gray and Ekstrom, 1998). NAT1 activity was determined in HeLa cells cultured at 40%, 80% and 100% confluence, both with and without TSA treatment. Induction of NAT1 by TSA was highest in the least confluent cells (4.1-fold) and decreased to less than 1.5-fold in 100% confluent cells (Fig. 1B).

TSA up-regulated NATb promoter activity. To determine if TSA up-regulated NATa or NATb promoter activity in HeLa cells, specific forward primers located in exon 3 (NATa transcripts) or exon 4 (NATb transcripts) were used with a common reverse primer located in the protein coding region to amplify cDNA following TSA treatment (Fig. 2A). NATb transcripts were present in both vehicle- and TSA-treated cells, whereas NATa transcripts were not detected in either group. Human liver tissue, which constitutively expresses NATa transcripts (Barker et al., 2006), was used as a positive control for the detection of transcripts derived from NATa. This result suggested that TSA had its effect via the NATb promoter. Quantification of mRNA derived from NATb by qPCR showed that TSA treatment caused a 25-fold increase in NAT1 mRNA (Fig. 2B).

HDAC inhibitors not only affect gene transcription by altering the interaction of histones with DNA, but they also affect mRNA stability (Spange et al., 2009). To determine whether TSA

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altered the stability of NAT1 mRNA, HeLa cells were treated with TSA in the presence of the transcriptional inhibitor actinomycin D (Fig. 2C). In the absence of TSA, the slope of the mRNA decay curve was -0.78 ± 0.09 hr^{-1} whereas, in the presence of the HDAC inhibitor, it was -0.46 ± 0.02 hr^{-1}. This suggests that TSA had a small, albeit significant, effect on NAT1 mRNA stability, but this effect was unlikely to account for the 25-fold increase in NAT1 mRNA observed following treatment.

Identification of the TSA responsive region in NATb. A series of NAT1 promoter reporter constructs (Butcher et al., 2007) was used to identify the TSA responsive region within the NATb promoter. The longest promoter sequence, a 3657 bp fragment of NATb immediately upstream of exon 4 (Butcher et al., 2007), was induced by TSA approximately 18-fold (Fig. 3A). Induction was seen with all constructs down to a promoter length of 34 bp. This final sequence is shown in Fig 3B along with the location of a previously identified Sp1 site (Husain et al., 2007). Sp1 binding sites have been implicated in the response of numerous genes to HDACs (Zhao et al., 2003; Yokota et al., 2004).

TSA recruited Sp1 to the NATb promoter. The functional relevance of the Sp1 site in the NAT1 promoter in response to TSA was investigated using the 34 bp reporter construct containing 2 mutations in the core Sp1 binding sequence (GC → TA). These mutations have previously been shown to prevent Sp1 binding (Lomberk and Urrutia, 2005; Husain et al., 2007). While the wild-type construct showed TSA induction of approximately 45-fold, induction of the mutant construct was less than 5-fold (Fig. 4A), indicating that this region of the NATb promoter was critical for TSA activity.

Next, electromobility shift assays (EMSA) were performed to identify whether Sp1 bound to the putative Sp1 binding sequence. Nuclear extracts from DMSO- and TSA-treated

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HeLa cells were incubated with ^{32}P-labelled oligonucleotide probe spanning the Sp1 site in NATb (Fig. 4B). Three complexes were observed, all of which were competed out with excess unlabelled probe. However, only the upper band (Fig. 4B, arrow) was not affected by excess probe containing mutations in the Sp1 site (Fig. 4B, lane 4), suggesting this band represented Sp1 binding. The presence of Sp1 was confirmed by a supershift using a Sp1-specific antibody (Fig. 4B, lane 8). When the probe was incubated with nuclear extracts from cells treated with TSA, a more intense band was observed, consistent with an increase in Sp1 binding (Fig. 4, lane 7). However, this increase was not due to an overall increase in Sp1 expression. Western blot analysis showed no change in the level of transcription factor over 24 hr of treatment with TSA (Fig. 4C).

