Objective: To investigate the effects of mitomycin C and the histone deacetylase inhibitors sodium butyrate and trichostatin on the viability and growth of conjunctival melanoma cell lines and Tenon capsule fibroblasts.

Methods: Cells were treated with a range of concentrations of sodium butyrate, trichostatin, and mitomycin C. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assays were performed 48 hours after treatment. Treated cells were stained with acridine orange/ethidium bromide to assess for cell death. Cell-cycle changes in histone deacetylase inhibitor–treated melanoma cells were quantified using flow cytometry.

Results: All agents induced dose-dependent cell death in the melanoma cell lines; however, sodium butyrate and trichostatin were relatively nontoxic to Tenon capsule fibroblasts. Acridine orange/ethidium bromide staining indicated that sodium butyrate and trichostatin induced apoptotic cell death. At low doses, sodium butyrate and trichostatin induced a G1 cell-cycle block in the melanoma cells.

Conclusions: Sodium butyrate and trichostatin induced cell death in melanoma cells, comparable with mitomycin C, with minimal effect on Tenon capsule fibroblasts. In addition, they induced a G1 cell-cycle block. These findings support the need for further investigation into the in vivo efficacy of these agents.


CONJUNCTIVAL MELANOMA is a rare condition with a 5-year survival between 68% and 93%. The current treatment involves en bloc surgical resection followed by double freeze-thaw cryotherapy to the surrounding conjunctiva or brachytherapy, with a recurrence rate of between 18% and 24%.

The accessibility of these lesions and the possibility of treating precancerous primary acquired melanosis with atypia provides a rationale for the use of topical agents. To date, the most promising agent is mitomycin C, with evidence supporting its topical use in the management of large, diffuse primary acquired melanosis with atypia or melanoma in situ not amenable to resection. In the primary treatment of conjunctival melanoma, the recurrence rate with mitomycin C alone is an unacceptably high 43%. Other agents such as fluorouracil and interferon alpha and beta have been tested in trials in primary acquired melanosis with some success.

Sodium butyrate and trichostatin A are members of a novel drug class, histone deacetylase (HDAC) inhibitors, which display low toxicity to normal cells and have the capacity to induce differentiation, growth arrest, and apoptotic cell death in tumor cells. These effects have been observed with cutaneous melanoma in cell culture, animal models, and early-phase clinical trials. Vorinostat (suberoylanilide hydroxamic acid) is the first HDAC inhibitor to be licensed by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma.

Sodium butyrate is a short-chain fatty acid that induces cell-cycle arrest, apoptosis, and differentiation of many tumor cell lines in vitro, including cutaneous and uveal melanoma. However, its short systemic half-life has limited its use as a systemic therapy. The hydroxamic acid derivative trichostatin A is the prototypical HDAC inhibitor, with a higher HDAC-inhibiting potency than sodium butyrate. To date, there has been little published work exploring the topical application of HDAC inhibitors for cutaneous or ocular surface malignancies.
which DNA is wound to form chromatin. Histone deacetylases and histone acetyltransferases antagonistically regulate the degree of condensation of chromatin to regulate transcription. Histone deacetylase inhibition increases the acetylation of the N-terminal histone lysine residues, which decreases the electrostatic attraction between the positively charged histones and the negatively charged DNA. This relaxes the dense chromatin structure and allows the cell’s transcriptional machinery to access DNA. It is increasingly evident that the state of histone acetylation also provides epigenetic codes that are recognized by nonhistone proteins to further regulate gene activation. In this study, we assessed the in vitro growth and viability of 2 conjunctival melanoma cell lines derived from recurrent epithelioid tumors and primary Tenon capsule fibroblasts in response to mitomycin C, sodium butyrate, and trichostatin A.

**METHODS**

**CELLS**

We used 2 conjunctival cell lines, CRMM-1 and CRMM-2 (Department of Ophthalmology, University of Duisburg-Essen, Essen, Germany). Both cell lines were derived from recurrent, predominantly epithelioid tumors located in the bulbar conjunctiva. Melanoma cell lines were grown in the Ham F12 medium supplemented with 10% heat-inactivated fetal bovine serum, 2mM l-glutamine, 50-IU/mL penicillin, and 50-µg/mL streptomycin. Primary human Tenon capsule fibroblasts (n = 3) were isolated and grown in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum, 2mM l-glutamine, 50-IU/mL penicillin, and 50-µg/mL streptomycin. Passage 2 through 5 fibroblasts were used for all experiments. All cells were maintained in a humidified incubator at 37°C and 5% carbon dioxide. The study was performed according to the Declaration of Helsinki and with approval from the University of Duisburg-Essen, Department of Ophthalmology.

