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The histone deacetylase inhibitor MGCD0103 has both deacetylase and microtubule inhibitory activity

KeeMing Chia, Heather Beamish, Kaneez Jafferi, Brian Gabrielli

University of Queensland Diamantina Institute, Princess Alexandra Hospital, Brisbane, Queensland. 4102 Australia.

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Running Title: MGCD0103 selectively targets mitosis

Corresponding Author: Dr Brian Gabrielli,

Diamantina Institute,

University of Queensland,

Princess Alexandra Hospital, Brisbane. Australia 4102

Email: brianG@uq.edu.au

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Abstract

Histone deacetylase inhibitors (HDACi) are currently being trialled or are in clinical use for treatment of a number of tumour 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 MGCD0103, a class I selective histone deacetylase inhibitor, on the cell cycle and cell killing. Surprisingly, MGCD0103 treatment failed to initiate a G1 phase arrest, but caused marked accumulation of cells in G2/M at 6 h and 12 h after treatment, and was cytotoxic 24 h post-treatment. These cell cycle effects were considerably distinct from the effects of suberic bishydroxamic acid (SBHA), 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. Similarly, it also specifically targeted tumor cells and was nontoxic to normal non-transformed cells. However, MGDC0103 also appeared to disrupt normal microtubule spindle formation whereas HDACi generally have only a minor effect on spindle formation. The effect of MGCD0103 on spindle formation was shown to be a consequence of microtubule destabilisation. This is the first example of a HDACi with microtubule destabilising activity and the combined effects of this drug has advantages for its therapeutic use.

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Introduction

Histone deacetylase inhibitors (HDACi) are an emerging class of anti-cancer drugs which possess tumour selective cytotoxicity. HDACi have been demonstrated to inhibit growth of tumour cells and induce them to undergo apoptosis or differentiation in vitro. They have also been shown to have anti-angiogenic properties (Bolden et al., 2006). These drugs are currently in clinical trials either as a monotherapy and in combination with other anti-cancer agents. The recent approval of Vorinostat (SAHA) for the treatment of T-cell lymphoma highlights the potential of these drugs as promising anti-cancer therapeutics.

The majority of HDACi are pan-isoform inhibitors of two classes of HDACs; class I HDACs, HDAC1-3 and HDAC8, and class II HDACs, HDAC4-7, HDAC9 and HDAC10. HDACs have been shown to regulate the acetylation state of nuclear histones and an increasing number of non-histone proteins. Inhibition of HDACs leads to changes in 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 HDACi utilized, $p21^{Waf1}$ is one gene which 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 primarily due to the inhibition of class I HDACs. Class I HDACs are aberrantly over-expressed in various tumours; HDAC1 in gastric cancer, HDAC2 in colorectal cancer and HDAC3 in colon cancer (Glozak and Seto, 2007; Ozdag et al., 2006; Wilson et al., 2006; Zhu et al., 2004). Depletion and knock out of individual HDACs has uncovered the unique biological roles of the individual HDACs. HDAC1 and HDAC3 appear to be involved in regulating proliferation (Bhaskara et al., 2008; Wilson et al., 2006), while HDAC2 appears to regulate

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apoptosis (Senese et al., 2007; Weichert et al., 2008). Class II HDACs appear not to regulate cell proliferation and are primarily involved in cellular development and differentiation (Verdin et al., 2003). In addition, 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 anti-proliferative 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). HDACi produce a number of cell cycle effects including G1/S phase arrest which is correlated with increased $p21^{Waf1}$ expression and is observed in a wide range of cell lines with all HDACi reported to date (Archer et al., 1998; Burgess et al., 2001); a G2 phase checkpoint arrest observed mainly in normal non-transformed cell lines (Qiu et al., 2000); and mitotic defects including mitotic arrest and mitotic slippage also observed in most cell lines in response to a broad range of HDACis (Stevens et al., 2008; Warrener et al., 2003). The HDACi sensitive G2 phase checkpoint appears to be the basis for the tumour selective cytotoxicity of these drugs with the majority of tumour cell lines being sensitive due to their defective checkpoint whereas normal tissues are protected by their intact G2 phase checkpoint response (Krauer et al., 2004; Qiu et al., 2000; Warrener et al., 2003). Progression through the HDACi induced aberrant mitosis induces rapid cell death (Blagosklonny et al., 2002; Dowling et al., 2005; Warrener et al., 2003), and this appears to be 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 MGCD0103 (Fournel et al., 2008), and compared it with an

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equipotent dose of a representative pan-isoform HDACi suberic bishydroxamic acid (SBHA) on a number of tumour cell lines in vitro. Similar to SBHA, we found that MGCD0103 promotes tumour 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 12h after MGCD0103 treatment or G1 phase arrest, which contributes to the increased cytotoxicity of this drug. MGCD0103 also possesses a novel activity, it destabilises microtubules in both interphase and mitosis, and this activity underlies the improved cytotoxicity of this drug.