Sp1 bound to the NATb promoter *in vivo*. Because EMSAs assess the binding of nuclear proteins to DNA probes, they may not necessarily represent protein binding to DNA elements in the context of the nuclear chromatin. To address this, a ChIP assay was performed. DNA from DMSO- and TSA-treated HeLa cells was immunoprecipitated with anti-Sp1 antibody and then amplified using primers that spanned the Sp1 binding site. Sp1 binding was observed for both the DMSO- and TSA-treated cells (Fig. 5A). Taken together, the results from the EMSAs, the supershift assay and ChIP assay show that Sp1 is recruited to the NATb promoter. However, the ChIP assay did not suggest that there was an increase in the amount of Sp1 associated with the NATb promoter *in vivo*. This may be explained by the poor quantitative nature of the ChIP assay, as reported elsewhere (Schroer et al., 2002).

TSA increased acetylated histones associated with NATb. Several studies have reported an increase in histone acetylation upon treatment with HDAC inhibitors (Zhao et al., 2003; Lopez-Soto et al., 2009). To determine whether acetylated histones are recruited to the

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NATb promoter in response to TSA treatment, a ChIP assay was performed using anti-acetylated histone 4 (H4) antibody. No acetylated H4 was associated with NATb in DMSO-treated cells. However, TSA treatment resulted in recruitment of acetylated H4 to the promoter (Fig. 5B). This result suggests that the chromatin in the region of the NATb promoter surrounding the Sp1 binding site is hypo-acetylated under normal conditions.

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Discussion

In eukaryotic cells, DNA exists tightly packaged with histone and histone-like proteins into chromatin (Spange et al., 2009). Post-translational acetylation of core histones associated with DNA influences chromatin structure and is central to the regulation of transcription (Senese et al., 2007; Smith and Workman, 2009). Inhibitors of HDACs can influence gene expression directly by promoting chromatin relaxation, or indirectly by increasing the expression of relevant transcription factors. In the present study, we found that the HDAC inhibitor TSA significantly increased NAT1 expression in a number of human cancer cell-lines. The mechanism appeared to involve chromatin relaxation in the vicinity of the proximal promoter NATb.

Interestingly, induction of NAT1 by TSA was greater in subconfluent cells, a phenomenon reported previously and related to differences in histone expression (Gray and Ekstrom, 1998) or activity of signalling pathways (Hatta and Cirillo, 2007). TSA increased NAT1 mRNA expression by more than 25-fold. This was mostly due to an increase in transcription, but some mRNA stabilisation was observed. The change in transcription required a Sp1 site located approximately 30 bp upstream of the most 5' transcription start site. Sp1 bound to this sequence both *in vitro* (EMSA) and *in vivo* (ChIP). However, TSA did not increase the cellular protein levels of Sp1, which has been reported as one mechanism for the increase in expression of Sp1 dependent genes. Rather, TSA appeared to recruit Sp1 to the NAT1 promoter, probably due to a relaxation of the chromatin surrounding this location of the genome. NAT1 expression was evident in the absence of TSA suggesting that histones associated with at least one of the alleles is sufficiently acetylated to allow gene expression. This does not appear to be H4 as it was not observed in ChIP assays of untreated cells. Allele-specific histone acetylation has been widely reported (Singh et al.) and is common in imprinted genes where a reciprocal

association between allelic acetylation and DNA methylation exists, both mechanisms for epigenetic regulation of gene expression. Methylation of the NAT1 gene has been reported in both mice and humans (Wakefield et al.; Kim et al., 2008), although which of the 2 promoters of the human gene is methylated remains unknown. Studies into the relationship between histone acetylation and DNA methylation of the NAT1 gene are warranted

The findings in the present study may have important clinical implications. HDAC inhibitors are currently under development as a treatment for cancer because of their ability to induce apoptosis. It is likely that this class of drug will increase NAT1 expression *in vivo*. Elevated NAT1 has been associated with increased proliferation of non-transformed cells and appears to be required for tumour invasion (Tiang et al.). HDAC inhibitors also show a synergistic cytotoxic effect in combination with a variety of anti-cancer drugs including cisplatin. Interestingly, cisplatin has recently been shown to inhibit NAT1 at physiological concentrations (Ragunathan et al., 2008). Thus, cisplatin may modulate any increases in NAT1 following HDAC inhibitor treatment, which may be a basis for the observed synergy (Fishel et al., 2005). Further studies are needed to elucidate the effects of these drugs on NAT1 and the possible effects on tumour growth *in vivo*.

