

Histone Deacetylase Inhibitors as Potential Anti-Skin Cancer Agents¹

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ABSTRACT

The regulation of squamous differentiation is a tightly regulated process involving transcriptional repression and activation. Previous studies have established that squamous carcinoma cell lines inappropriately regulate the transcription of genes important to the control of squamous differentiation. Histone deacetylase inhibitors such as trichostatin A (TSA) and butyrate disrupt normal chromatin structure and cause alterations in gene expression/regulation. For these reasons, we examined the effects of both butyrate and TSA on the growth and differentiation of human keratinocytes or squamous carcinoma cells in tissue culture. We found that treatment of keratinocytes or squamous carcinoma cells with butyrate induced a reversible growth arrest. TSA, on the other hand, induced an irreversible growth arrest in both keratinocytes and squamous carcinoma cells. The growth arrest of keratinocytes induced by TSA or butyrate was accompanied by a reduction in the mRNA levels for proliferation gene *cdk1* and an induction of the mRNA for the differentiation-specific transglutaminase type I gene (*TGI*). In contrast, the squamous carcinoma cells had decreased *cdk1* and *TGI* mRNA in response to TSA or butyrate. Both of these agents produced transient increases in the acetylation of histone H4 in keratinocytes and squamous carcinoma cells. These data indicated that TSA may have potential as a topical treatment for epidermal malignancies.

INTRODUCTION

Squamous differentiation of epithelial cells, in particular, the epidermal keratinocyte, is a carefully orchestrated process that is initiated by the inhibition of proliferation followed by the induction of squamous-specific functions. Aberrant control of this process occurs in many epithelial tissues in which squamous differentiation may not normally occur (*e.g.*, squamous metaplasia of the trachea). Furthermore, defective regulation of this process in stratifying epithelia (*e.g.*, esophagus and skin) is often associated with various pathological states such as cancer (1). Examination of the molecular events controlling squamous differentiation in normal and abnormal conditions indicates that many of these processes may be regulated by alterations in the expression of genes controlling proliferation or differentiation. This is controlled, to a large extent, by transcriptional mechanisms.

In the initial phase of differentiation, keratinocytes undergo irreversible growth arrest. This growth arrest is associated with an irreversible down-regulation of genes associated with proliferation such as *E2F1* (1, 2), *cdk1* (3), or *p53* (4). The down-regulation of these genes seems to be a complex process mediated by transcriptional (5–7) and posttranscriptional mechanisms (2). E2F is a transcription factor complex that is required for the initiation of DNA synthesis (8).

In its active form, “free” E2F binds its cognate response element and induces gene expression. In its inactive form, E2F is complexed with inhibitory pocket proteins. During growth arrest, E2F activity is lost or inhibited, which results in the decrease of mRNA expression for E2F-responsive genes such as *cdk1* and *E2F1* (5–7). In the instance of *cdk1*, this down-regulation is also mediated by the activation of a transcriptional repressor (9). Combined, these data indicate that keratinocyte growth arrest is mediated by the loss of *trans*-activators and the induction of *trans*-repressors. These data take on more significance when contrasted with keratinocyte-derived squamous carcinoma cell lines. These cell lines are generally resistant to normal growth-inhibitory stimuli (3, 4) and are characterized by a failure to down-regulate *cdk1* and *E2F1* mRNA expression (1, 3). The growth inhibitor insensitivity seems to correlate with the inability to regulate the expression of proliferation genes in an appropriate fashion.

The induction of growth arrest is followed by the induction of genes required for squamous differentiation-specific functions (*e.g.*, maintenance of the impermeable barrier). The barrier function of the epidermis is bestowed, in large part, by the formation of the cross-linked envelope (10). The cross-linked envelope is formed by the transglutaminase-catalyzed cross-linking of precursor proteins such as cornifin, involucrin, and fillagrin (10, 11). The activation of this differentiation pathway seems to be regulated by several transcription factors such as activator protein 1 (12), activator protein 2 (13), cAMP-responsive element binding protein/activating transcription factor³, and Skn1a/i (14). Thus, the activation of the later phases of the epithelial squamous differentiation pathway is also associated with the coordination of transcriptional events.

Transcriptional regulation is a complex process that is associated with the interaction of the basal transcription machinery with positive/negative upstream regulatory factors. This process has been complicated by the reports that alterations in the tertiary structure of the DNA such as nucleosomal integrity also affect transcription (for a review, see Refs. 15–18). The nucleosomal integrity is regulated by the association of the core histones (H2A, H2B, H3, and H4) and the linker histone H1. This nucleosomal integrity is maintained, in part, by the acetylation status of the core histones (15–18). In the hypoacetylated state, a tight nucleosomal structure is maintained (15–18) that is nonpermissive for transcription (15–18). When the core histones are acetylated, the nucleosomal integrity is relaxed, and the DNA becomes permissive to transcriptional regulation (15–18). The acetylation status of the histones is governed by the combined activities of the histone acetylases and histone deacetylases (15–18); consequently, the disruption of these enzymes has been implicated in alterations in transcriptional regulation (19–21). This has been further supported by recent studies in which various transcription factors such as c-myc or hormone receptors exist as DNA-bound complexes with histone acetylase or deacetylase enzymes (for a review, see Ref. 17).

