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The Histone Deacetylase Inhibitor MGCD0103 Has Both Deacetylase and Microtubule Inhibitory Activity[S]

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ABSTRACT

Histone deacetylase inhibitors (HDACis) are currently in trial or are in clinical use for the treatment of a number of tumor types. The clinical efficacy of HDACis can be partly attributed to the modulation of the cell cycle by the HDACis. Here, we have examined the effects of *N*-(2-aminophenyl)-4-((4-pyridin-3-ylpyrimidin-2-ylamino)methyl)benzamide (MGCD0103), a class I-selective histone deacetylase inhibitor, on the cell cycle and cell killing. Surprisingly, MGCD0103 treatment failed to initiate a G_1-phase arrest but caused marked accumulation of cells in G_2/M at 6 and 12 h after treatment and was cytotoxic 24 h after treatment. These cell cycle effects were considerably distinct from the effects of suberic bishydroxamic acid, a representative of the pan-isoform HDACi used in this study. MGCD0103 shared the ability of the pan-isoform HDACi to trigger defective mitosis and promote mitotic slippage. Likewise, it also specifically targeted tumor cells and was nontoxic to normal nontransformed cells. However, MGDC0103 also seemed to disrupt normal microtubule spindle formation, whereas HDACis generally have only a minor effect on spindle formation. The effect of MGCD0103 on spindle formation was shown to be a consequence of microtubule destabilization. This is the first example of an HDACi with microtubule destabilizing activity, and the combined effects of this drug have advantages for its therapeutic use.

Introduction

Histone deacetylase inhibitors (HDACis) are an emerging class of anticancer drugs that possess tumor-selective cytotoxicity. HDACis have been demonstrated to inhibit the growth of tumor cells and induce them to undergo apoptosis or differentiation in vitro. They have also been shown to have antiangiogenic properties (Bolden et al., 2006). These drugs are currently in clinical trials, either as a monotherapy or in combination with other anticancer agents. The recent approval of suberoylanilide hydroxamic acid (vorinostat), for the treatment of T-cell lymphoma highlights the potential of these drugs as promising anticancer therapeutics.

The majority of HDACi are pan-isoform inhibitors of two classes of HDACs: class I HDACs, which include HDACs 1 to 3 and HDAC8; and class II HDACs, which include HDACs 4 to 7, HDAC9, and HDAC10. HDACs have been shown to regulate the acetylation state of nuclear histones and an increasing number of nonhistone proteins. Inhibition of HDACs leads to changes in the expression of genes involved in the regulation of apoptosis, proliferation, and the cell cycle. Although changes in gene expression in HDACi-treated cells are dependent on the cell lines tested and the class of HDACis used, $p21^{Waf1}$ is one gene that is commonly up-regulated (Burgess et al., 2001; Glaser et al., 2003; Peart et al., 2005).

Several lines of evidence suggest that the cytotoxicity of HDACi may be due primarily to the inhibition of class I HDACs. Class I HDACs are aberrantly overexpressed in various tumors: HDAC1 in gastric cancer, HDAC2 in colorectal cancer, and HDAC3 in colon cancer (Zhu et al., 2004; Ozdag et al., 2006; Wilson et al., 2006; Glozak and Seto, 2007). Depletion and knockout of individual HDACs has uncovered the unique biological roles of the individual HDACs. HDAC1 and HDAC3 seem to be involved in regulating proliferation (Wilson et al., 2006; Bhaskara et al., 2008), whereas HDAC2 seems to regulate apoptosis (Senese et al., 2007; Weichert et al., 2008). Class II HDACs seem not to regulate cell proliferation and are primarily involved in cellular development and differentiation (Verdin et al., 2003). In addition, *N*-(2-aminophenyl)-

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ABBREVIATIONS: HDACis, histone deacetylase inhibitors; HDAC, histone deacetylase; SBHA, suberic bishydroxamic acid; MGCD0103, *N*-(2-aminophenyl)-4-((4-pyridin-3-ylpyrimidin-2-ylamino)methyl)benzamide; MS-275, *N*-(2-aminophenyl)-4-[*N*-(pyridine-3-ylmethoxy-carbonyl) aminomethyl]benzamide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; PARP, poly-(ADP-ribose) polymerase; NFF, neonatal foreskin fibroblast; siRNA, small interfering RNA.

4-[*N*-(pyridine-3-ylmethoxy-carbonyl)aminomethyl]benzamide (MS-275), a reported class I HDAC-specific inhibitor, triggers effects reminiscent of those achieved by pan-isoform HDACi, including cell cycle effects, $p21^{Waf1}$ up-regulation, and initiation of apoptosis.

The cell autonomous antiproliferative activity of HDACi results from a combination of inhibiting cell cycle progression and promoting cell death (Bernhard et al., 2001; Peart et al., 2003). However, HDACi can also promote cell death in arrested cells, although the drugs require a longer exposure to achieve the same effects (Burgess et al., 2004). HDACis produce a number of cell cycle effects, including G_1/S-phase arrest, which is correlated with increased $p21^{Waf1}$ expression and is observed in a wide range of cell lines with all HDACis reported to date (Archer et al., 1998; Burgess et al., 2001); a G_2-phase checkpoint arrest observed mainly in normal nontransformed cell lines (Qiu et al., 2000); and mitotic defects, including mitotic arrest and mitotic slippage, was also observed in most cell lines in response to a broad range of HDACis (Warrener et al., 2003; Stevens et al., 2008). The HDACi-sensitive G_2-phase checkpoint seems to be the basis for the tumor-selective cytotoxicity of these drugs, with the majority of tumor cell lines being sensitive because of their defective checkpoint, whereas normal tissues are protected by their intact G_2 phase checkpoint response (Qiu et al., 2000; Warrener et al., 2003; Krauer et al., 2004). Progression through the HDACi-induced aberrant mitosis induces rapid cell death (Blagosklonny et al., 2002; Warrener et al., 2003; Dowling et al., 2005), and this is a significant contributor to the cytotoxicity of these drugs.