**AGENTS**

Sodium butyrate and mitomycin C were prepared as 100mM and 1-mg/mL stock solutions in water, respectively, made fresh for each experiment. Trichostatin A was dissolved in dimethylsulphoxide as a 1-mg/mL stock solution and stored in aliquots at -30°C. All reagents were subsequently diluted in the appropriate culture medium immediately before use.

**CELL GROWTH AND SURVIVAL**

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays were used to assess cell viability with a range of concentrations of sodium butyrate, trichostatin A, and mitomycin C. Initial experiments used a range of doses that killed most cells (≤16mM sodium butyrate, ≤4µM trichostatin A, and ≤16-µg/mL mitomycin C). Final experimental concentrations that resulted in comparable melanoma cell death rates were chosen: 0mM to 8mM sodium butyrate, 0µM to 2µM trichostatin A; and 0- to 2-µg/mL mitomycin C.

**DOSE-DEPENDENT GROWTH INHIBITION AND CELL DEATH IN CONJUNCTIVAL MELANOMA CELL LINES**

Initial experiments demonstrated a more than 60% decrease in cell viability for more than 8mM sodium butyrate, more than 2µM trichostatin A, and more than 4-µg/mL mitomycin C at 48 hours (not shown). Treatment with trichostatin A and sodium butyrate resulted in significant growth inhibition in CRMM-1 and CRMM-2 cell lines at an earlier time point compared to mitomycin C. For each experiment, CRMM-1 and CRMM-2 cells and fibroblasts were initially seeded at 5 × 10^4 cells/well (n=6) in 96 well plates and grown for 36 hours. The growth medium was then changed to variable concentrations of mitomycin C, sodium butyrate, or trichostatin A; medium alone; or a dimethylsulphoxide vehicle (control for trichostatin A) and incubated for an additional 48 hours. The MTT reagent was then added to each well and cells were incubated for an additional 4 hours at 37°C. After addition of a solubilization solution (10% sodium dodecyl sulfate in 0.01M hydrogen chloride), cells were incubated for an additional 18 hours at 37°C. Absorbance at 570 nm was detected using a Tecan Safire microplate reader (Tecan Group Ltd, Männedorf, Switzerland), with which changes in absorbance were proportional to cell viability. Mean absorbances and standard error of the mean were calculated and the results expressed as the percent change in absorbance from the mean control (untreated) absorbance.

For melanoma cells, 6 wells per treatment were used and dose-response experiments performed twice (n = 2). For Tenon capsule fibroblasts (n = 3), 6 wells per treatment were used for sodium butyrate, trichostatin A, and high-dose mitomycin C experiments. Low-dose mitomycin C experiments were performed on Tenon capsule fibroblasts (n = 2), with 6 wells per treatment.

**CELL MORPHOLOGY**

The CRMM-1 and CRMM-2 cells and fibroblasts were seeded in 6 well plates for 48 hours and treated with a range of sodium butyrate, trichostatin A, and mitomycin C concentrations. At 48 hours posttreatment, cell morphology was assessed and imaged digitally using an inverted-phase contrast microscope. In separate experiments, treated and control cells were also stained with acridine orange/ethidium bromide, an RNA/DNA–intercalating agent used to assess cell death, including apoptosis.

**CELL-CYCLE STUDIES**

The effects of sodium butyrate and trichostatin A on cell-cycle progression of CRMM-1 and CRMM-2 cells were analyzed by flow cytometry. Cells were plated at a density of 5 × 10^4 per 35-mm dish for 24 hours, then treated (Ham F12 medium as a control for 1mM or 2mM sodium butyrate; and dimethylsulphoxide as a control for 0.1µM or 1µM trichostatin A). The adherent and nonadherent cells were collected at 24 and 48 hours after treatment. Cells were washed in phosphate-buffered saline and subsequently fixed by drop-wise addition of 70% ice-cold ethanol. Cells were stored at 4°C in 70% ethanol until the day of analysis. Cells were pelleted, washed in phosphate-buffered saline, and incubated for 30 minutes at room temperature in 50-µg/mL propidium iodide with 100-µg/mL ribonuclease before flow cytometry analysis. Data acquisition (10,000 cells) was performed using a FACS Calibur (Becton Dickinson, Franklin Lakes, New Jersey) and analyzed by FlowJo software, version 7.25 (Tree Star Inc, Ashland, Oregon).
Sodium butyrate and trichostatin A were proportionately less toxic to Tenon capsule fibroblasts than mitomycin C. For example, doses of mitomycin C that decreased CRMM-2 cell viability by approximately 30% also decreased Tenon capsule fibroblasts by approximately 30% (Figure 1C). In contrast, doses of sodium butyrate or trichostatin A that induced an approximately 30% decrease in CRMM-2 cell viability decreased viability of Tenon capsule fibroblasts by approximately 10% (Figure 1A and B).