Given that the regulation of squamous differentiation involves the coordination of various transcriptional activities, and because histone deacetylase inhibitors may induce global changes in chromatin structure (and presumably in transcription), we initiated a series of exper-

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³A. Medvedev, N. A. Saunders, A. Chistokhina, H. Matsuura, and A. M. Jetten. Regulation of transglutaminase type I: identification of DNA elements involved in transcriptional control, submitted for publication.

iments to: (a) determine the consequences of altering histone acetylation status on the regulation of the squamous differentiation pathway in normal human keratinocytes; and (b) determine the potential of inhibiting histone deacetylases as a basis for treating skin cancers. Earlier studies have used the histone deacetylase inhibitor sodium butyrate to alter histone acetylation as well as induce epithelial differentiation. Sodium butyrate has previously been reported to induce differentiation and growth arrest in keratinocytes (22, 23). However, sodium butyrate is also known to alter protein kinase C activity and mitochondrial function (24, 25). Thus, a definitive association between histone acetylation status and differentiation status is not possible. Recently, specific histone deacetylase inhibitors (TSA⁴ and trapoxin) have been synthesized (25). These compounds now allow a more detailed examination of the role of histone acetylation status in various biological systems.

The present study demonstrates that both sodium butyrate and TSA induce growth arrest and differentiation of normal human keratinocytes that are characterized by a coordinated decrease in the expression of a proliferation marker gene (*cdk1*) and the induction of a differentiation specific marker gene (*TGI*). In contrast, these agents also induce growth arrest in a squamous carcinoma cell line, but without activating the squamous differentiation pathway. Our data suggest that TSA may have potential as a topical treatment of squamous carcinomas of the skin.

MATERIALS AND METHODS

Cell Culture, Treatments, and DNA Synthesis Assays. HEKs were isolated from neonatal foreskins and cultured as described previously (1). The keratinocyte-derived squamous carcinoma cell lines SCC25 and SCC15 were obtained from American Type Culture Collection and cultured as described previously (1). HPV16 E6/E7-expressing HEKs were obtained after retroviral infection of HEKs as described previously (1). The retroviral construct was a generous gift from Dr. Denise Galloway (Fred Hutchison Cancer Center, Seattle, WA). Infection, selection, and confirmation of E6/E7 status have been described previously (1). Proliferation was estimated using a previously reported [³H]thymidine incorporation assay (26). Sodium butyrate was purchased from Sigma (New South Wales, Australia) and kept as a 1 M solution in H₂O at -20°C. TSA was a generous gift from Prof. Minoru Yoshida (University of Tokyo, Tokyo, Japan) and was kept as a 3 mM solution in DMSO at -20°C.

RNA Isolation and Analysis. Total RNA was isolated from HEK cultures using the chaotropic agent Trizol (Life Technologies, Inc.), and single-stranded cDNA was synthesized (1). Expression of transglutaminase type I (*TGI*), *cdk1*, and *actin* was detected by PCR using specific primers (1, 9) under linear conditions with respect to the cycle number. PCR reactions were fractionated on a 1% agarose gel, blotted to nylon, and UV fixed. Blots were then prehybridized, hybridized (28), and probed with the appropriate ³²P-labeled oligonucleotide. The final stringency for washing the blots used in 0.5× SSC, 0.1% SDS at 45°C for 10 min. Blots were then exposed to Kodak XAR5 film or phosphorimager screens. Quantitation was performed using Imagequant software from Molecular Biosciences (New South Wales, Australia).

Histone Isolation and Immunodetection of Acetylated Histone H4. Keratinocytes were either left untreated or treated with NaB (1 mM) or TSA (300 nM) for varying times. In one experiment, histones were isolated from confluent differentiated HEKs. Histones were also isolated from untreated SCC25 cells or from SCC25 cells treated with NaB (3 mM) or TSA (300 nM). At varying times after treatment, cells were trypsinized, and histones were isolated by using established techniques (29). Histone proteins were quantified by the Bio-Rad protein assay. Each sample (5 μg) was then dried down in a centrifugal vacuum dryer (Radiometer Pacific), and the samples were resuspended in protein sample buffer (1), boiled, and run on a 7.5% SDS-PAGE.

⁴ The abbreviations used are: TSA, trichostatin A; HEK, human epidermal keratinocyte; HPV, human papillomavirus; TGF, transforming growth factor; RT-PCR, reverse transcription-PCR; TPA, 12-O-tetradecanoylphorbol-13-acetate; SCC, squamous cell carcinoma.

Proteins were then transferred to nitrocellulose (Amersham), and the acetylated histone H4 was detected with a rabbit polyclonal antibody (Upstate Biotechnology Inc.) against acetylated histone H4 (1:2000 dilution in 3% skim milk powder in PBS containing 0.1% Tween 20 (vol/vol)). Visualization was by means of enhanced chemiluminescence detection (Amersham). The specificity of the antibody was confirmed by the detection of purified histone H4 (Boehringer Mannheim) run on the same gel.