The cell cycle effects of HDACi significantly contribute to the selective cytotoxicity of these drugs. Here, we have investigated the cell cycle effects of a novel class I-specific histone deacetylase inhibitor, *N*-(2-aminophenyl)-4-((4-pyridin-3-ylpyrimidin-2-ylamino)methyl)benzamide (MGCD0103) (Fournel et al., 2008), and compared it with an equipotent dose of a representative pan-isoform HDACi suberic bishydroxamic acid (SBHA) on a number of tumor cell lines in vitro. Similar to SBHA, we found that MGCD0103 promotes tumor cell-selective cytotoxicity. It also triggered mitotic failure, characterized by a delay in mitosis and subsequent mitotic slippage. However, in contrast to SBHA treatment, there was no $p21^{Waf1}$ induction until at least 12 h after MGCD0103 treatment or G_1-phase arrest, which contributes to the increased cytotoxicity of this drug. MGCD0103 also possesses a novel activity, it destabilizes microtubules in both interphase and mitosis, and this activity underlies the improved cytotoxicity of this drug.

Materials and Methods

Materials. MGCD0103 and 001 (inactive analog of MGCD0103) were kindly provided by MethylGene Inc (Montreal, QC, Canada). SBHA was purchased from Sigma-Aldrich (St. Louis, MO). All compounds were dissolved in dimethyl sulfoxide (DMSO) with MGCD0103 and 001 dissolved as 10 mM stock solutions and SBHA dissolved as 500 mM solution.

Cell Culture, Synchrony, and Drug Treatment. All cells were cultured in a humidified incubator at 37°C with 5% CO_2. HeLa cells, HeLa cells overexpressing Bcl-2 (HeLa-Bcl2), and neonatal foreskin fibroblast (NFF) were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) Serum Supreme (Lonza Walkersville, Inc., Walkersville, MD). A2058, A02, and MM604 melanoma cell lines were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) Serum Supreme. HDACis were added to either asynchronous cells or cells synchronized by simple thymidine block (2 mM for 17 h) in early S phase or late G_2 phase (7-h release from thymidine) and harvested at the indicated time points. For early S-phase addition, HDACis were added immediately after release, whereas for G_2-phase addition, HDACis were added 7 h after release. For the mitotic shake-off experiments, HDACis were added in early S phase, and 0.25 μg/ml nocodazole was added 7 h after release. Mitotic cells were collected by mechanical shake-off 3 h later. In parallel experiments, HDACis were added in combination with the microtubule-disrupting agents paclitaxel (Taxol; Bristol-Meyers Squibb Co., Stamford, CT) (100 nM) or nocodazole (0.25 μg/ml) to asynchronous cell populations for 24 h. $p21^{Waf1}$ knockdown was achieved by transfection with 5 nM p21 antisense oligonucleotides (Ambion, Austin, TX; and Dharmacon Smart Pool; Dharmacon RNA Technologies, Lafayette, CO) using Lipofectamine LF2000 transfection reagent (Invitrogen), according to the manufacturer's recommendations. Silencer Select Negative Control siRNA 1 (Ambion) was used as scrambled siRNA.

Flow Cytometry. For flow cytometric analysis, both floating and attached cells were harvested. Cells were fixed in ice-cold 70% ethanol and then stained with 4 μg/ml propidium iodide (Sigma-Aldrich) and 400 μg/ml RNase A (Invitrogen) in PBS. The stained cells were subsequently filtered through a 37-μm gauze and analyzed on a FACSCalibur using CellQuestPro (both from BD Biosciences, San Jose, CA). The different subpopulations were quantified using ModFit LT program (Verity Software House, Topsham, ME). Experiments were performed in triplicate, and the results are presented as the mean and S.D. *P* values were calculated by two-tailed *t* test.

Immunoblotting. Cell pellets were lysed in 1× SDS lysis buffer (0.4% SDS, 2% glycerol, 2.5 mM Tris, pH 6.7 and 0.3 M 2-mercaptoethanol), and total cell lysates were quantified using RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Samples (20–40 μg) were resolved by 10 or 12% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to Hybond-C Extra Membrane (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Membranes were immunoblotted with primary antibodies against histone H3, acetylated H3 (Lys9), acetyl lysine, phospho-B23, phosho-H3 (Ser10), PARP (Cell Signaling Technology, Danvers, CA), $p21^{Waf1}$ (Calbiochem, San Diego, CA), α-tubulin (Sigma-Aldrich), BubR1 (Abcam Inc., Cambridge, MA), centromere protein A, phospho-centromere protein A (Millipore Corporation, Billerica, MA), cyclin E, and cyclin A (Santa Cruz Biotechnology, Santa Cruz, CA). These were detected with anti-mouse or -rabbit horseradish peroxidase-conjugated secondary antibodies (Zymed Laboratories, South San Francisco, CA) and Western Lightning Plus-ECL reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA). Quantification of protein bands was carried out using the Bio-Profile Bio1D software (Vilber Lourmat, Marne-la-Vallée, France).

Immunofluorescent Staining. Cells grown on poly(L-lysine)-coated coverslips were fixed in ice-cold methanol overnight at −20°C. Where cells were permeabilized before fixation, cells were washed with 50 μg/ml digitonin, 130 mM sucrose, 50 mM KCl, 50 mM Na acetate, 20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, and 2 mM EGTA for 90 s before washing twice with PBS and fixing with −20°C methanol. Coverslips were washed twice in PBS and then incubated in cell-blocking buffer [30 μg/ml bovine serum albumin (Sigma Aldrich) in PBS with 0.05% Tween 20] for 30 min at room temperature before immunostaining with anti α-tubulin antibody for 1 h at room temperature. Coverslips were washed twice in PBS and immunostained with an fluorescein isothiocyanate-conjugated secondary antibody for 30 min at room temperature. DNA was counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich). Coverslips were mounted onto microscopic glass slides with ProLong Gold antifade reagent (Invitrogen). Fluorescent mi-

croscopy was carried out with Axioskop 2 Plus (Carl Zeiss Inc., Thornwood, NY).

Time-Lapse Microscopy. Synchronized HeLa-Bcl2 cells and neonatal foreskin fibroblasts treated with HDCAi were followed by time-lapse microscopy using a Zeiss Live Cell Observer in a 37°C incubator and 5% CO_2 hood. Images were captured every 15 min for 20 h.