Materials and Methods

Materials

MGCD0103 and 001 (inactive analogue of MGCD0103) were kindly provided by MethylGene Inc (Montreal Canada). Suberic bishydroxamic acid (SBHA) was purchased from Sigma Aldrich. All compounds were dissolved in dimethyl sulphoxide (DMSO) with MGCD0103 and 001 dissolved as 10mM 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 over-expressing Bcl-2 (HeLa-Bcl2), and neonatal foreskin fibroblast (NFF) were maintained in Dulbecco's modified Eagle's Medium (Gibco) supplemented with 10% (v/v) Serum Supreme (Biowhittaker). A2058, A02 and MM604 melanoma cell lines were maintained in RPMI Medium 1640 (Gibco) supplemented with 10% (v/v) Serum Supreme.

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HDACi were added to either asynchronous cells or cells synchronized by simple thymidine block (2 mM for 17 h) in early S phase or late G2 phase (7 h release from thymidine), and harvested at the indicated time points. For early S phase addition, HDACi were added immediately after release, whereas for G2 phase addition, HDACi were added 7 h after release. For the mitotic shake-off experiments, HDACi were added in early S phase and 0.25μg/ml nocodazole was added 7 h after release. Mitotic cells were collected by mechanical shake-off 3h later. In parallel experiments, HDACi were added in combination with the microtubule-disrupting agents taxol (100 nM) or nocodazole (0.25 μg/ml) to asynchronous cell populations for 24 h. $p21^{Waf1}$ knockdown was achieved by transfection with 5nM p21 anti-sense oligonucleotides (Ambion and Dharmacon Smart Pool) 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 4μg/ml propidium iodide (Sigma) and 400 μg/ml RNase A (Invitrogen) in PBS. The stained cells were subsequently filtered through a 37 μm gauze and analyzed on a FACSCailbur (BD Biosciences) using CellQuestPro® (BD Biosciences). The different subpopulations were quantified using ModFit LT® program (Verity Software House Inc). Experiments were performed in triplicate and the presented as mean and standard deviation. P values were calculated by 2 tailed T-test.

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Immunoblotting

Cell pellets were lysed in 1X SDS lysis buffer (0.4% SDS, 2% glycerol, 2.5mM Tris pH6.7 and 0.3 M 2-mercaptoethanol) and total cell lysates were quantified using Bio-Rad RC DC Protein Assay. 20-40 µg of samples were resolved by 10% or 12% SDS-PAGE, transferred electrophoretically to Hybond^{TM}-C Extra Membrane (Amersham Biosciences). Membranes were immunoblotted with primary antibodies against histone H3, acetylated H3 (Lys9), acetyl lysine, phospho-B23, phosho-H3 (Ser10), PARP (Cell Signalling), p21^{Waf1} (Calbiochem), α-tubulin (Sigma), BubR1 (Abcam), CENP-A, phospho-CENP-A (Upstate Biotechnology), cyclin E and cyclin A (Santa Cruz). These were detected with anti-mouse or –rabbit HRP conjugated secondary antibodies (Zymed) and Western Lightning™ Plus-ECL reagent (PerkinElmer). Quantification of protein bands was carried out using the Bio-Profile Bio1D software (Vilber Lourmat).

Immunofluorescent staining

Cells grown on poly-L-lysine coated coverslips were fixed in ice cold methanol overnight at -20°C. Where cells were permeablised prior to fixation, cells were washed with 50 µg/ml digitonin, 130 mM sucrose, 50 mM KCl, 50 mM Na acetate, 20 mM Hepes pH7.5, 5 mM $MgCl_2$, 2 mM EGTA for 90 sec before washing twice with PBS and fixing with -20^{o}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 30mins at room temperature before immunostaining with anti α-tubulin antibody for one hour at room temperature. Coverslips were washed twice in PBS and immunostained with a FITC-conjugated secondary antibody for 30min at room temperature. DNA was counterstained with 4'-6'Diamindino-2-phenylindole (DAPI; Sigma Aldrich). Coverslips were mounted onto

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microscopic glass slides with ProLong® Gold anti-fade reagent (Invitrogen). Fluorescent microscopy was carried out with Axioskop 2 plus (Carl Zeiss).