RESULTS

Histone Deacetylase Inhibitors Induce Growth Arrest and Differentiation of Keratinocytes. Treatment of proliferating keratinocytes with 1 mM NaB resulted in a time-dependent inhibition of keratinocyte proliferation, with a 50% inhibition of proliferation occurring within 4 h, and a maximal inhibition occurring by 48 h (Fig. 1A). We next examined whether the growth inhibition induced by NaB was reversible or irreversible. This point is of interest because agents capable of inducing irreversible growth arrest can also induce differentiation, whereas agents such as TGF-β1, which induce a reversible growth arrest, do not induce differentiation in keratinocytes (3). Therefore, NaB was added to keratinocytes, and the keratinocytes were rinsed thoroughly with fresh media at various times thereafter

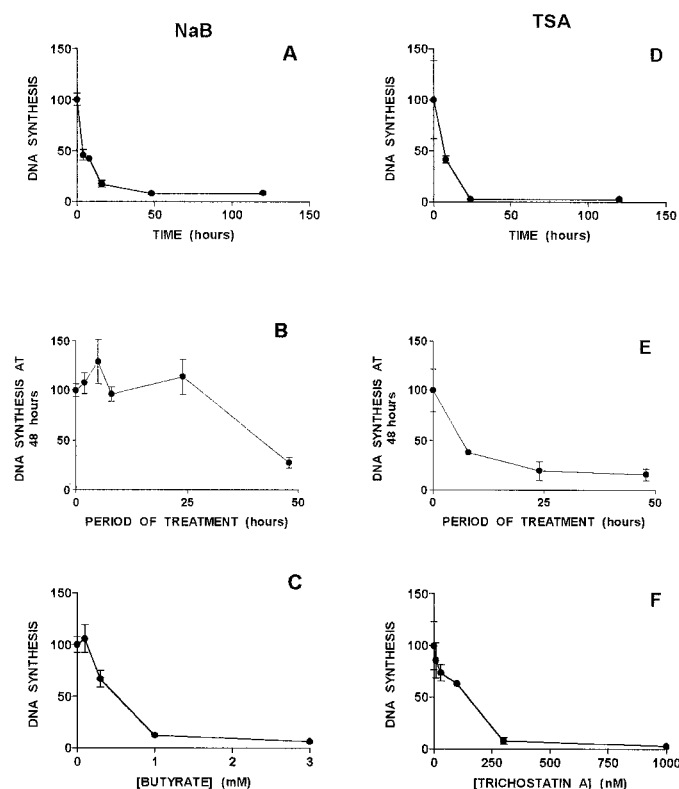


Fig. 1. Histone deacetylase inhibitors induce a dose- and time-dependent growth arrest in human keratinocytes. Proliferating keratinocytes were treated with NaB or TSA, and proliferation was determined by incubation with 10^5 dpm [³H]thymidine/ml media as described in "Materials and Methods." Keratinocytes were incubated for various periods of time with either 1 mM NaB (A) or 300 nM TSA (D) before estimating DNA synthesis. The reversibility of action for NaB and TSA was determined. Keratinocytes were incubated with either 1 mM NaB (B) or 300 nM TSA (E) starting at 0 h and extending to the time indicated. At this time point, media were removed, cells were washed thoroughly with PBS, and media without NaB or TSA were added back. Cells were then allowed to continue their incubation until the end of the 48-h period, at which time DNA synthesis was determined. It is important to note that all cells had a 48-h incubation that varied only in the amount of time they were initially exposed to NaB or TSA. The dose-dependent inhibition of DNA synthesis by NaB or TSA was determined. Keratinocytes were incubated with varying concentrations of either NaB (C) or TSA (F) for 48 h before assaying proliferation. All data were presented as dpm [³H]thymidine incorporated/μg cellular protein expressed as a percentage of the untreated cells. Data presented are the mean ± SE of triplicate determinations from two experiments.

and allowed to continue their incubation for 48 h. Fig. 1B clearly shows that to obtain growth inhibition, NaB had to be continuously present. Combined, these data indicate that the effects of butyrate are mediated through a pathway that requires continuous activation by butyrate. Examination of the dose dependency of butyrate action (Fig. 1C) indicates that it has an EC_{50} value of approximately 0.5 mM and is maximal at 1 mM.

Because NaB is not a specific inhibitor of histone deacetylase (25, 30), we examined the effect of the specific histone deacetylase inhibitor TSA. The time course of action of TSA was similar to that for NaB, with half-maximal inhibition occurring by 8 h, and maximal inhibition occurring by 24 h. However, the action of TSA differed from that of NaB in that after an 8-h incubation with TSA, a significant fraction of the keratinocytes did not resume DNA synthesis (Fig. 1E). Proliferating keratinocytes were growth inhibited by TSA with an EC_{50} value of approximately 120 nM and a maximal effective concentration of 300 nM. A similar dose-response profile has been reported for the induction of tissue-type plasminogen activator in human endothelial cells (33). Similar to butyrate, TSA was not toxic at any of these doses (<95% viability as estimated by trypan blue dye exclusion).