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Legend to Figures

FIG. 1. Induction of NAT1 activity by TSA. A, A number of different cell lines were treated with either 0.5 μM TSA (closed columns) or vehicle (DMSO; open columns) for 24 hr. Cells were then lysed and assayed for NAT1 activity. B, HeLa cells were seeded at 2×10^5, 4×10^5, or 6×10^5 cells/well in a 6-well plate (40, 80, or 100% confluence at treatment, respectively), treated with 0.5 μM TSA or vehicle for 24 hr and then lysed and assayed for NAT1 activity. * denotes $p < 0.05$ (Student's *t*-test) compared to vehicle-treated cells. Data are presented as mean ± SEM (n = 3).

FIG. 2. Effect of TSA on NAT1 mRNA expression. A, The presence of NAT1 transcript in HeLa cells treated with either 0.5 μM TSA or vehicle for 24 hr was assessed by PCR using specific primers that detect transcripts derived from either the NATb or NATa promoter. Human liver cDNA (L) was used as a positive control for NATa transcripts. B, NAT1 transcript originating from the NATb promoter was quantified by qPCR. NAT1 mRNA levels were normalized to b-actin. * denotes $p < 0.05$ (Student's *t*-test) compared to vehicle-treated cells (Control). Data are presented as mean ± SEM (n = 6). C, HeLa cells were treated with 0.5 μM TSA (open circles) or vehicle (closed circles) for 24 hr and then treated with 5 μg/ml actinomycin D for up to 4 hr. RNA was isolated, converted to cDNA and NATb transcripts quantified by qPCR. NATb mRNA levels were normalized to β-actin and then expressed relative to T = 0 using the ΔΔCT method. Linear regression was used to determine the rate of decay of NAT1 mRNA. Data are presented as mean ± SEM (n = 3).

FIG. 3. Identification of the TSA responsive region in NATb. A, HeLa cells were transiently transfected with a deletion series of NATb luciferase reporter constructs and treated with either 0.5 μM TSA (closed bars) or vehicle (DMSO; open bars) for 24 hr. Cells were then lysed and assayed for luciferase activity. Promoter length indicates bp upstream of exon 4. * denotes $p < 0.05$ (Student's *t*-test) compared to vehicle-treated cells. Data are presented as mean ± SEM (n = 3). B, Sequence of the minimal NATb promoter required for TSA responsiveness. A consensus Sp1 binding motif is boxed. Exon 4 is underlined and reported transcription start sites are indicated in bold.

FIG. 4. TSA recruits Sp1 to the NATb promoter. A, HeLa cells were transiently transfected with a luciferase reporter construct containing either the wild-type (WT Sp1) or mutated (Mut Sp1) Sp1 binding motif located -40 to -29 bp relative to exon 4 of the NAT1 promoter NATb. Cells were then treated with 0.5 mM TSA or vehicle (DMSO) for 24 hr, lysed and assayed for luciferase activity. * denotes $p < 0.05$ (Student's *t*-test) compared to vehicle-treated cells. Data are presented as mean ± SEM (n = 3). B, EMSA using nuclear extracts from HeLa cells and a ^{32}P-labeled probe spanning the Sp1 binding site immediately upstream of exon 4 (see Table 1 for probe sequences). Lanes 1 and 5 are probe only. Lanes 2 and 6 contain nuclear extract from vehicle-treated HeLa cells. Arrow indicates Sp1 containing complex. Cold competition (100× molar excess) with either consensus Sp1 or mutated Sp1 cold probes are shown in Lanes 3 and 4, respectively. Lane 7 contains nuclear extract from TSA-treated HeLa cells. Lane 8 contains Sp1 antibody and supershifted complex is indicated by an asterisk. C, Cell lysates were prepared from HeLa cells treated with 0.5 μM TSA for up to 24 hr and Western blotted for Sp1. Sp1

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protein levels were normalized to α-tubulin. Numbers in parentheses are fold-change relative to T = 0.