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 15min for 20 h.

Results

MGCD0103 induces delay increase in p21^{Waf1} and fails to G1 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 previously demonstrated to have similar cell cycle effects as other HDACi including SAHA (suberoylanilide hydroxamic acid), TSA (trichostatin A) and sodium butyrate (Stevens et al., 2008; Warrener et al., 2003). 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 (Figure 1A and B) and were used as equipotent doses throughout this work. These doses of the drugs also induced H3 Lys 9 acetylation with very similar kinetics, although only SBHA increased α-tubulin acetylation indicating that it also inhibited the class II HDAC6 (Figure 1C).

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Using the equipotent concentrations of MGCD0103 (10 μM) and SBHA (500 μM) which induced maximal histone acetylation (Figure 1A), the effects on cell cycle were analysed. MGCD0103 produced two immediately noticeable differences from SBHA treatment. There was a striking accumulation of cells with 4n DNA content from 6 h post-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 G1 phase (Figure 2A 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 treatment, whereas all HDACi, exemplified by SBHA rapidly induced its expression (Figure 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, Figure 2C). The marked accumulation of phosphorylated B23 (pB23), a marker of mitosis, at 6 and 12 h post-treatment only in the MGCD0103 treated cells (Figure 2C), indicated that the 4n DNA peak observed corresponded to cells accumulating in mitosis at 6 and 12 h after MGCD0103 treatment (Figure 2A). Interestingly, the 4 n peak persisted to 24 h whereas the pB23 signal was lost at this time. Both HDACi also induced PARP cleavage, a marker of caspase 3/7 activation and apoptosis, to a similar extent at 24 h although only the MGCD0103 treated cells had a readily detectible sub diploid 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 Figure S1 and data not shown).

The loss of the S phase population and G1 phase arrest observed with the majority of HDACi including SBHA, was demonstrated to be a consequence of HDACi induced $p21^{Waf1}$

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expression. siRNA mediated depletion of the induced $p21^{Waf1}$ completely overcame the loss of S phase and correspondingly increased G1 phase populations after SBHA treatment (Figure 3A). To test whether increased p21 expression could reduce the 4 n accumulation and cell death induced by MGCD0103 at 24 h, cells were pretreated with a relatively non-toxic dose of SBHA (100 μM) for 12 h prior to treatment with 10 μM MGCD0103 for a further 24 h. Treatment with the low dose of SBHA alone induced strong $p21^{Waf1}$ expression and G1 phase arrest, with a corresponding decrease in S phase, but little cell death as determined by either sub diploid cells or PARP cleavage (Figure 3B). SBHA pretreatment imposed a G1 phase arrest in the subsequently MGCD0103 treated cells, and decreased the 4 n accumulation and markers of cell death. Thus, the lack of increased $p21^{Waf1}$ expression in the MGCD0103 treated cells was underlies the lack of effect on G1 or S phase compartments with MGCD0103 treatment. The absence of G1 phase arrest alone cannot be responsible for the 4n accumulation observed with MGCD0103 treatment, as there was no 4n accumulation in SBHA treated, $p21^{Waf1}$depleted cells (Figure 3A).

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 over expressing Bcl-2 (HeLa-Bcl2) that are relatively refractory to HDACi-induced apoptosis were used (Supplementary Figure S2; (Warrener et al., 2003)). When MGCD0103 was added to thymidine block release synchronised HeLa-Bcl2 cells and followed using time lapse microscopy, it delayed entry into mitosis by < 2 h, a little longer than SBHA treatment (Supplementary Figure S3). MGCD0103 treated cells also delayed in mitosis for an average of 402 min compared to an average of 49 min for the inactive control, similar to the effect of SBHA which delayed cells