Treatment of HEKs with either NaB or TSA was accompanied by alterations in keratinocyte morphology (data not shown). After a 5-day treatment with 1 mM NaB or 300 nM TSA, keratinocytes took on a flattened appearance reminiscent of the squamous differentiated phenotype. This prompted us to examine the effects of NaB or TSA on the expression of genes specific for proliferation (*cdk1*) or differentiation (*TG1*). The expression of *cdk1* mRNA was reduced within 8 h and was maximally reduced by 24 h with both NaB or TSA (Fig. 2, A and B). The level of *cdk1* mRNA remained low for up to 5 days after NaB treatment (Fig. 2A), whereas in the TSA-treated cells, the *cdk1* mRNA levels returned to basal levels by 48 h (Fig. 2B). This rebound in *cdk1* mRNA levels in TSA-treated cells was not associated with a return to proliferation (see Fig. 1D). A similar response to both NaB and TSA was observed with another proliferation-associated gene, *E2F1* (data not shown). Treatment with NaB (Fig. 2A) or TSA (Fig. 2B) was associated with an increase in the differentiation-specific marker *TG1*. This was a late event that occurred 2–5 days after NaB or TSA treatment. These data indicate that both NaB and TSA can induce the growth arrest and differentiation of keratinocytes, which is associated with a decrease in *cdk1* (albeit transiently in the TSA-treated cells) followed by an increase in *TG1* mRNA levels.

Keratinocytes were treated with 1 mM NaB or 300 nM TSA for varying times to determine the effects of the inhibitors on histone H4 acetylation status. This study yielded some surprising results (Fig. 2C). In the first instance, proliferating HEKs had minimal levels of acetylated H4. Furthermore, when cells differentiated at confluence, there was no change in the levels of acetylated H4. More surprising was the observation that both NaB and TSA treatment of HEKs resulted in a rapid accumulation of acetylated H4 by 8 h that returned to basal levels 48 h after treatment. These data are consistent with the inhibition of histone deacetylase by NaB or TSA. However, these data suggest that even in the continued presence of inhibitor, there is only a transient accumulation of acetylated histone H4.

Histone Deacetylase Inhibitors Are Potent Inhibitors of Squamous Carcinoma Cell Growth. Previous studies with keratinocyte-derived squamous carcinoma cells have shown they are unable to respond to normal growth inhibitors such as phorbol esters, IFN- γ , confluence (1, 3), or TGF- β 1 (31). We examined the effects of NaB and TSA on a well-characterized squamous carcinoma cell line, SCC25 (Fig. 3). The responses of the SCC25 cell line in response to NaB or TSA were similar to those of HEKs. Butyrate (3 mM) caused a time-dependent decrease in DNA synthesis that was maximal by 48 h (Fig. 3A) and reversible (Fig. 3B). An examination of the

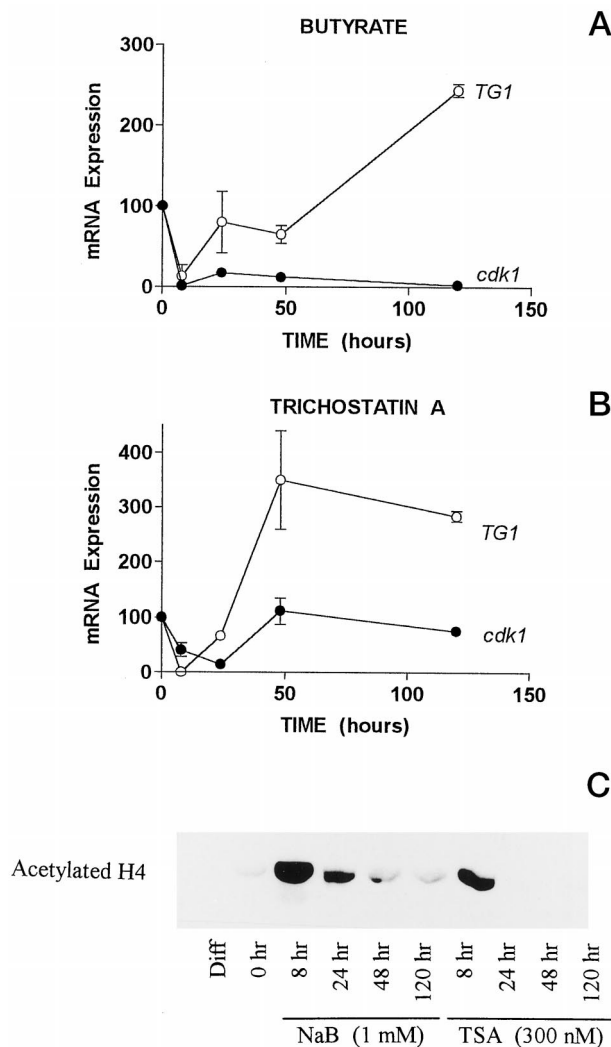


Fig. 2. NaB and TSA induce an accumulation of acetylated histones and induce squamous differentiation in human keratinocytes. The mRNA expression of proliferation-associated (*cdk1*) and differentiation-specific (*TG1*) genes was examined in keratinocytes after treatment with 1 mM NaB (A) or 300 nM TSA (B) for varying times. RNA expression was estimated by semiquantitative RT-PCR. The data represent mean \pm range from two independent experiments. C, proliferating keratinocytes were treated with either 1 mM NaB or 300 nM TSA. At various times, histones were isolated, and 5 μ g were run on a 7.5% SDS-PAGE gel, blotted, and probed with an antibody against acetylated histone H4. In one condition, the acetylation of H4 in confluent/differentiated keratinocytes was examined. This experiment has been performed in three independent experiments.