**CELL MORPHOLOGY AND CELL DEATH**

At low concentrations of sodium butyrate (<1mM) and trichostatin A (<1µM), melanoma cells displayed reduced growth and evidence of morphologic differentiation compared with controls (Figure 2A and C). CRMM-1 and CRMM-2 cells showed increased numbers of branching processes, some with extensive elongation (Figure 2B). Melanoma cells treated with more than 0.5mM sodium butyrate, more than 0.5µM trichostatin A, and more than 0.5-µg/mL mitomycin C displayed evidence of apoptosis, and as concentrations increased, detached, rounded cells were prominent (Figure 2D-F).

With acridine orange/ethidium bromide staining, melanoma cells treated with sodium butyrate, trichostatin A (Figure 3), or mitomycin C (not shown) displayed obvious apoptotic morphology with condensed and fragmented nuclear chromatin and cytoplasmic condensation (Figure 3C, E, and F). Control (Figure 3B) and sodium butyrate– or trichostatin A–treated Tenon capsule fibroblasts appeared similar (Figure 3B cf Figure 3D, 4mM sodium butyrate).

**CELL-CYCLE EFFECTS**

Flow cytometry showed cell-cycle inhibition at G1 for melanoma cells treated with low-dose sodium butyrate and trichostatin A. Representative examples are shown in Figure 4. Cell death was induced at higher doses (>2mM sodium butyrate and >1µM trichostatin A), evidenced by a pre-G1 peak (apoptotic cells) that increased in a dose-dependent fashion (Figure 4). Both CRMM-1 and CRMM-2 cell lines displayed similar effects.

In this study, we demonstrated that the HDAC inhibitors sodium butyrate and trichostatin A can selectively kill melanoma cells in a dose-dependent fashion and/or induce morphologic differentiation while being relatively nontoxic to normal Tenon capsule fibroblasts. The efficacy of cell killing with sodium butyrate and trichostatin A compared well with the current topical treatment of choice, mitomycin C, but had reduced toxicity for normal cells. The low toxicity of HDAC inhibitors to normal cells, compared with tumor cells, is a hallmark of conjunctival melanoma cell lines with sodium butyrate, trichostatin A, or mitomycin C resulted in a dose-dependent decrease in cell viability and increased cell death (mostly apoptosis) (Figure 1). Mitomycin C induced a greater than 50% decrease in cell viability for the melanoma cells at the final doses used (Figure 1C).
of this class of drug,\textsuperscript{32,33} though the reasons for this differential cytotoxicity remain to be fully established.

Apoptosis was induced with sodium butyrate and trichostatin A, consistent with findings in cutaneous and uveal melanoma cell lines.\textsuperscript{14,34-38} Histone deacetylase inhibitors are known to be potent differentiating agents,\textsuperscript{27} and at lower doses of sodium butyrate and trichostatin A, conjunctival melanoma cells showed some features of morphologic differentiation. Because most conjunctival melanomas arise from primary acquired melanosis with atypia,\textsuperscript{4} it may be possible to inhibit or slow malignant transformation using HDAC inhibitors.