Results

MGCD0103 Induces a Delay Increase in p21^{Waf1} and Fails to Induce G_1-Phase Arrest. The effects of MGCD0103 on the cell cycle and on cell killing were compared with the representative pan-HDAC inhibitor SBHA, which we have demonstrated previously to have cell cycle effects similar to those of other HDACis, including suberoylanilide hydroxamic acid, trichostatin A, and sodium butyrate (Warrener et al., 2003; Stevens et al., 2008). The potency of MGCD0103 and SBHA in promoting histone acetylation and apoptosis was determined using the level of histone H3 Lys9 acetylation and caspase 3 and 7 activation, respectively. MGCD0103 at 10 μM and SBHA at 500 μM produced maximal H3 Lys9 acetylation and activated caspase 3/7 to a similar extent (Fig. 1, A and B) and were used as equipotent doses throughout this work. These doses of the drugs also induced H3 Lys9 acetylation with very similar kinetics, although only SBHA increased α-tubulin acetylation, indicating that it also inhibited the class II HDAC6 (Fig. 1C).

Using the equipotent concentrations of MGCD0103 (10 μM) and SBHA (500 μM), which induced maximal histone acetylation (Fig. 1A), the effects on cell cycle were analyzed. MGCD0103 produced two immediately noticeable differences from SBHA treatment. There was a striking accumulation of cells with 4N DNA content from 6 h after treatment, which continued to increase to 24 h in the MGCD0103-treated cells, whereas in the SBHA-treated cells there was a loss of the S-phase population and accumulation of cells in G_1 phase (Fig. 2, A and B). Analysis of cell cycle proteins reported to be regulated by HDACi treatment revealed striking difference between the effects of MGCD0103 and SBHA. Surprisingly, MGCD0103 did not increase p21^{Waf1} expression until 24 h of treatment, whereas all HDACis, exemplified by SBHA, rapidly induced its expression (Fig. 2C). There was also a lack of both increased cyclin E expression and down-regulation of cyclin A that are normally associated with HDACi treatment (as observed with SBHA, Fig. 2C). The marked accumulation of phosphorylated B23 (pB23), a marker of mitosis, at 6 and 12 h after treatment only in the MGCD0103-treated cells (Fig. 2C), indicated that the 4N DNA peak observed corresponded to cells accumulating in mitosis at 6 and 12 h after MGCD0103 treatment (Fig. 2A). It is noteworthy that the 4N peak persisted to 24 h, whereas the pB23 signal was lost at this time. Both HDACis also induced PARP cleavage, a marker of caspase 3/7

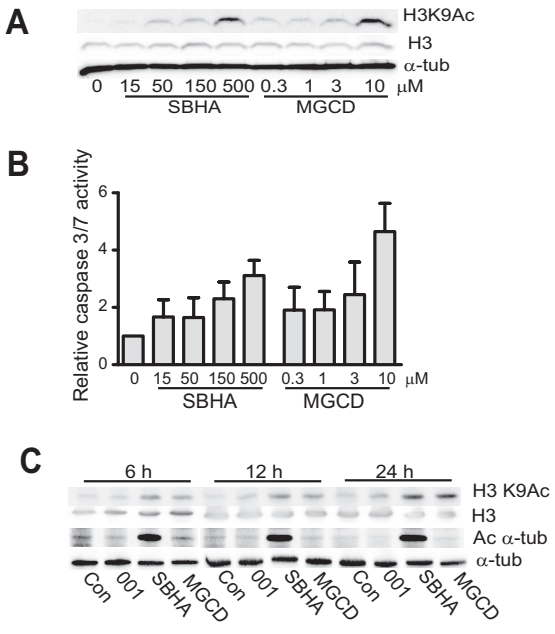

Fig. 1. Defining equipotent concentrations for MGCD0103 and SBHA. A, asynchronously growing HeLa cells were treated with the indicated dose of either SBHA or MGCD0103 for 8 h. Cell lysates were immunoblotted for acetylated H3 K9 (H3K9Ac), total H3, and α-tubulin (α-tub) as loading controls. B, cells from an experiment similar to that in A were assayed for caspase 3/7 activity. The data represent the mean from triplicate experiments. C, asynchronously growing HeLa cells were treated with either 10 μM MGCD0103 or inactive analog 001, 500 μM SBHA, or an equal volume of DMSO as control (Con), for the indicated times. Cells were lysed and analyzed for the level of histone H3 Lys9 acetylation (H3K9Ac), total H3 protein, or acetylated α-tubulin (Ac α-tub).

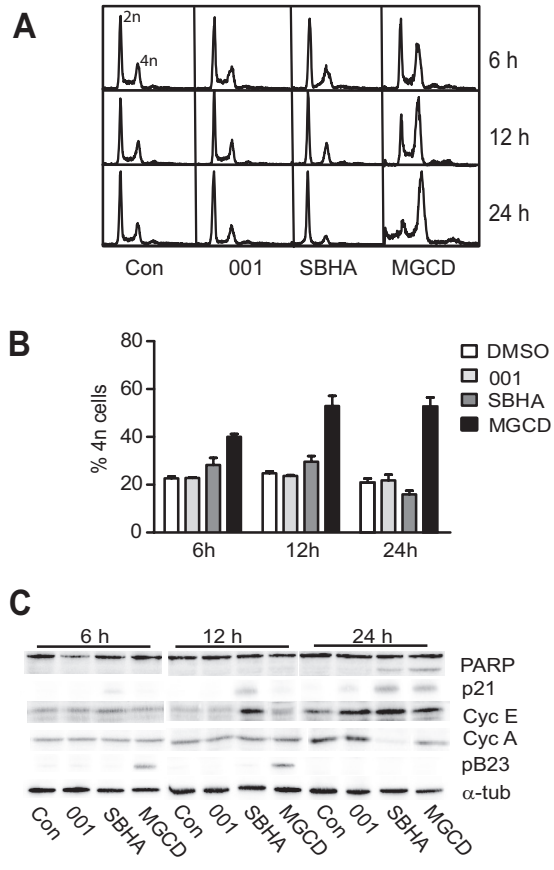

Fig. 2. MGCD0103 induces mitotic accumulation and delayed p21 expression. A, asynchronously growing HeLa cells were treated with either 10 μM MGCD0103 or inactive analog 001, 500 μM SBHA, or an equal volume of DMSO as control for the indicated times. Cells were analyzed by FACS for their DNA content. B, quantitation of the 4N populations from at least three replicate experiments identical with that shown in B. open bars, control DMSO; light gray bars, 001; dark gray bars, SBHA; filled bars, MGCD0103. C, cells from the same experiment were lysed and immunoblotted for PARP cleavage as a marker of caspase 3 activation and apoptosis, p21^{Waf1} (p21), cyclin E (Cyc E), cyclin A (Cyc A), phospho-B23 (pB23), and α-tubulin (α-tub) as a loading control.

