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Short communication

HDAC inhibitor M344 suppresses MCF-7 breast cancer cell proliferation

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ABSTRACT

Histone deacetylase (HDAC) inhibitors represent a novel class of drugs that selectively induce cell cycle arrest and apoptosis in transformed cells. This study examined, for the first time, the effects of the relatively new HDAC inhibitor, M344 [4-dimethylamino-N-(6-hydroxycarbamoylhexyl)-benzamide], on the proliferation of MCF-7 breast cancer cells. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays revealed significant concentration- and time-dependent decreases in MCF-7 cell proliferation following treatment with M344 (1-100 μM). In contrast to the significant induction of $p21^{waf1/cip1}$ mRNA expression following treatment with M344 (10 μM) for 1 or 3 days, there was a significant decrease in p53 mRNA expression, although p53 protein levels were unchanged. Similar treatment with M344 also induced expression of the pro-apoptotic genes, Puma and Bax, together with the morphological features of apoptosis, in MCF-7 cells. The results of this study reinforce previous findings indicating that HDAC inhibitors are an important group of oncostatic drugs, and show that M344 is a potent suppressor of breast cancer cell proliferation.

1. Introduction

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are essential enzymes involved in the regulation of gene expression. HATs are responsible for the post-translational addition of acetyl moieties to the ε-amino group of lysine residues in histone proteins. Acetylation interferes with the positive charge of lysine residues that allow tight binding with the negatively charged DNA, thereby promoting gene transcription. Conversely, HDACs reverse this modification and act as transcription repressors [1–3]. HDAC inhibitors are a novel class of anti-cancer agents that have been shown to selectively inhibit cancer cell growth, by altering gene transcription with consequent changes in the expression of oncogenes and/or tumour suppressor genes [3]. Possible mechanisms by which HDAC inhibitors inhibit cancer progression include cell cycle arrest at the G_1 or G_2/M phases of the cycle, induction of differentiation and/or apoptosis [4–6].

A range of antiproliferative potencies have been reported for various HDAC inhibitors in both in vitro and in vivo studies [7]. For example, suberoylanilide hydroxamic acid and valproic acid, inhibit human MCF-7 breast cancer cell proliferation in micromolar and millimolar concentrations, respectively [8,9]. This study examined the effects of a relatively novel HDAC inhibitor, M344 [4-dimethylamino-N-(6-hydroxycarbamoylhexyl)-benzamide], an amide analog of trichostatin A [10], on this cell line. This drug has shown promise as an anti-cancer agent in other cell lines, including endometrial and ovarian cell lines [11], as well as a number of medulloblastoma, neuroblastoma and rhabdoid tumour cell lines [12]. In addition, M344 was shown to increase the response to radiation in SQ-20B and SCC-35 human squamous carcinoma lines, which were otherwise resistant to radiation [13], suggesting its potential in combination cancer therapies. Therefore, it was of interest to examine the effects of M344 on human breast cancer cell proliferation for the first time.

2. Materials and methods

2.1. Cell culture

Human MCF-7 breast cancer cells were grown on 10-cm Corning culture dishes at 37 °C in a 5% CO_2 incubator. They were incubated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen Canada Inc., Burlington, ON), and supplemented with penicillin/streptomycin (100 IU/ml/100 μg/ml) and fungizone (1.25 μg/ml). In order to examine the effects of M344 on gene expression, cells were subcultured in DMEM/1% FBS for 24 h and then treated with vehicle or M344 for indicated periods.

2.2. M344 treatment and MTT Assay

After hemocytometer counting, cells were seeded in quadruplet on a 96-well plate at about 2500 cells for 1 day and 3-day

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treatments, 1500 cells for 5-day treatments, and 1000 cells for 7-day treatments. Cells were incubated at 37 °C in 200 μl of culture medium, allowed to attach overnight and then treated with M344 (Tocris Bioscience, Ellisville, Missouri) in doses ranging from 1 μM to 100 μM for 1–7 days. For 5-day experiments, cells were retreated after day 3; for 7-day experiments, cells were retreated after day 3 and day 5. Following the treatment period, 20 μl of a 5 mg/ml solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Oakville, ON] in phosphate-buffered saline (PBS) was added to each well. After 2 hours of incubation, medium and MTT were removed and cells were lysed using 100 μl of a 20% SDS/50% formamide solution. Plates were left to incubate overnight and read the following day using a 595 nm filter in a Titertek Multiskan Plus microplate reader.

2.3. Reverse transcription PCR

MCF-7 cells were treated with vehicle (0.1% DMSO) or M344 (10 μM) for 1day or 3 days. Total RNA was extracted using Trizol, as described by the supplier (Invitrogen Canada Inc.). Following DNase treatment, cDNA was synthesized from 2.2 μl of total RNA using the Omniscript reverse transcriptase kit (Qiagen Inc., Mississauga, Ontario, Canada) and oligo dT primers. Changes in the mRNA expression of p53, p21, Puma and Bax, were assessed by amplification using 2 μl of the RT product and the following primers: p53 – forward: 5′-cctcaccatcatcacactgg-3′; reverse: 5′-tctgagtcaggcccttctgt-3′; p21-forward: 5′-gacac-cactggagggtgact-3′; reverse: 5′-ggattagggcttcctcttgg-3′; Puma-forward: 5′-gcccagactgtgaatcctgt-3′; reverse: 5′-tcctccctcttccga-gattt-3′; Bax-forward: 5′-tttgcttcagggtttcatcc-3′; reverse: 5′-cagttgaagttgccgtcaga-3′. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control with forward: 5′-ttcaccaccatggagaaggc-3′ and reverse: 5′-ggcatggactgtggtcatga-3′ primers.