FIG. 5. TSA increases acetylated histones associated with NATb. HeLa cells were treated with 0.5 μM TSA or vehicle (DMSO) and then ChIP assays were performed using antibodies to Sp1 (A), Ac-H4K8 (B) or IgG (negative control). Immunoprecipitated DNA was amplified by PCR using specific primers for NATb or GAPDH (negative control; see Table 1 for primer sequences). Input PCR reactions are performed on DNA prior to immunoprecipitation (positive PCR controls). NTC denotes no template control (negative PCR control).

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Table 1. Oligonucleotides used in this study

Oligonucleotide	Sequence
NATa transcripts (F)[a]	GGGGATAATCGGACAATACAACTC
NATa transcripts (R)	TCCAAGTCCAATTTGTTTCTAGACT
NATb transcripts forward	TCCCCTGCAAGCTTTGGAAGTGAGAGC
NATb transcripts (R)	AGTGCTCTCAAAGCTTGTCCAACTGG
β-Actin forward (F)	CCTCGCCTTTGCCGATCC
β-Actin (R)	GGATCTTCATGAGGTAGTCAGTC
ChIP assay NATb (F)	AGGAGCAACCTGAGTCCCTGCAAC
ChIP assay NATb (R)	GGCAACCAGGAACTAGACTGAATGC
ChIP assay GAPDH (F)	ATGGTTGCCACTGGGGATCT
ChIP assay GAPDH (R)	TGCCAAAGCCTAGGGGAAGA
EMSA probe Wt	5'-GACTCTGGGGGCGGGGCCAGTTGGAC-3'
EMSA probe mutant[b]	5'-GACTCTGGGGTAGGGGCCAGTTGGAC-3'