activation and apoptosis, to a similar extent at 24 h, although only the MGCD0103-treated cells had a readily detectible subdiploid population at 24 h. An inactive analog of MGCD0103, 001, had no effect on either histone H3 or α-tubulin acetylation. These effects of MGCD0103 on both $p21^{Waf1}$ expression and accumulation in mitosis compared with SBHA were also observed in four other cell lines tested (Supplementary Fig. S1 and data not shown).

The loss of the S-phase population and G_1-phase arrest observed with the majority of HDACis, including SBHA, was demonstrated to be a consequence of HDACi-induced $p21^{Waf1}$ expression. siRNA-mediated depletion of the induced $p21^{Waf1}$ completely overcame the loss of S phase and correspondingly increased G_1-phase populations after SBHA treatment (Fig. 3A). To test whether increased p21 expression could reduce the 4N accumulation and cell death induced by MGCD0103 at 24 h, cells were pretreated with a relatively nontoxic dose of SBHA (100 μM) for 12 h before treatment with 10 μM MGCD0103 for a further 24 h. Treatment with the low dose of SBHA alone induced strong $p21^{Waf1}$ expression and G_1-phase arrest, with a corresponding decrease in S phase, but little cell death as determined by either subdiploid cells or PARP cleavage (Fig. 3B). SBHA pretreatment imposed a G_1-phase arrest in the subsequently MGCD0103-treated cells, and decreased the 4N accumulation and markers of cell death. Thus, the lack of increased $p21^{Waf1}$ expression in the MGCD0103-treated cells underlies the lack of effect on G_1 or S phase compartments with MGCD0103 treatment. The absence of G_1-phase arrest alone cannot be responsible for the 4N accumulation observed with MGCD0103 treatment, because there was no 4N accumulation in SBHA-treated, $p21^{Waf1}$-depleted cells (Fig. 3A).

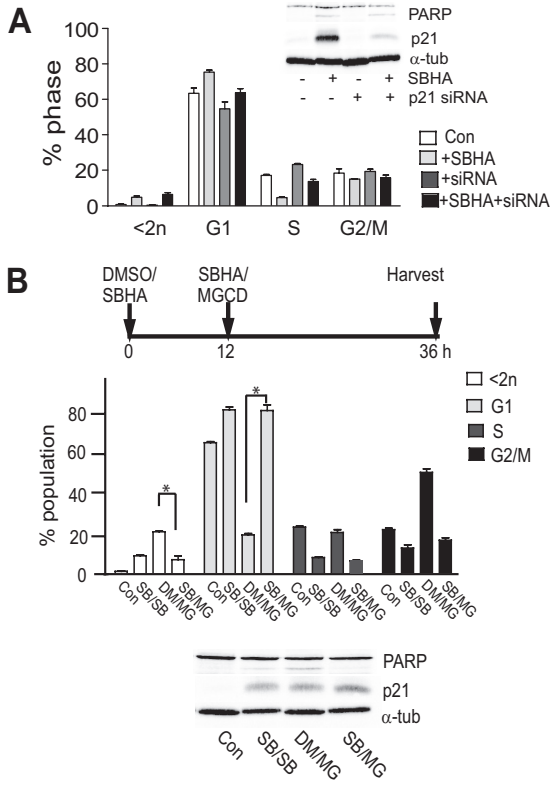

Fig. 3. Increased p21 expression is responsible for SBHA induced G_1-phase arrest. A, HeLa cells were transfected with either a scrambled (−) or p21-directed siRNA and then treated with or without 100 μg/ml SBHA for 24 h. Cells were analyzed for either DNA content by FACS, or immunoblotted for $p21^{Waf1}$, PARP as marker of apoptosis, and α-tubulin as a loading control (inset). The FACS data are the average and S.D. of triplicate determinations. Similar data were obtained with a different p21 siRNA and in three other cell lines. Open bars, control; light gray bars, +SBHA; dark gray bars, +siRNA; black bars, +SBHA+siRNA. B, HeLa cells were treated for 12 h with either 20 μg/ml SBHA or equal volume of DMSO as a control and then treated with either 20 μg/ml SBHA or 10 μM MGCD0103 for a further 24 h as shown in the scheme. DMSO only (Con), SBHA only (SB/SB) or MGCD0103 (DM/MG), and SBHA for 12 h and then MGCD0103 (SB/MG). Cells were analyzed by FACS for DNA content and immunoblotted for PARP as a marker of apoptosis, $p21^{Waf1}$ and α-tubulin as a loading control. The asterisk indicates P values <0.0001.

MGCD0103 Induces Mitotic Defects and Mitotic Slippage. To examine the effects of drug treatment on entry into and progression through mitosis without the complication of cell death, HeLa cells overexpressing Bcl-2 (HeLa-Bcl2) that are relatively refractory to HDACi-induced apoptosis were used (Supplementary Fig. S2) (Warrener et al., 2003). When MGCD0103 was added to thymidine block release-synchronized HeLa-Bcl2 cells and followed using time-lapse microscopy, it delayed entry into mitosis by <2 h, a little longer than SBHA treatment (Supplementary Fig. S3). MGCD0103-treated cells also delayed in mitosis for an average of 402 min compared with an average of 49 min for the inactive control, similar to the effect of SBHA, which delayed cells in mitosis for 304 min (Fig. 4A). Examination of the cells revealed more than 90% of MGCD0103-treated cells arrested in a prophase-like state, with failure of the chromosomes to congress, similar to other HDACis (Fig. 4B) (Qiu et al., 2000; Warrener et al., 2003; Stevens et al., 2008). There was also a surprising disruption of the normal mitotic spindle structure with MGCD0103 treatment, in which <90% of MGCD0103-treated cells failed to form a clearly discernible spindle structure, although strong microtubule foci were observed in the mitotic cells (Fig. 4B). This was not normally observed with HDACis, with the most common spindle defect reported being minor secondary spindle pole formation in 30% of mitotic cells (Stevens et al., 2008).