dose-dependent growth arrest by NaB indicated that SCC25 cells were less responsive to NaB (an EC_{50} value of approximately 1 mM and a maximal effective dose of 3 mM) than the HEKs (an EC_{50} value of approximately 0.5 mM and a maximal dose of 1 mM). TSA treatment decreased DNA synthesis (Fig. 3D) in an apparently irreversible (Fig. 3E) and dose-dependent manner (Fig. 3F). TSA treatment of SCC25 cells had an EC_{50} value of 100 nM and a maximal effective dose of 300 nM (Fig. 3F). These values are similar to those of normal keratinocytes; similarly, there was no toxicity (by dye exclusion test) at any of the concentrations tested (data not shown). These data are significant because with few exceptions, inducers of growth arrest in keratinocytes do not normally inhibit the growth of keratinocyte-derived carcinoma cells. The inhibition of DNA synthesis by NaB and TSA was not restricted to the SCC25 cells because another keratinocyte-derived squamous carcinoma cell line, SCC15, was also growth inhibited by NaB or TSA (Table 1). Furthermore, we also examined the effects of NaB or TSA on normal HEKs expressing the HPV16 E6 and E7 proteins. The expression of the E6 and E7 genes in these cells

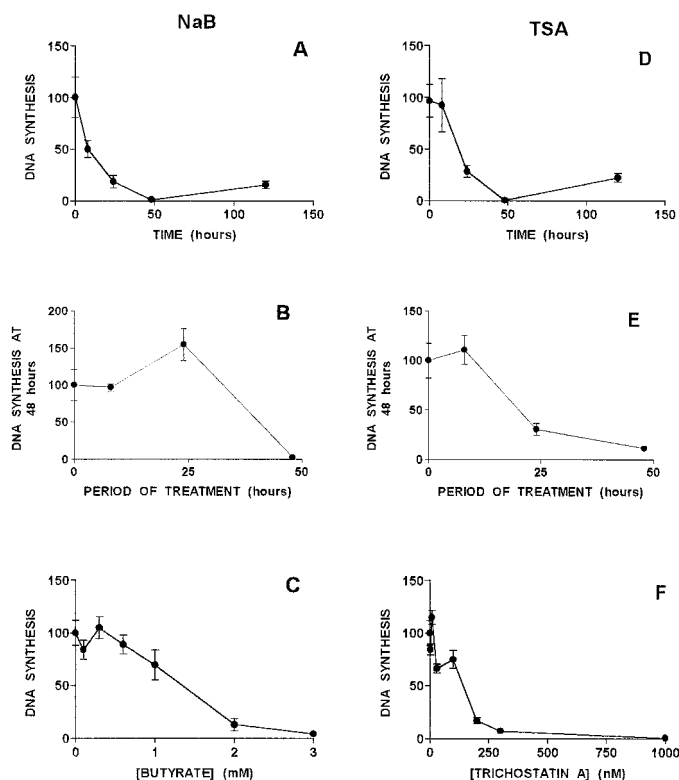


Fig. 3. Histone deacetylase inhibitors induce a dose- and time-dependent growth arrest in SCC25 cells. Proliferating SCC5 cells were treated with NaB or TSA, and proliferation was determined by incubation with 10^5 dpm [3 H]thymidine/ml media as described in "Materials and Methods." SCC25 cells were incubated for various periods of time with either 3 mM NaB (A) or 300 nM TSA (D) before estimating DNA synthesis. The reversibility of action of NaB and TSA was determined. SCC25 cells were incubated with either 3 mM NaB (B) or 300 nM TSA (E) for the period of time shown. At this point, media were removed, cells were washed thoroughly with PBS, and media without NaB or TSA were added back. Cells were then allowed to incubate until the end of the 48-h period, and DNA synthesis was determined. The dose-dependent inhibition of DNA synthesis by NaB or TSA was determined. SCC5 cells were incubated with varying concentrations of either NaB (C) or TSA (F) for 48 h before assaying proliferation. All data are presented as dpm [3 H]thymidine incorporated/ μ g cellular protein expressed as a percentage of the untreated cells. Data presented are the mean \pm SE of triplicate determinations from two experiments.

was confirmed by RT-PCR analysis (Ref. 1; data not shown). These cells exhibit deregulated growth characteristics but were still growth inhibited by NaB or TSA (Table 1). These data established that the growth inhibition induced by TSA and NaB also occurred in another SCC cell line and in HEKs with deregulated growth.