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in mitosis for 304 min (Figure 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 HDACi (Figure 4B; (Qiu et al., 2000; Stevens et al., 2008; Warrener et al., 2003)). There was also a surprising disruption of the normal mitotic spindle structure with MGCD0103 treatment, <90% of MGCD0103 treated cells failed to form a clearly discernible spindle structure, although strong microtubule foci were observed in the mitotic cells (Figure 4B). This was not normally observed with HDACi, 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 (Figure 2), suggested that MGCD0103 shares the ability to promote mitotic slippage in common with other HDACi (Dowling et al., 2005; Stevens et al., 2008). This was clearly demonstrated by the ability of MGCD0103 treatment to overcome the spindle assembly checkpoint imposed by nocodazole treatment (Figure 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 addition of the drug to cells in G2 phase resulted in these cells arresting in mitosis for an extended period (533 min; Figure 4A), whereas SBHA was previously shown not to affect mitotic progression when added in G2 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 to nocodazole arrested cells where BubR1 was exclusively in activated, lower mobility form, despite both nocodazole and MGCD0103 treated population containing

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>90% aberrant mitotic cells (Figure 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 (Figure 4A 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 (Figure 4C), or the pericentric CenpA phosphorylation which is also catalysed by Aurora B (Supplementary Figure S4). Thus, MGCD0103 does initiate a mitotic checkpoint arrest but prematurely exits, similar to other HDACi, although it disrupts the normal mitotic spindle formation in a manner unique to this drug.

Interestingly, 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 undergo 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 G2 phase (Figure 4D). In contrast, SBHA does not affect mitosis when added in G2 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 G2 phase arrest in normal cells

The mitotic effects described above were previously only observed in HDACi treated tumour and immortalised cell lines which had a defect in an HDACi sensitive G2 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 G2 checkpoint in normal cells, asynchronous cultures of primary neonatal foreskin fibroblasts

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(NFF), previously characterised to have an intact G2 checkpoint response, were treated with either SBHA or MGCD0103 and analysed by FACS and time lapse microscopy. FACS revealed the expected 4n accumulation with both MGCD0103 (Figure 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 (Figure 5B). This indicated that the drugs were required to be present throughout S phase for the G2 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 G2 phase cells arrested cells in mitosis suggested that MGCD0103 was targeting the microtubules directly in a manner not previously observed with HDACi. MGCD0103 treated mitotic cells often appeared 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 HDACi, although only MGCD0103 treated cells failed to produce a bipolar spindle (Figure 6A). The lack of spindle structure was reminiscent of treatment with microtubule depolymerising agents such as nocodazole. To examine whether MGCD0103 was disrupting normal microtubule polymerisation, drug treated mitotic cells were permeablised briefly to extract the cytosolic components, including the unpolymerised tubulins, prior to 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

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microtubule staining remaining in the MGCD0103 treated cells (Figure 6B). A similar loss of microtubule staining was observed in the MGCD0103 treated interphase cells (Figure 6C), and was also observed in another cell line (Supplementary Figure S5). The effect of MGCD0103 on microtubules appears to be either destabilising microtubules or blocking polymerisation. To examine this, the effect of MGCD0103 treatment on taxol induced microtubule polymerisation and bundling was examined. Taxol induced microtubule bundling was unaffected by MGCD0103 treatment, indicating that it was not affecting taxol binding to β-tubulin and stabilising microtubules (Figure 6D).

The ability of HDACi to induce mitotic slippage would suggest that they should combine with microtubule disrupting agents to promote cell death. This was observed with combination of SBHA with both nocodazole and taxol, where the increase in the sub diploid population for each combination exceeded the additive effects of the individual drugs by 2.5 to 3 fold, with each combination (SBHA + nocodazole or taxol) producing a similar degree of synergy (Figure 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 sub diploid population over the additive effect. This combination produced the same level of sub diploid cells as the SBHA combinations (Figure 7B). By contrast, the combination of MGCD0103 with taxol had a 2 fold increase in the sub diploid 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 (Figure 6B). The relatively weak effect of the microtubule destabilising drug nocodazole in combination with MGCD0103 compared to the effect with the stabilising drug taxol, provides evidence that some of the increased cytotoxicity of MGCD0103 is likely to be based on the combination of HDACi and microtubule destabilising activities of the drug. The

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equivalence of the effects of the SBHA-nocodazole and MGCD0103-nocodazole effects strongly supports this conclusion.

Discussion

Previous studies have demonstrated that HDACi possess tumour selective cytotoxicity based on the functional status of a G2 phase checkpoint response that is sensitive to HDACi, 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 (Krauer et al., 2004; Qiu et al., 2000). A majority of tumour cell lines are defective for this checkpoint (Qiu et al., 2000). MGCD0103 is capable of initiating this same G2 phase checkpoint arrest in primary fibroblasts, and it has been reported to have little cytotoxicity towards normal cells (Fournel et al., 2008).