The loss of the mitotic marker pB23 by 24 h (Fig. 2) suggested that MGCD0103 shares the ability to promote mitotic slippage in common with other HDACis (Dowling et al., 2005; Stevens et al., 2008). This was demonstrated clearly by the ability of MGCD0103 treatment to overcome the spindle assembly checkpoint imposed by nocodazole treatment (Fig. 4A). Similar to SBHA treatment, MGCD0103-treated cells delayed in mitosis for a similar time with and without nocodazole treatment (302 and 327 min, 497 and 402 min, respectively), and this arrest in mitosis was much shorter than in the presence of nocodazole alone (822 min). A surprising feature of MGCD0103 treatment was that the addition of the drug to cells in G_2 phase resulted in these cells arresting in mitosis for an extended period (533 min; Fig. 4A), whereas SBHA was shown previously not to affect mitotic progression when added in G_2 phase (Warrener et al., 2003).

The failure to maintain the spindle assembly checkpoint has been ascribed to the lack of activation of BubR1 in HDACi-treated mitotic cells (Shin et al., 2003; Stevens et al., 2008). MGCD0103-treated mitotic cells similarly had little of the slower migrating, activated BubR1 compared with nocodazole-arrested cells in which BubR1 was exclusively in the activated, lower mobility form, despite both nocodazole-

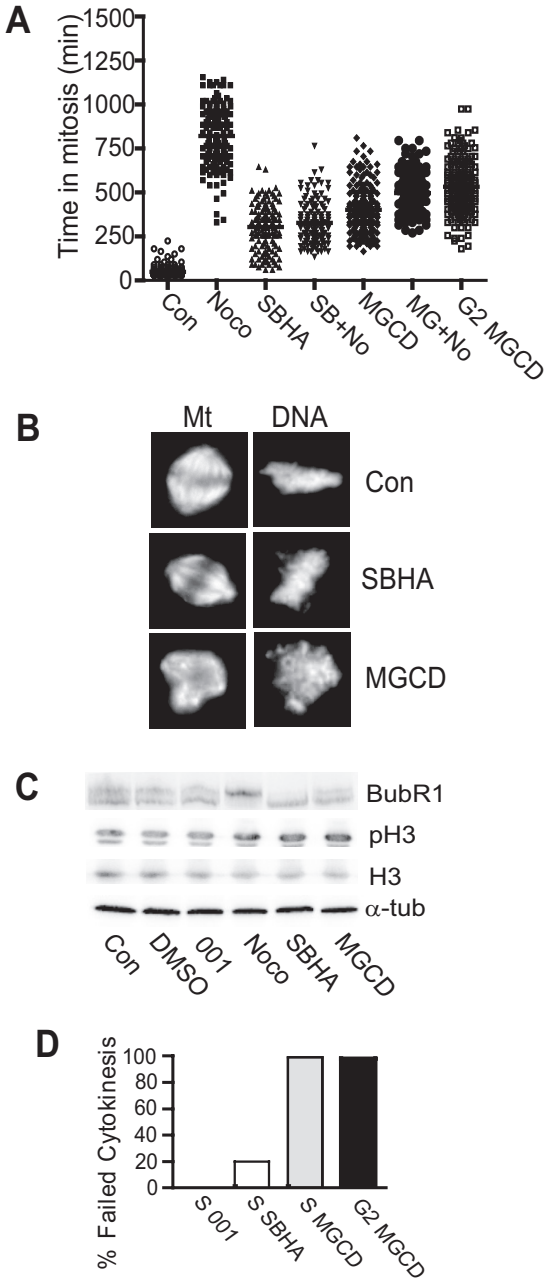

Fig. 4. MGCD0103 disrupts normal spindle checkpoint function. A, synchronized HeLa cells were treated in either early S phase with 500 μM SBHA or 10 μM MGCD0103 or an equal volume of DMSO as a control (Con), with and without the addition of nocodazole (Noco) 7 h after release from the synchrony arrest as they progressed through G_2 phase into mitosis. Cells were followed by time-lapse microscopy, and the time that cells stayed in mitosis was measured. In each case, >100 cells were analyzed. MGCD0103 (0 μM) was also added at 7 h after release from the synchrony arrest when they had entered G_2 phase (G2 MGCD). B, synchronized HeLa cells were treated in G_2 phase with 10 μM MGCD0103 or 500 μM SBHA and then allowed to enter into mitosis and fixed and stained with anti-α-tubulin for microtubules (Mt) and counterstained with 4,6-diamidino-2-phenylindole for DNA. The images are representative of the mitotic cells observed after these treatments. C, mitotic cells were collected by mechanical shake-off from synchronized population treated with either SBHA or MGCD0103 in early S phase or nocodazole in late G_2 phase and indicated controls. Cell lysates were immunoblotted for BubR1, phosphorylated H3 Ser10 (pH3), total histone H3 protein, and α-tubulin as a loading control. D, synchronized HeLa cells were treated in either S or G_2 phase with SBHA or MGCD0103 as in A and followed by time-lapse microscopy. Cells were analyzed for their ability to form two daughter cells (cytokinesis) or resume an interphase phenotype within a single cytoplasm (failed cytokinesis). More than 100 cells were analyzed for each treatment.

and MGCD0103-treated populations containing >90% aberrant mitotic cells (Fig. 4C). There was more activated BubR1 in the MGCD0103 than SBHA-treated cells, in line with the longer delay in mitosis of the MGCD0103-treated cells (Fig. 4, A and C). Inhibition of the class I HDAC3 has been reported to reduce the ability of Aurora B to phosphorylate histone H3 Ser10 in mitosis (Li et al., 2006). However, MGCD0103 treatment did not affect histone H3 Ser10 phosphorylation (Fig. 4C) or the pericentric CenpA phosphorylation, which is also catalyzed by Aurora B (Supplementary Fig. S4). Thus, MGCD0103 does initiate a mitotic checkpoint arrest but prematurely exits, similar to other HDACis, although it disrupts the normal mitotic spindle formation in a manner unique to this drug.