\textbf{Figure 2.} Photomicrographs of control and treated conjunctival melanoma cells. Control CRMM-1 (A) and CRMM-2 (C) cells (Department of Ophthalmology, University of Duisburg-Essen, Essen, Germany)\textsuperscript{29} appeared similar to dimethylsulphoxide controls (not shown) at 48 hours. Cells cultured with low-dose sodium butyrate (0.5mM, CRMM-1) (B) or trichostatin A (<0.5µM) (not shown) showed long, sometimes multiple extensive cell processes, suggesting cell differentiation (*); some cell death also occurred in these cultures. These elongated processes occurred more often in CRMM-1 cells (normally a spindlelike morphology) (B). Higher doses of sodium butyrate (2mM) (CRMM-2) (D), trichostatin A (2µM, CRMM-2) (E), and mitomycin C (4 µg/mL, CRMM-2) (F) induced marked loss of cell attachment and cell death, with numerous floating dead cells apparent in cultures (*) by 48 hours.
Low concentrations of both sodium butyrate and trichostatin A inhibited the melanoma cell cycle at G1. Previous studies demonstrated a G1 and occasionally a G2/M block in melanoma cell lines treated with trichostatin A or sodium butyrate. G1 inhibition is also seen in most other HDAC-treated malignant cell lines and is attributed in part to upregulation of the cyclin-dependent kinase inhibitor p21WAF1/CIP1.

Figure 3. Acridine orange/ethidium bromide staining of CRMM-1 and CRMM-2 cells (Department of Ophthalmology, University of Duisburg-Essen, Essen, Germany) and Tenon capsule fibroblasts. Control melanoma cells (A) and Tenon capsule fibroblasts (B) are seen with flattened morphology and no evidence of ethidium bromide staining (dead cells). With more than 1mM sodium butyrate, acridine orange/ethidium bromide staining showed melanoma cells with nuclear condensation and fragmentation characteristic of apoptosis (*) (CRMM-1, 2mM sodium butyrate) (C) compared with Tenon capsule fibroblasts (4mM sodium butyrate) (D). Melanoma cells treated with trichostatin A also showed evidence of apoptosis (CRMM-2, 0.5µM trichostatin A) (E). Cells at various stages of degeneration are also seen at higher concentrations (CRMM-2, 2µM trichostatin A) (F).
To date, only 1 study has examined HDAC inhibitors in conjunction with mitomycin C. The study found a synergistic decrease in viability in colorectal adenocarcinoma cell lines treated with mitomycin C and the HDAC inhibitor valproic acid. The use of HDAC inhibitors combined with mitomycin C to treat conjunctival melanocytic lesions is a promising therapy for further investigation. For diffuse conjunctival melanoma or focal melanoma with surgical margins positive for melanoma, there is also some evidence to support resection with plaque brachytherapy. Interestingly, sodium butyrate and trichostatin A show potential to selectively sensitize melanoma cell lines to ionizing radiation at doses equivalent to those used in our study. As such, pre-treatment of conjunctival lesions with sodium butyrate or trichostatin A prior to radiation therapy may increase the efficacy of this treatment.

The adverse effects of mitomycin C are well known, and the concentrations of mitomycin C used in the present study are approximately 200 times less than 0.4 mg/mL, which is commonly used to treat melanocytic lesions in clinical practice. This emphasizes the need for further in vivo investigation of sodium butyrate and trichostatin A to develop safe dosing regimens. Both trichostatin A and sodium butyrate have been largely abandoned in clinical trials of other malignancies in favor of derivatives with more favorable systemic pharmacokinetics.

However, the short systemic half-lives of trichostatin A and sodium butyrate may be an advantage for topical use. Our study indicates a need for further investigation of the in vivo safety and efficacy of topical HDAC inhibitors, including trichostatin A and sodium butyrate, either as primary or adjunctive agents for the management of conjunctival melanoma and primary acquired melanosis with atypia.

Submitted for Publication: August 22, 2008; final revision received January 8, 2009; accepted January 21, 2009.

Correspondence: Thomas S. Cunneen, MBBS, MM(OphthSc), Save Sight Institute, GPO Box 4337, Sydney, New South Wales, Australia 2001 (tomcunneen@hotmail.com).

Author Contributions: Dr Cunneen, the principal investigator, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Disclosure: None reported.

Additional Contributions: Dr Gerasimos Anastassiou and Dr Gordon Nareyecek (Department of Ophthalmology, University of Duisburg-Essen, Essen, Germany) developed and supplied the CRMM-1 and CRMM-2 cell lines.

Figure 4. Representative examples of cell-cycle analysis in CRMM-1 conjunctival melanoma cells (Department of Ophthalmology, University of Duisburg-Essen, Essen, Germany) following treatment with sodium butyrate at 24 (A) and 48 (B) hours. The CRMM-2 cells displayed similar responses to sodium butyrate and trichostatin A (not shown). cv indicates peak width; FL2-A, fluorescence channel 2; RMS, root-mean-square; S, S (cells in) phase.

REFERENCES