[a]F = forward, R = reverse

[b]Mutations in Sp1 site underlined

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Figure 1

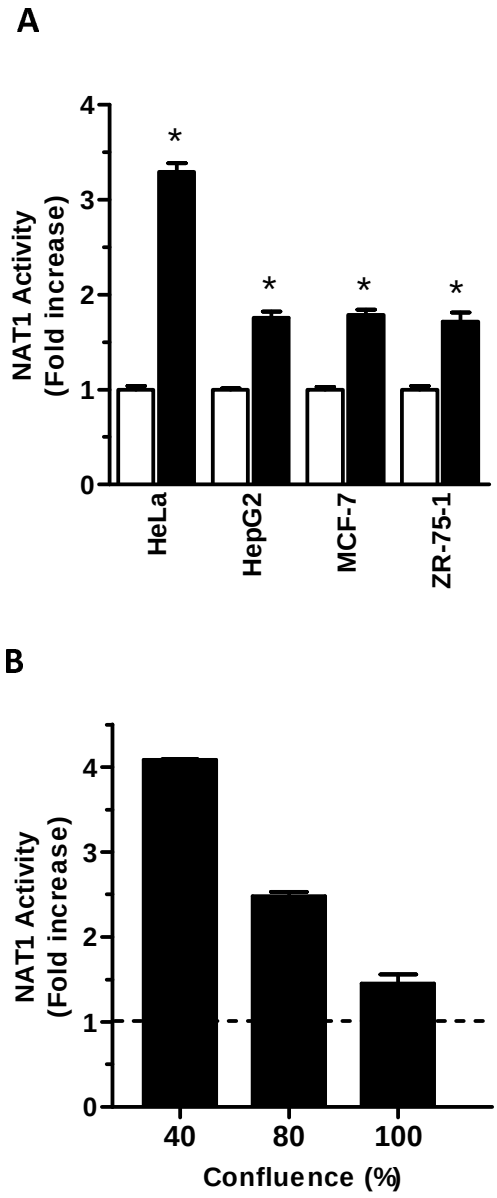

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Figure 2

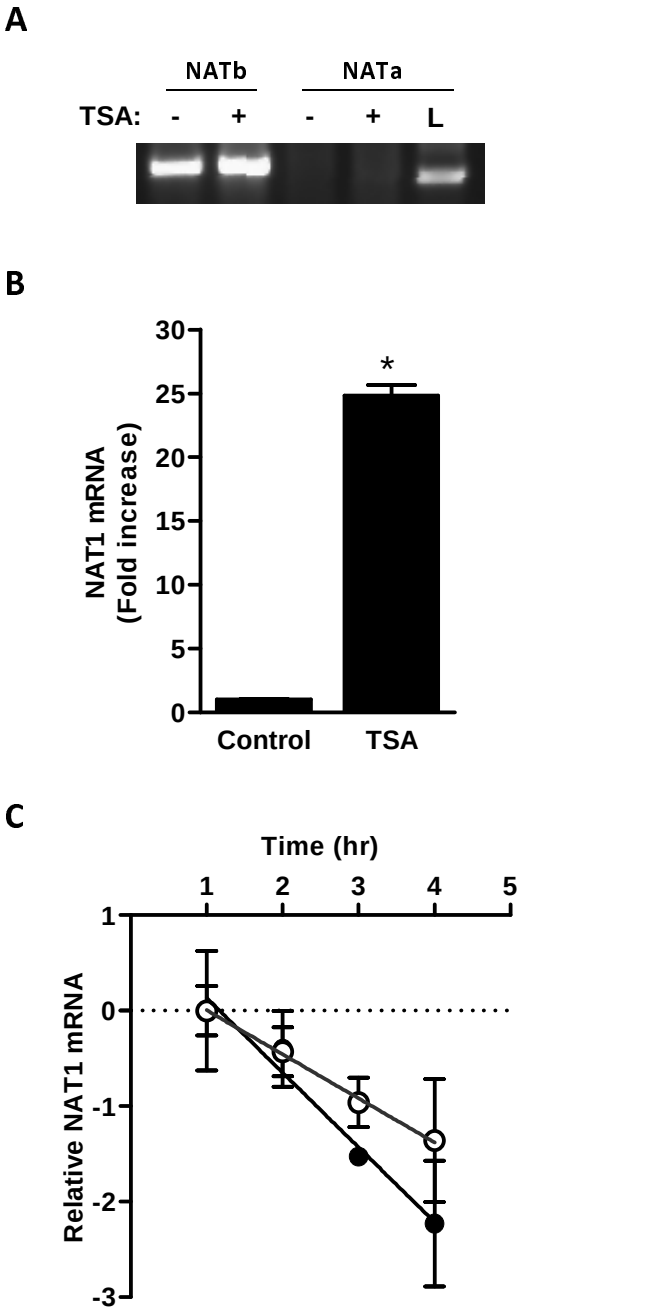

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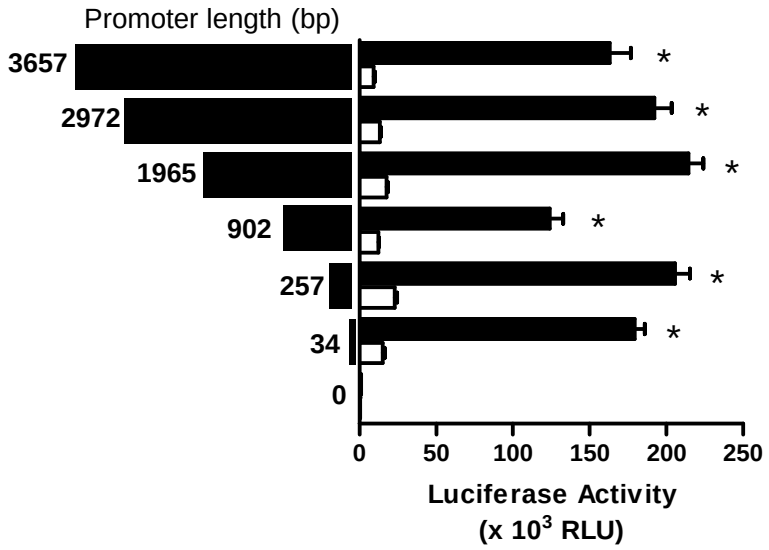
Figure 3