It is noteworthy that the effect on cytokinesis was another feature that discriminated between MGCD0103 and SBHA treatment. When HDACi-treated cells exited mitosis by mitotic slippage, a high proportion of cells that did not die underwent an aberrant form of cytokinesis (Stevens et al., 2008). With MGCD0103 treatment, all cells failed cytokinesis, resulting in cells that have exited mitosis with 4N DNA content, irrespective of whether the drug was added in S phase or G_2 phase (Fig. 4D). In contrast, SBHA does not affect mitosis when added in G_2 phase (Warrener et al., 2003). The presence of this very high proportion of failed cytokinesis accounts for the 4N population with no pB23 24 h after MGCD0103 treatment.

MGCD0103 Induces a G_2-Phase Arrest in Normal Cells. The mitotic effects described above were only observed previously in HDACi-treated tumor and immortalized cell lines that had a defect in an HDACi-sensitive G_2-phase checkpoint. Normal cells competent for the checkpoint were relatively insensitive to HDACi-induced cytotoxicity (Qiu et al., 2000). To determine whether MGCD0103 triggered the G_2 checkpoint in normal cells, asynchronous cultures of primary NFFs, characterized previously to have an intact G_2 checkpoint response, were treated with either SBHA or MGCD0103 and analyzed by FACS and time lapse microscopy. FACS revealed the expected 4N accumulation with

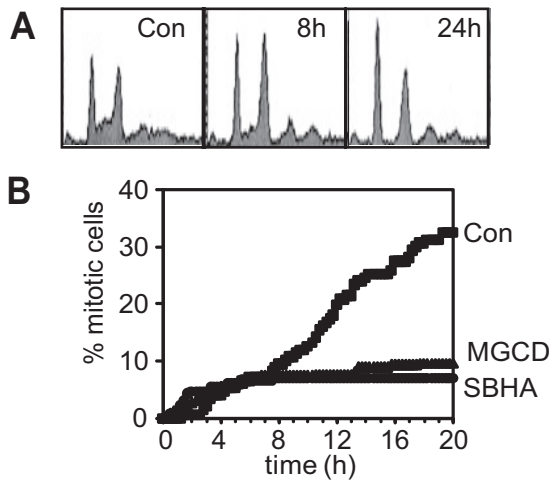

Fig. 5. MGCD0103 initiates a G_2 arrest in normal cells. Asynchronously growing NFF cultures were treated with either 500 μM SBHA, 10 μM MGCD0103, or equal volume of DMSO as control (Con) and analyzed by FACS at the indicated times (A) or followed by time-lapse microscopy (B). Cells were scored for entry into mitosis, and the cumulative score is shown. More than 100 cells were analyzed for each treatment.

both MGCD0103 (Fig. 5A) and SBHA (data not shown) treatment as early as 8 h after treatment. Time-lapse microscopy also revealed a block of entry into mitosis 6 h after drug treatment (Fig. 5B). This indicated that the drugs were required to be present throughout S phase for the G_2 arrest to be initiated. MGCD0103 treatment did not induce significant cell death in NFF cultures over the time course of these experiments, similar to the lack of cytotoxicity observed with SBHA and other pan-HDACi treatment.

MGCD0103 Disrupts Microtubule Stability. The surprising observations that MGCD0103 treatment disrupted mitotic spindle assembly, completely blocked cytokinesis, and, when added to G_2-phase cells, arrested cells in mitosis suggested that MGCD0103 was targeting the microtubules directly in a manner not observed previously with HDACi. MGCD0103-treated mitotic cells often seemed to have <2 microtubule foci that could possibly represent multiple centrosomes. However, only two γ-tubulin-stained centrosomes were detected in mitotic cells after treatment with any of the HDACis, although only MGCD0103-treated cells failed to produce a bipolar spindle (Fig. 6A). The lack of spindle structure was reminiscent of treatment with microtubule depolymerizing agents such as nocodazole. To examine whether MGCD0103 was disrupting normal microtubule polymerization, drug-treated mitotic cells were permeabilized briefly to extract the cytosolic components, including the unpolymerized tubulins, before fixation. Whereas this had little effect on the mitotic spindles in either controls, SBHA-treated cells or cells treated with another class I-selective inhibitor, sodium butyrate, there was little microtubule staining remaining in the MGCD0103-treated cells (Fig. 6B). A similar loss of microtubule staining was observed in the MGCD0103-treated interphase cells (Fig. 6C) and was also observed in another cell line (Supplementary Fig. S5). The effect of MGCD0103 on microtubules seems to be either destabilizing microtubules or blocking polymerization. To examine this, the effect of MGCD0103 treatment on paclitaxel-induced microtubule polymerization and bundling was examined. Paclitaxel-induced microtubule bundling was unaffected by MGCD0103 treatment, indicating that it was not affecting paclitaxel binding to β-tubulin and stabilizing microtubules (Fig. 6D).