HDACi induced disruption of mitosis and subsequent mitotic slippage have been demonstrated to be critical contributors to the apoptosis observed in many solid tumour derived cell lines (Dowling et al., 2005; Stevens et al., 2008; Warrener et al., 2003). 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, shown to be cells arresting in mitosis and cells that have exited mitosis but failed cytokinesis. The time lapse microscopy supports the biochemical analysis and demonstrates that cells are undergoing mitotic slippage. In contrast to other HDACi such as SBHA, there

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was no evidence for a G1 arrest with MGCD0103 or increased $p21^{Waf1}$ expression during the first 12 h of treatment. The absence of a G1 arrest accounts for the higher proportion of an MGCD0103 treated, asynchronously growing population to enter mitosis whereas other HDACi which induce rapid $p21^{Waf1}$ up regulation block a high proportion of cells from exiting G1 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 HDACi is not clear. Another class I selective HDACi MS275 has been reported to increase $p21^{Waf1}$ expression and impose a G1 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 appear to be cell line specific (Dejligbjerg et al., 2008; Senese et al., 2007). HDAC3 has been shown to be directly involved in mitosis. HDAC3 localises 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 appeared 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, as microtubule disruption with nocodazole or taxol co-treatment had no effect on the level of $p21^{Waf1}$ expression or the G1 phase arrest induced by SBHA (unpublished observations).

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The ability of MGCD0103 to destabilise microtubules is a unique activity of this drug and has not previously been reported for other HDACi, 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 G2 phase, and failure of cytokinesis, properties normally associated with anti-microtubule drugs. Its weak combination with the microtubule destabilising drug nocodazole compared to pronounced effect in combination with taxol, and the strong combinations of SBHA with both microtubule drugs, points to MGCD0103 possessing intrinsic microtubule destabilising activity. It is not clear whether MGCD0103 directly binds tubulins to disrupt the polymers as other tubulin binding drugs such as nocodazole and taxol (Pasquier and Kavallaris, 2008), although it appears that MGCD0103 does not block taxol binding to tubulin, or if it has an indirect effect though 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 destabilising activity of the drug is likely to be independent of HDAC inhibition by the drug (Warrener, Chia and Gabrielli, manuscript submitted). However, the combination of HDACi with microtubule drugs does produce a useful synergy of action. The presence of both activities in a single drug, particularly when combined with the lack of G1 arrest presents a potent combination. Normal tissue toxicity of the anti-microtubule effects of the drug would be minimised by the G2 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 anti-tumour effects of HDACi. It also supports the view that HDACi induced aberrant mitosis and subsequent mitotic slippage as key contributors to the tumour selective cytotoxicity of this class of drugs. MGCD0103 possesses many of the useful anti-

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cancer properties of HDACi but also have a number of unique features including delayed up regulation of $p21^{Waf1}$, and anti-microtubule effects that do not appear 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 tumours where anti-microtubule drugs already have demonstrated clinical efficacy.

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Footnotes

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Figure Legends

Figure 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 a similar experiment to 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 analysed for the level of histone H3 Lys 9 acetylation (H3 K9Ac), total H3 protein, or acetylated α-tubulin (Ac α-tub).

Figure 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 analysed by FACS for their DNA content.

B: Quantitation of the 4n populations from at least three replicate experiments identical to that shown in B. Open bars, control DMSO; light grey, 001; dark grey, SBHA; black, MGCD0103.

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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.

Figure 3: Increased p21 expression is responsible for SBHA induced G1 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 analysed 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 average and SD (Standard Deviation) of triplicate determinations. Similar data was obtained with a different p21 siRNA, and in three other cell lines. Open bars, control; light grey bars, +SBHA; dark grey 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, 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), SBHA for 12h then MGCD0103 (SB/MG). Cells were analysed by FACS for DNA content and immunoblotted for PARP as a marker of apoptosis, $p21^{Waf1}$ and α-tubulin as a loading control. The asterix indicates p values <0.0001.

Figure 4: MGCD0103 disrupts normal spindle checkpoint function

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A: Synchronised 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 addition of nocodazole (Noco) 7 h after release from the synchrony arrest as they progressed through G2 phase into mitosis. Cells were followed by time lapse microscopy and the time cells stayed in mitosis was measured. In each case >100 cells were analysed. .

0 μM MGCD0103 was also added at 7 h after release from the synchrony arrest when they had entered G2 phase (G2 MGCD).