The growth inhibition of the SCC25 cells was surprising and prompted us to also examine the consequences of NaB or TSA treatment on the expression of *cdk1* and *TG1* in the SCC25 cells (Fig. 4, A and B). In contrast to HEKs, the treatment of SCC25 cells did not result in the initiation of the differentiation program. SCC25 cells are a partially differentiated squamous carcinoma cell line and consequently express *TG1*. Treatment of SCC25 cells with either NaB or TSA resulted in a decrease in *cdk1* mRNA expression in SCC25 cells (Fig. 4, A and B). However, both NaB and TSA also resulted in a decrease in the differentiation-specific marker gene, *TG1*. These data demonstrate that treatment of the SCC25 cells with histone deacetylase inhibitors does not result in the reinstatement of a normal squamous differentiation program. Similar to the HEKs, NaB and TSA treatment resulted only in a transient accumulation of acetylated histone H4 (Fig. 4C). In the instance of NaB treatment, histone acetylation was maximal by 24 h and had decreased to basal levels by 48 h (Fig. 4C). TSA treatment, on the other hand, resulted in a rapid accumulation of acetylated histone H4 by 8 h that decreased to basal levels by 24 h (Fig. 4C).

Because NaB and TSA were able to cause the growth arrest of SCC25 cells in the short term, we examined the long-term reversibility of action of TSA and NaB on SCC25 proliferation. SCC25 cells were treated with either 3 mM NaB or 300 nM TSA for 5 days. After this treatment, cells were rinsed and left untreated for an additional 2 days or treated for an additional 2 days with NaB or TSA. This study established that if SCC25 cells were treated for a period of time that allowed growth arrest to occur, then the cells resumed DNA synthesis after NaB withdrawal (44 ± 11 -fold greater than continuously treated NaB cells), whereas those treated with TSA did not (2.2 ± 0.2 -fold greater than continuously treated TSA cells; Fig. 5).

DISCUSSION

The present study demonstrates that: (a) the histone deacetylase inhibitors TSA and sodium butyrate are potent inhibitors of squamous carcinoma cell growth and may have therapeutic potential as topical anticancer agents; (b) the mechanism of action of TSA or NaB may not be attributed simply to the induction of squamous differentiation of the cancer cells; and (c) the induction of growth arrest by TSA or NaB may not be due simply to an accumulation of acetylated histone H4.

This study provides strong evidence supporting the trial of TSA in the treatment of topical neoplasms such as SCCs. For instance, *in vitro* studies with TSA indicated that it produces an irreversible growth arrest (at least in the short term) in SCC cells. Previous studies using butyrate and various structural analogues of butyrate have proved disappointing for a number of reasons. The most significant of these is the short half-life of butyrate when given systemically (approximately 6 min; Ref. 29). In this study we show that NaB is relatively stable *in vitro* in keratinocytes. This is most clearly shown by the observation that keratinocytes and SCC25 cells must be exposed to NaB continuously to mediate growth arrest. If NaB is required continuously to mediate growth inhibition, then it must be biologically active for at least 48 h in keratinocyte cultures. However, because the action of NaB seems to be reversible in nature, we believe that TSA may be a more favorable compound to trial in the treatment of topical neoplasms. TSA induces an apparently irreversible growth arrest, and given the lipophilicity of TSA, it may be possible to deliver high concentrations of TSA specifically to the skin lesion.

The present study clearly shows that treatment of normal or transformed keratinocytes with histone deacetylase inhibitors initiates a growth-inhibitory pathway that is dissimilar to that of normal squamous differentiation. Superficially, the effects of NaB and TSA on normal HEKs seem to be similar to those of other differentiation-inducing agents (e.g., TPA or IFN- γ ; Refs. 1 and 3) in that growth arrest is accompanied by decreases in *cdk1* mRNA expression and the induction of *TG1* expression. However, closer examination of normal keratinocytes suggested differences between the induction of squamous differentiation mediated by other agents and the responses associated with TSA or NaB treatment. For instance, in contrast to phorbol ester-treated keratinocytes,

Table 1 Growth inhibition of keratinocytes and keratinocyte-derived cell lines by NaB or TSA

Proliferating (untreated) HEKs, the squamous carcinoma cell lines SCC25 and SCC15, or a HPV16 E6/E7-expressing keratinocyte cell line were treated with varying concentrations of the histone deacetylase inhibitors NaB or TSA for 48 h. At the end of 48 h, the DNA synthesis rate was determined and is expressed as a percentage of the value of the untreated cells.

	HEKs	SCC25	SCC15	HPV16 E6/E7
Untreated	100 \pm 38	100 \pm 12.5	100 \pm 2.9	100 \pm 19
1 mM NaB	7.2 \pm 4.4	72 \pm 10	57 \pm 7	52 \pm 10
3 mM NaB		1 \pm 0.3	3.2 \pm 0.2	16 \pm 2
0.3 μ M TSA	2.6 \pm 0.4	0.8 \pm 0.2	16.2 \pm 0.7	57 \pm 6
1 μ M TSA		0.5 \pm 0.1	5.8 \pm 3.3	37 \pm 14