The ability of HDACi to induce mitotic slippage suggests

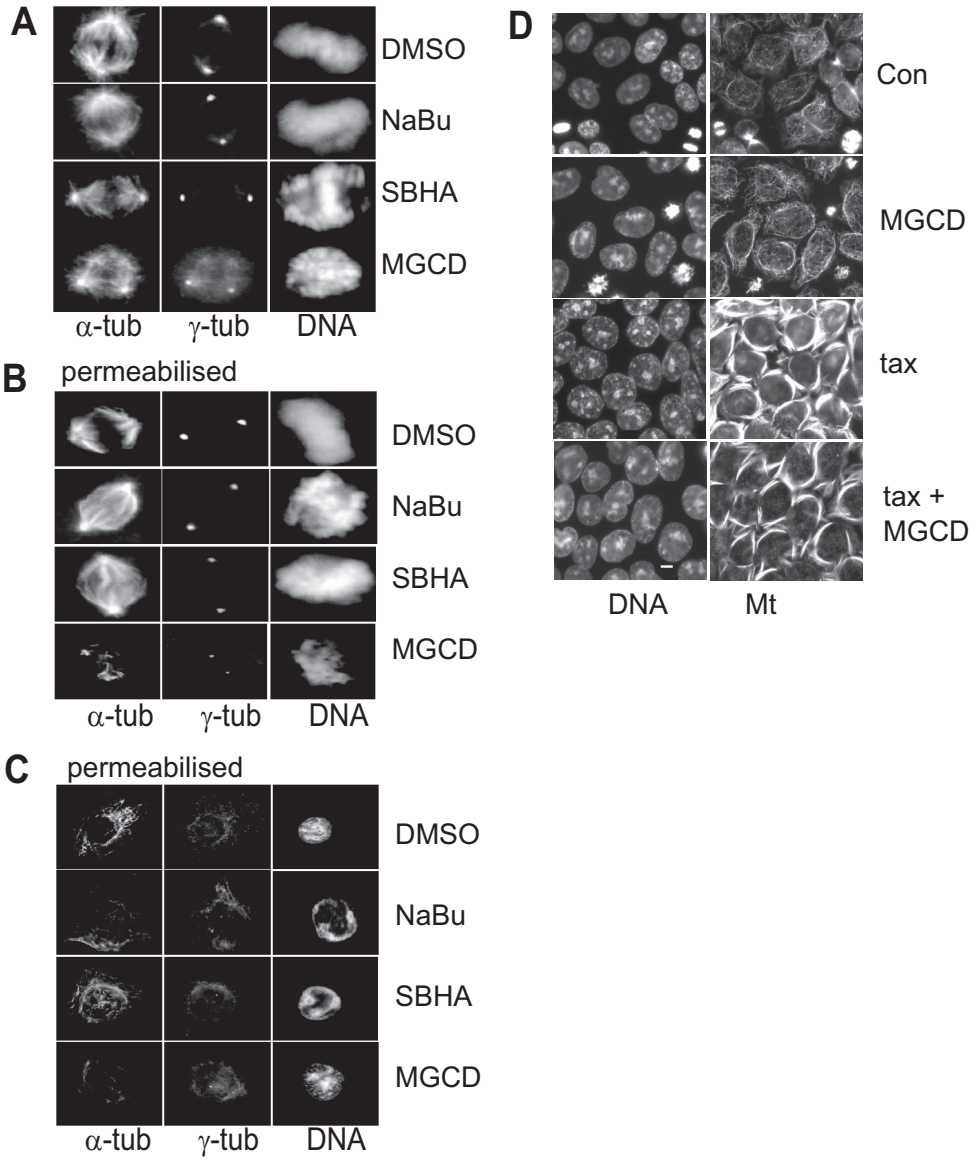

Fig. 6. MGCD0103 destabilizes microtubules. A, HeLa cells were treated with the either DMSO as control, 20 mM sodium butyrate (NaBu), 500 μM SBHA, or 10 μM MGCD0103. Cells were fixed after overnight drug treatment and immunostained for microtubules (α-tub), centrosomes (γ-tub), and DNA. B, in a similar experiment to that shown in A, cells were first permeabilized before fixing and immunostaining. Treatment with only MGCD0103 consistently resulted in a loss of the spindle microtubules. The other HDACi treatment had no effect on spindle microtubules. C, interphase cells from the same coverslips as in B showed a loss of the microtubules in only the MGCD0103-treated samples. D, HeLa cells were treated for 7 h without (Con) or with MGCD0103, paclitaxel (tax) or MGCD0103 and paclitaxel then fixed and immunostained for DNA and α-tubulin to detect microtubules (Mt).

that they should combine with microtubule-disrupting agents to promote cell death. This was observed with a combination of SBHA with both nocodazole and paclitaxel, in which the increase in the subdiploid population for each combination exceeded the additive effects of the individual drugs by 2.5- to 3-fold, with each combination (SBHA + nocodazole or paclitaxel) producing a similar degree of synergy (Fig. 7). Although the combinations with MGCD0103 produced the higher levels of cell death, the effect of combination with nocodazole was little more than additive, producing a 50% increase in the subdiploid population over the additive effect. This combination produced the same level of subdiploid cells as the SBHA combinations (Fig. 7B). By contrast, the combination of MGCD0103 with paclitaxel had a 2-fold increase in the subdiploid population relative to the additive effect of the individual drug when used alone. This effect is similar to the degree of apparent synergy obtained with SBHA and both microtubule drugs (Fig. 6B). The relatively weak effect of the microtubule destabilizing drug nocodazole in combination with MGCD0103 compared with the effect with the stabilizing drug paclitaxel, provides evidence that some of the increased cytotoxicity of MGCD0103 is likely to be based on the combination of HDACi and microtubule-destabilizing activities of the drug. The equivalence of the effects of the SBHA-nocodazole and MGCD0103-nocodazole effects strongly supports this conclusion.

Discussion

Previous studies have demonstrated that HDACis possess tumor-selective cytotoxicity based on the functional status of a G_2-phase checkpoint response that is sensitive to HDACis and their ability to disrupt normal mitosis and to promote mitotic slippage. The checkpoint blocks cells from entering the aberrant mitosis in the presence of drugs, protecting them from the cytotoxic effects of these drugs (Qiu et al., 2000; Krauer et al., 2004). A majority of tumor cell lines are defective for this checkpoint (Qiu et al., 2000). MGCD0103 is capable of initiating this same G_2-phase checkpoint arrest in primary fibroblasts, and it has been reported to have little cytotoxicity toward normal cells (Fournel et al., 2008).