B: Synchronised HeLa cells were treated in G2 phase with 10 μM MGCD0103 or 500 μM SBHA then allowed to enter into mitosis, and fixed and stained with anti-α-tubulin for microtubules (Mt) and counterstained with DAPI for DNA. The images are representative of the mitotic cells observed following these treatments.

C: Mitotic cells were collected by mechanical shake-off from synchronised population treated with either SBHA or MGCD0103 in early S phase or nocodazole in late G2 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: Synchronised HeLa cells were treated in either S or G2 phase with SBHA or MGCD0103 as in A and followed by time lapse microscopy. Cells were analysed 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 analysed for each treatment.

Figure 5: MGCD0103 initiates a G2 arrest in normal cells

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Asynchronously growing NFF cultures were treated with either 500 μM SBHA, 10 μM MGCD0103 or equal volume of DMSO as control (Con), and A, analysed by FACS at the indicated times or B, followed by time lapse microscopy. Cells were scored for entry into mitosis and the cumulative score is shown. Over 100 cells were analysed for each treatment.

Figure 6: MGCD0103 destabilises 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 A, cells were first permeablised before fixing and immunostaining. Treatment with only MGCD0103 consistently resulted in loss of the spindle microtubules. The other HDACi treatment had no effect on spindle microtubules.

C: Interphase cells from the same coverslips a B showed loss of the microtubules in only the MGCD0103 treated samples.

D: HeLa cells were treated for 7 h without (Con) or with MGCD0103, taxol (tax) or MGCD0103 and taxol then fixed and immunostained for DNA and α-tubulin to detect microtubules (Mt).

Figure 7: MGCD0103 combines with taxol

A: HeLa cells were treated with 125 μM SBHA or 2.5 μM MGCD0103 either with or without added nocodazole (+Noco), then harvested after 24 h and analysed by FACS for DNA content.

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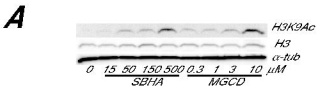
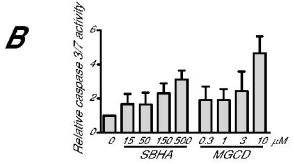
B: Quantification of the sub diploid population (<2n cells) as a marker of cell death from three independent experiment of combinations or SBHA and MGCD0103 as in A, with either nocodazole or taxol.