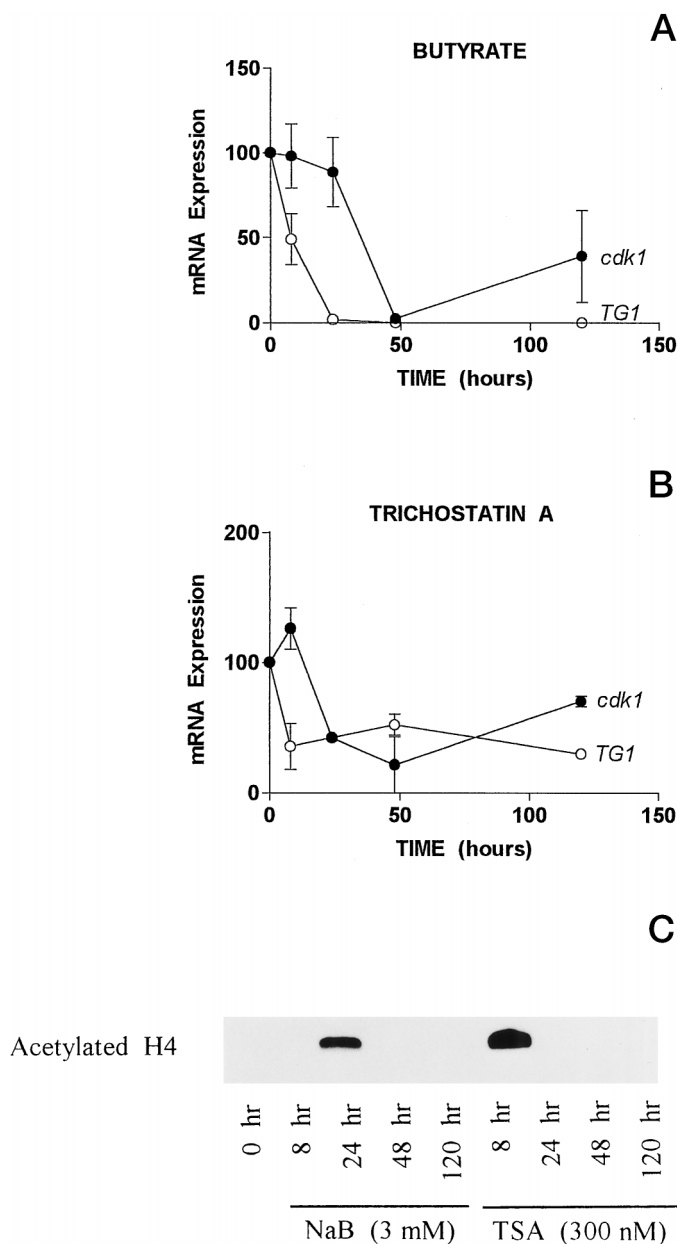


Fig. 4. NaB and TSA induce an accumulation of acetylated histones but do not induce squamous differentiation in SCC25 cells. The mRNA expression of proliferation-associated (*cdk1*) and differentiation-specific (*TG1*) genes were examined in SCC25 cells after treatment with 3 mM NaB (A) or 300 nM TSA (B) for varying times. RNA expression was estimated by semiquantitative RT-PCR. The data represent the mean \pm range from two independent experiments. C, proliferating SCC25 cells were treated with either 3 mM NaB or 300 nM TSA. At various times, histones were isolated, and 5 μ g were run on a 7.5% SDS-PAGE gel, blotted, and probed with an antibody against acetylated histone H4. This experiment has been performed in two independent experiments.

the induction of *TG1* in NaB- or TSA-treated keratinocytes was only moderate (2.5–3.5-fold compared with 10-fold for TPA-treated cells; Ref. 2). Furthermore, the reduction in *cdk1* mRNA in TSA-treated cells was only transient and did not correlate with the apparent irreversibility of action of TSA or DNA synthesis data. This is in contrast to the tight correlation between *cdk1* mRNA expression and DNA synthesis reported for keratinocytes undergoing growth arrest in response to senescence, confluence, or TGF- β_1 , TPA, or IFN- γ treatment (3, 26). Our studies with the SCC25 cells also support a novel mechanism of growth inhibition for NaB and TSA. Both TSA and NaB induced a similar dose- and time-dependent growth arrest in the absence of an increase in the differentiation-specific marker gene *TG1* in squamous carcinoma cells. This

A difference was significant at two levels: (a) the SCC25 cells are resistant to other growth-inhibitory stimuli that we have tested (1); and (b) the growth arrest was not associated with the induction of differentiation. Combined, these data would suggest that the mechanism of action of TSA and NaB in keratinocytes is not mediated by the activation of the normal squamous differentiation pathway.

A possible explanation for the effects of NaB or TSA may be derived from the observation that the inhibition of histone deacetylases leads to hyperacetylation of histones (as exemplified by H4 in this study) and an alteration in chromatin structure. Such an alteration in the chromatin may be perceived by the cell as akin to a DNA-damaging event (as postulated in Ref. 25). In this circumstance, normal keratinocytes may undergo growth arrest/retardation. Supporting this proposition are reports that substituting the four lysines in the amino-terminal tail of H4 with glutamate results in a histone with the same characteristics as tetra-acetylated H4, which, in yeast, results in a G₂-M-phase block (15, 32). A G₁- and a G₂-M-phase block has previously been reported for keratinocytes and colonic epithelial cells treated with NaB (23, 24). However, the mechanism by which these checkpoints are executed is unknown. Arguing against this proposition is the observation that most carcinoma cells have lost the ability to undergo growth inhibition in response to DNA-damaging events, suggesting that a novel pathway of growth inhibition would have to be present in the carcinoma cells.