A



B

TGGGGGCGGGGCCAGTTGGACATCCAATGAGAGC**A**CTTCC
-1
CCTGC**AG**ACTTTGGAAGTGAGAGCACTTCCTCATAGACCTTG

GATGTGGG**A**GGATTGC**A**TT**CA**GTCTAGTTCCTG ……

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Figure 4

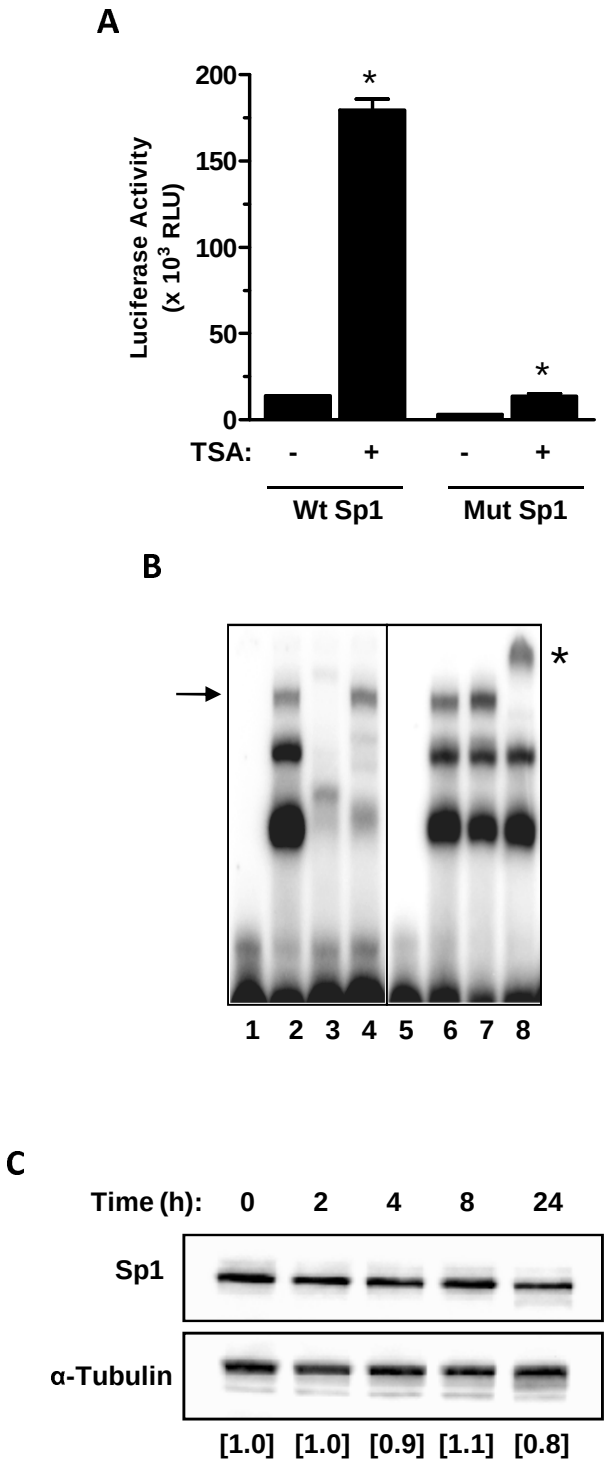

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Figure 5