Figure 1


A
H3K9Ac
H3
α-tub
0 15 50 150 500 0.3 1 3 10 μM
SBHA
MGCD


B
Relative caspase-3,7 activity
0 15 50 150 500 0.3 1 3 10 μM
SBHA
MGCD

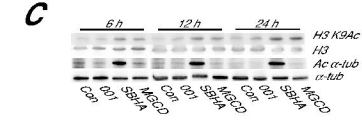

C
6 h
12 h
24 h
H3 K9Ac
H3
Ac α-tub
α-tub
Con
001
SBHA
MGCD

Figure 2

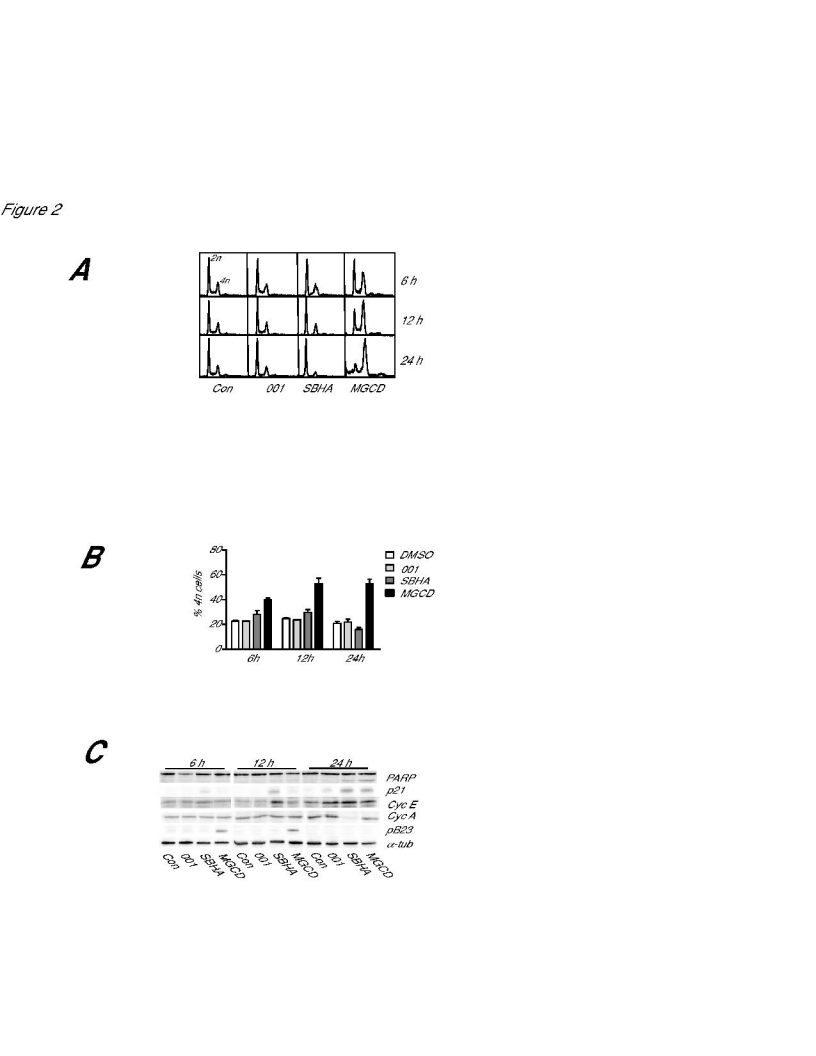

A
2n
4n
6 h
12 h
24 h
Con
001
SBHA
MGCD
B
% 4n cells
80
60
40
20
0
8h
12h
24h
DMSO
001
SBHA
MGCD
C
6 h
12 h
24 h
PARP
p21
Cyc E
Cyc A
pB23
α-tub
Con
001
SBHA
MGCD

Figure 3

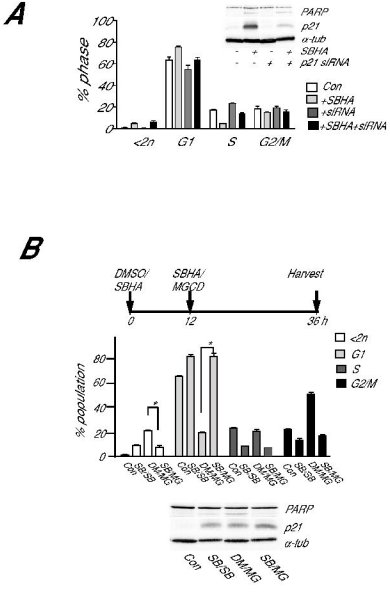
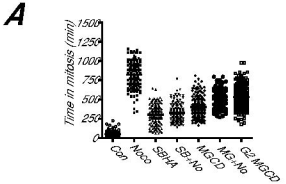
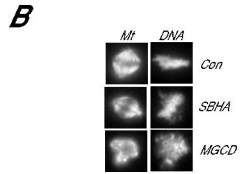
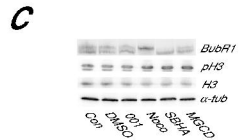

A
% phase
100
80
60
40
20
0
<2n
G1
S
G2/M
PARP
p21
α-tub
SBHA
p21 siRNA
Con
+SBHA
+siRNA
+SBHA+siRNA
B
DMSO/
SBHA
SBHA/
MGCD
Harvest
0
12
36 h
% population
<2n
G1
S
G2/M
Con
SB-SB
DM/MG
SB/MG
PARP
p21
α-tub

Figure 4


A
Time in mitosis (min)
1500
1250
1000
750
500
250
0
Con
Noco
SBHA
SB+No
MGCD
MG+No
G2-MGCD