PCR parameters were as follows; 94 °C for 30 s, 57 °C (p53 and p21) or 55 °C (Bax, Puma and GAPDH) for 30 s, 72 °C for 1 min for 27 cycles. All PCR reactions included a 15-min preincubation at 95 °C and a final extension step at 72 °C for 10 min. PCR products were run on 1.5% agarose gels stained with ethidium bromide and visualized under UV light. Optical density (OD) data were used for semiquantitation of mRNA expression.

2.4. Western blotting

After treatment of MCF-7 cells with M344 (10 μM) or vehicle (0.1% DMSO) for 24 h, proteins were extracted in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100), supplemented with phenylmethylsulphonyl fluoride (PMSF; 1 mM), leupeptin (1 μg/ml), sodium orthovanadate (1 mM) and a protease inhibitor cocktail (Roche Diagnostics Canada, Laval, Quebec). Proteins (30 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted overnight at 4 °C. Membranes were incubated with a 1:1000 dilution of anti-human p53 antibody (Cell Signaling Tech., Danvers, MA), overnight at 4 °C. After subsequent incubation with a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 h, proteins were detected by enhanced chemiluminescent (ECL)-autoradiography. For internal controls, blots were stripped and reprobed with a 1:2000 dilution of monoclonal anti-mouse β-actin antibody (Sigma-Aldrich, Oakville, ON) and then incubated with a 1:10,000 dilution of HRP-conjugated anti-mouse secondary antibody (Sigma-Aldrich, Oakville, ON).

2.5. Hoechst staining

In order to detect possible drug-induced apoptotic changes, MCF-7 cells (100,000) were seeded overnight on a 24-well plate in DMEM/10% FBS, and then treated with M344 (10 μM) or vehicle (0.1% DMSO) for 24 h. Cells were fixed in a 1% paraformaldehyde solution and washed with PBS, followed by staining with 0.2 mM of the DNA dye Hoechst 33258, for15 min. Nuclear morphological changes were observed under a fluorescence microscope.

2.6. DNA fragmentation

Following treatment of MCF-7 cells with M344 (10 μM) or vehicle (0.1% DMS), DNA was extracted as described by Laird et al. [14]. Cells were incubated in 3.7 ml lysis buffer (100 mM Tris–HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) for 2 h at 37 °C. An equal volume of isopropanol was added to the lysate and samples were swirled until DNA precipitation was complete. Isolated DNA was dissolved by overnight incubation at 37 °C, in 100–300 μl of 10 mM Tris–HCl, 0.1 mm EDTA, pH 7.5. DNA samples were run on an agarose (1% w/v) gel stained with ethidium bromide and visualized under UV light.

3. Results

3.1. Effects of M344 on MCF-7 cell growth

M344 caused significant concentration-dependent decreases in MCF-7 cell proliferation at all times examined, as shown on Fig. 1. Two-way ANOVA revealed a significant treatment × time interaction ($P < 0.0001$), along with significant treatment ($P < 0.0001$) and time ($P < 0.0001$) effects. Bonferroni posthoc analysis indicated that treatment of MCF-7 cells with M344 for 1 day caused a significant inhibition at 50 μM ($P < 0.01$), whereas treatment for 3 days showed significant inhibition at 10 μM, 50 μM and 100 μM, with a maximal inhibition of 40% at 100 μM ($P < 0.001$). After 5 days, all concentrations of M344 (1 μM, 10 μM, 50 μM and 100 μM) caused a significant suppression of MCF-7 cell growth, with a maximal inhibition of 60% observed at 10 μM ($P < 0.001$). Treatment for 7 days also showed significant inhibition at all doses of M344 examined, with a maximal inhibition of 65% at 10 μM ($P < 0.0001$).

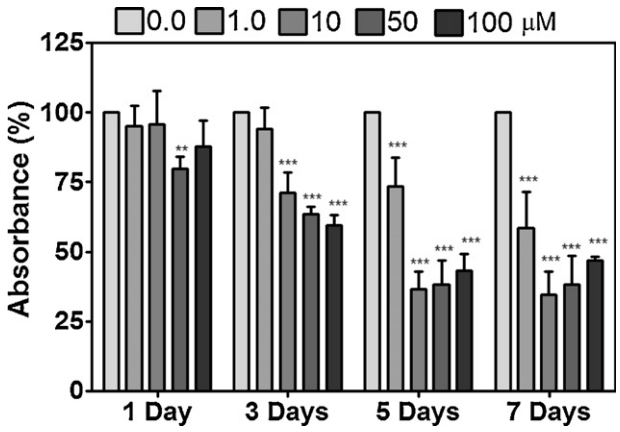

Fig. 1. Concentration- and time-dependent inhibition of MCF-7 cell proliferation by M344. Cells were treated with M344 (1.0, 10, 50 and 100 μM) or vehicle (0.1% DMSO) for 1, 3, 5 or 7 days, as shown. Two-way ANOVA indicated a significant treatment × time interaction ($P < 0.0001$) as described under Results. Bonferroni posthoc analysis revealed significant differences between controls and treated cells: $^{**}P < 0.01$, $^{**}P < 0.001$ versus control.