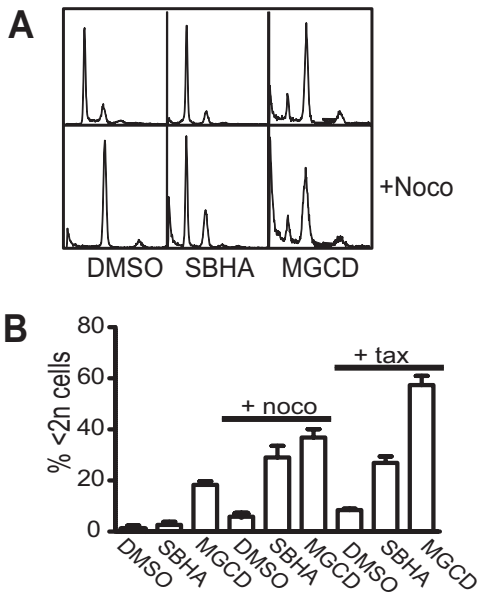

Fig. 7. MGCD0103 combines with paclitaxel. A, HeLa cells were treated with 125 μM SBHA or 2.5 μM MGCD0103 either with or without added nocodazole (+Noco) and then harvested after 24 h and analyzed by FACS for DNA content. B, quantification of the subdiploid population (<2N cells) as a marker of cell death from three independent experiment of combinations or SBHA and MGCD0103 as in A, with either nocodazole or paclitaxel.

HDACi-induced disruption of mitosis and subsequent mitotic slippage have been demonstrated to be critical contributors to the apoptosis observed in many solid tumor-derived cell lines (Warrener et al., 2003; Dowling et al., 2005; Stevens et al., 2008). A critical feature of HDACi-induced aberrant mitosis is that it promotes spindle checkpoint activation, demonstrated by the extended time in mitosis. Activation of the spindle assembly checkpoint is necessary for generating a death signal at the premature exit (Gabrielli et al., 2007), and blocking the activation of this checkpoint inhibits this death signal (Nitta et al., 2004; Sudo et al., 2004; Vogel et al., 2005). MGCD0103 induced rapid accumulation of cells with 4N DNA content over the first 12 h of treatment, which was maintained until at least 24 h, at which time cells arresting in mitosis and cells that have exited mitosis but failed cytokinesis are observed. The time-lapse microscopy supports the biochemical analysis and demonstrates that cells are undergoing mitotic slippage. In contrast to other HDACis such as SBHA, there was no evidence for a G_1 arrest with MGCD0103 or increased $p21^{Waf1}$ expression during the first 12 h of treatment. The absence of a G_1 arrest accounts for the higher proportion of an MGCD0103-treated, asynchronously growing population to enter mitosis, whereas other HDACis that induce rapid $p21^{Waf1}$ up-regulation block a high proportion of cells from exiting G_1 phase. MGCD0103 treatment also failed to affect the expression of other genes commonly affected by HDACi treatment, namely cyclins E and A. This suggests that MGCD0103 may not affect gene expression significantly over the first 12 h of treatment. The basis of the difference in the transcriptional effects of MGCD0103 and other HDACis is not clear. Another class I-selective HDACi, MS-275, has been reported to increase $p21^{Waf1}$ expression and impose a G_1-phase arrest (Saito et al., 1999), indicating that the effect of MGCD0103 may be related to the spectrum of HDAC inhibited by the drug rather than a normal consequence of generally inhibiting class I HDAC. SiRNA depletion of individual class I HDACs has less effect on gene expression than pan-isoform HDACi, but the effects also seem to be cell line-specific (Senese et al., 2007; Dejligbjerg et al., 2008). HDAC3 has been shown to be directly involved in mitosis. HDAC3 localizes to the spindle poles in association with the nuclear receptor corepressor N-Cor, and depletion of HDAC3, but not HDAC1 or HDAC2, induced mitotic defects that seemed similar to those observed with HDACi treatment (Ishii et al., 2008). MGCD0103 is an efficient inhibitor of HDAC1, HDAC2, HDAC3, and HDAC11 in vitro (Fournel et al., 2008); however, this spectrum of HDAC inhibition does not readily explain the lack of immediate expression changes observed here in a number of cell lines. The delayed $p21^{Waf1}$ expression is also not a consequence of the microtubule-disrupting activity of this MGCD0103, because microtubule disruption with nocodazole or paclitaxel cotreatment had no effect on the level of $p21^{Waf1}$ expression or the G_1 phase arrest induced by SBHA (K. M. Chia and B Gabrielli, unpublished observations).

The ability of MGCD0103 to destabilize microtubules is a unique activity of this drug and has not been reported previously for other HDACis, including other class I-selective

inhibitors such as sodium butyrate used in this work. This unique activity accounts for many of the properties unique to MGCD0103, including the disordered mitotic spindle, mitotic arrest when added to cells during transit through G_2 phase, and failure of cytokinesis, properties normally associated with antimicrotubule drugs. Its weak combination with the microtubule-destabilizing drug nocodazole compared with a pronounced effect in combination with paclitaxel, and the strong combinations of SBHA with both microtubule drugs, points to MGCD0103 possessing intrinsic microtubule-destabilizing activity. It is not clear whether MGCD0103 directly binds tubulins to disrupt the polymers as other tubulin-binding drugs such as nocodazole and paclitaxel (Pasquier and Kavallaris, 2008), although it seems that MGCD0103 does not block paclitaxel binding to tubulin, or if it has an indirect effect though the interaction with microtubule-associated stability factors. In addition, siRNA depletion of HDAC1, HDAC2, and HDAC3, individually or together, had no effect on microtubule spindle formation, indicating that the microtubule-destabilizing activity of the drug is likely to be independent of HDAC inhibition by the drug (Warrener et al., 2010). However, the combination of HDACi with microtubule drugs produces a useful synergy of action. The presence of both activities in a single drug, particularly when combined with the lack of G_1 arrest presents a potent combination. Normal tissue toxicity of the antimicrotubule effects of the drug would be minimized by the G_2-phase checkpoint arrest initiated in normal tissue by the drug.

In conclusion, observations from this study support the claim that inhibition of class I HDACs is sufficient for antitumor effects of HDACi. It also supports the view that HDACi induced aberrant mitosis and subsequent mitotic slippage as key contributors to the tumor-selective cytotoxicity of this class of drugs. MGCD0103 possesses many of the useful anticancer properties of HDACi but also have a number of unique features including delayed up-regulation of $p21^{Waf1}$, and antimicrotubule effects that do not seem to be associated with the HDACi activity of the drug. The combination of these properties suggests that MGCD0103 will potentially be clinically useful in treating solid tumors in which antimicrotubule drugs already have demonstrated clinical efficacy.

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