B
Mt
DNA
Con
SBHA
MGCD


C
BubR1
pH3
H3
α-tub
Con
DMSO
ZM
Noco
SBHA
MGCD

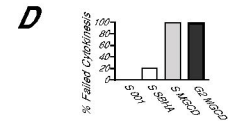

D
% Failed Cytokinesis
100
80
60
40
20
0
S-ZM
S-SBHA
S-MGCD
G2-MGCD

Figure 5

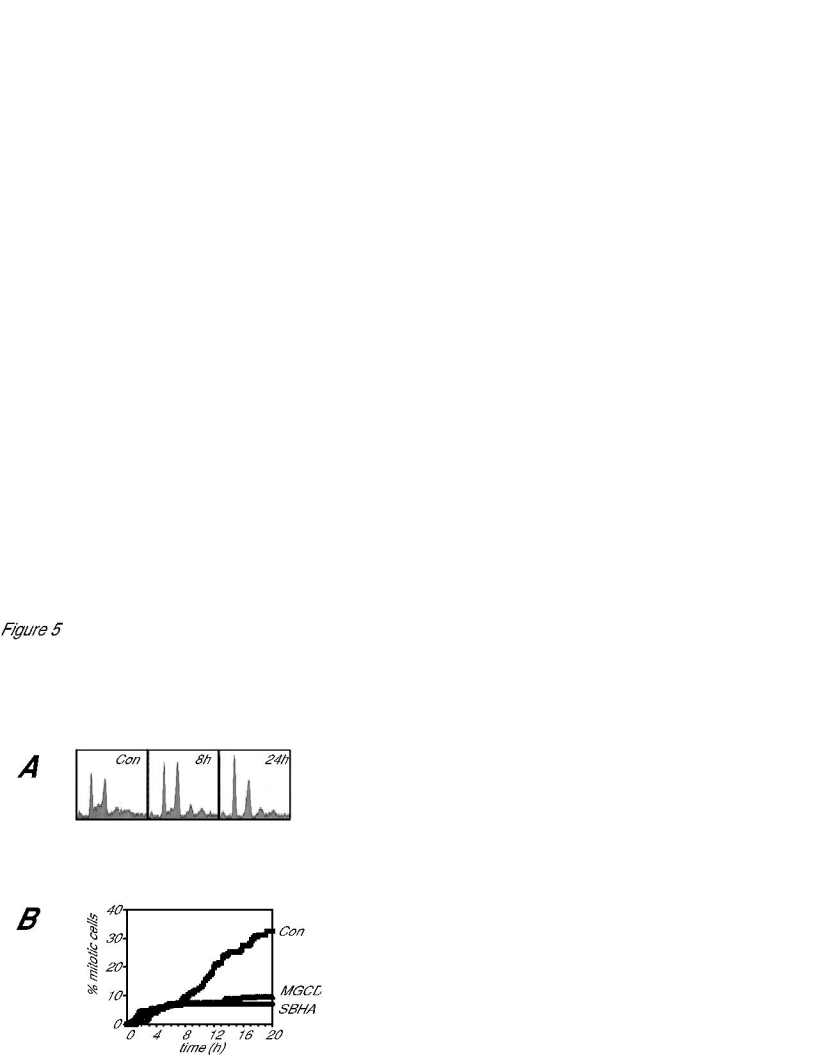

A
Con
8h
24h
B
40
30
20
10
0
% mitotic cells
0
4
8
12
16
20
time (h)
Con
MGCD
SBHA

Figure 6

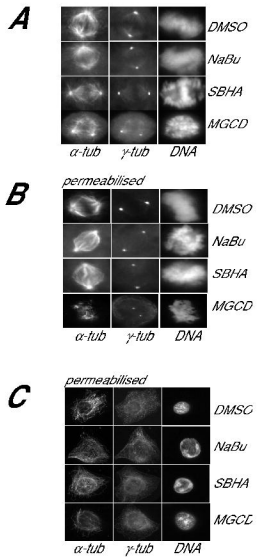

A
DMSO
NaBu
SBHA
MGCD
α-tub
γ-tub
DNA
permeabilised
B
DMSO
NaBu
SBHA
MGCD
α-tub
γ-tub
DNA
permeabilised
C
DMSO
NaBu
SBHA
MGCD
α-tub
γ-tub
DNA

Figure 6

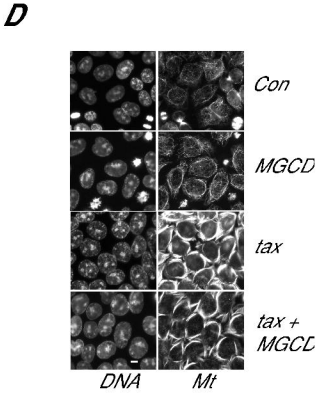
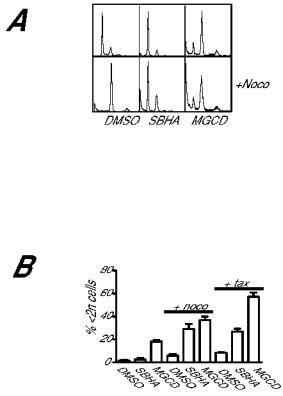


Figure 7


A
+Noco
DMSO
SBHA
MGCD
B
% <2n cells
80
60
40
20
0
+ noco
+ tax
DMSO
SBHA
MGCD
DMSO
SBHA
MGCD
DMSO
SBHA
MGCD