The present study demonstrates that the induction of growth arrest in normal keratinocytes and the inhibition of tumorigenicity in keratinocyte-derived carcinoma cells are not simply due to the accumulation of acetylated histones and the deregulation of gene expression. This is supported by the observation that *TG1* mRNA is induced in normal keratinocytes and repressed in carcinoma cells in response to both NaB or TSA. On one hand, it was found that acetylated histone H4 accumulated rapidly in both normal and cancer cells after NaB or TSA treatment. However, in all instances, this accumulation was transient. Evidence suggesting that histone H4 acetylation was not directly required for growth arrest stems from the observation that in HEKs, both NaB and TSA cause the induction of *TG1* at a time at which the H4 acetylation has returned to basal levels. Furthermore, the reversibility of TSA action in SCC25 cells after an 8-h treatment (maximal H4 acetylation) and the reversibility of NaB in HEKs at 24 h after treatment (H4 acetylation was decreased) is not consistent with a direct role of histone acetylation in this process. However, these data are consistent with histone hyperacetylation initiating a

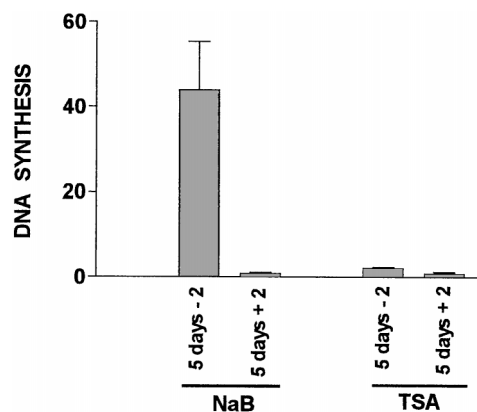


Fig. 5. Long-term reversibility of growth arrest induced by histone deacetylase inhibitors in SCC25 cells. Proliferating SCC25 cells were treated with 3 mM NaB or 300 nM TSA for 5 days. Some cells were then treated for an additional 2 days (5 days + 2) or were washed with PBS and left untreated for 2 days (5 days - 2). Data presented are the means \pm SE from two experiments performed in triplicate. Data are expressed as the fold increase over the 5 day + 2.

cascade of events that ultimately triggers growth arrest and modulation of *TG1* mRNA expression. The transient accumulation of acetylated histones after NaB or TSA treatment has not been reported before and contrasts with that reported in human endothelial cells (33). Although the mechanism underlying the transient hyperacetylation in keratinocytes is unknown, it is possible that NaB or TSA may decrease the activity/expression of the histone acetylases or may increase the activity of histone deacetylases such that the inhibitor concentrations are no longer effective.

Although this study focuses primarily on biological end points such as growth and differentiation, it raises some interesting observations on how changes in histone acetylation status (exemplified by H4 acetylation) may affect normal cellular processes. Previous reports on how histone acetylation may be implicated in the control of transcription suggest a complicated model in which histone acetylation bestows transcriptional competence to a gene (16). It is clear that the appropriate regulation of histone acetylation is essential to normal cellular physiology. For example, histone H3 and H4 mutants, which were unable to be acetylated, proved to be lethal mutations (32). Furthermore, the acetylation of histones is essential for DNA replication (15). Although histone acetylation is essential for cell survival, it is only recently that we have become aware of some of the mechanisms by which it mediates its effects. For instance, recent studies have provided compelling evidence that positive or negative transcriptional regulation by established transcription factors such as Mad/Max, E2F, YY1, or members of the steroid/thyroid receptor superfamilies requires interactions with either histone acetylases or deacetylases (34–39). Thus, the transcription factors remain the major regulators of transcriptional control, whereas the acetylase and deacetylase enzymes contribute to the activation or repressive functions of the DNA-bound transcription factor. There is some evidence in the HEKs that this may occur because both the *cdk1* and *TG1* promoters may be accessible to normal transcriptional activators or repressors due to the acetylation of the histones. This explanation, however, would not be consistent with the presumed general activating properties of acetylated histones because *cdk1* mRNA is decreased and *TG1* is increased. Furthermore, *TG1* is increased in normal keratinocytes and repressed in the carcinoma cells. A recent study reported that the inhibition of cellular proliferation may be mediated by the binding of Rb to a histone deacetylase that results in transcriptional repression of the E2F transcription factor and, consequently, growth arrest (39). This may provide an explanation for the growth inhibition we observed in both HEKs and SCC25 cells because both the HEKs and the SCC25 cells contain E2F (1), and the *cdk1* gene is an E2F-responsive gene (5), and the level of *cdk1* mRNA was decreased in response to NaB or TSA in both HEKs and SCC25 cells.

In conclusion, the present study demonstrates that NaB and TSA are potent growth inhibitors for both normal HEKs and transformed keratinocytes. We also show that TSA may have potential as a topical therapeutic for use in epidermal neoplasms.

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