The anti-leukemic effect and molecular mechanisms of novel hydroxamate and benzamide histone deacetylase inhibitors with 5-aza-cytidine

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Abstract. Histone deacetylase inhibitors (HDACi) demonstrate considerable *in vitro* and *in vivo* activity and clinical efficacy in the treatment of hematological malignancies. Pre-clinical and early phase clinical trials identify therapeutic activity using a combination of HDACi and demethylating agents which may be more efficacious than single agent treatment. Our studies aimed to determine the effects and molecular mechanisms of action of novel hydroxamate (MCT-3) and benzamide [MGCD0103 (MG)] HDACi's in the HL-60 cell line alone and in combination with the demethylating agent 5-aza-cytidine (AZA). MG, MCT-3 and AZA treatment significantly inhibited HL-60 cell growth *in vitro* with MG being the most potent agent. MG in combination with AZA demonstrated no significant increase in inhibition of cell growth over MG treatment alone whilst MCT-3 in combination with AZA demonstrated increased inhibition of cell growth over either agent alone although no more significant than MG alone. MG alone or MCT-3 in combination with AZA significantly increased p15 and caspase-3 expression. MG and MCT-3 significantly attenuated AZA-induced MMP-9 mRNA expression and proteolytic activity. Interestingly, MCT-3, MG and AZA alone and in combination increased expression of the novel tumour suppressor gene Nur77, important in leukemogenesis, with MG a more potent inducer as a single agent. These observations suggest the enhanced anti-leukemia activity of the combination of AZA and HDACi may only reside with certain HDACi classes and may be in-part explained by regulation of genes associated with cell cycle arrest, apoptosis and tumour suppression.

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Introduction

Histone deacetylase inhibitors (HDACi) represent a novel, structurally heterogeneous class of compounds. The four chemical classes of HDACi, include the benzamides (e.g., MGCD0103), the hydroxamates (e.g., MCT-3, SAHA), the short-chain fatty acids (e.g., valproic acid), and the cyclic tetrapeptides (e.g., Depsipeptide/FK228) (1-4). The hydroxamates are frequently referred to as 'broad-spectrum' or 'pan'-HDACi resulting from inhibition of several HDAC isoforms). The remaining chemical classes of HDACi selectively inhibit class I HDACs (3,4).

HDACi have recently been described to have significant therapeutic activity in the treatment of hematological malignancies including cutaneous T-cell lymphoma, Hodgkin's lymphoma, myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (5). *In vitro* HDACi have been shown to up-regulate the expression of p15[INK4b], p21[WAF1/CIP1], caspase-3 and the inducible orphan nuclear receptor Nur-77, whose expression is important in the induction of apoptosis and has recently been demonstrated to be abrogated in acute myeloid leukemia, whilst also down regulating Bcl-XL and MMPs in cell lines (6-14).

Numerous *in vitro* studies together with recent pre-clinical and early phase clinical studies identify significant anti-leukemic activity of a combination of HDACi with demethylating agents with greater inhibition of growth and DNA synthesis, and a greater loss of clonogenicity than that observed by single agent treatment in *in vitro* studies (14-20). However, the mechanisms of HDACi-induced cell death and underlying the anti-cancer synergy between HDACi and other agents are not completely understood. As a consequence of the complexity of the cellular actions of HDACi, it is currently unclear as to whether pan-HDACi or specific HDACi will provide the most clinical benefit to patients when used in combination with demethylating agents (1).

MGCD0103, an orally available class I benzamide HDACi, has been reported to have broad spectrum anti-tumour activity *in vitro* and anti-leukemia activity in a phase I study (21,22). Based on the known hypomethylating activity and clinical

anti-leukemia activity of AZA and the reported HDACi activity of MGCD0103, we studied the effect of these agents alone or in combination and compared them with a novel hydroxamate HDACi, MCT-3 (4), in the HL-60 leukemia cell line and explored the molecular mechanisms responsible for these effects.

Materials and methods

Cell culture. HL-60 cells were cultured in RPMI-1640 (Gibco BRL) containing 10% heat inactivated fetal calf serum and kept in a 5% CO_2 incubator at 37°C. Agents: AZA (kind gift of Celgene Pty Ltd., Australia), MG (kind gift of MethylGene, Pty Ltd., Quebec, Canada) and MCT-3, (synthesized as a meta-substituted biphenyl analogue of Oxamflatin in the Department of Chemistry, Monash University, Melbourne, Victoria, Australia (4,23) were added to plates for 24 h. AZA was dissolved in 1% DMSO and used at a final concentration of 1.0 μM. MCT-3 and MG were dissolved in PBS with 1% DMSO and used at a final concentration of 0.5 μM.

Growth inhibition assay. HL-60 cells in log phase were plated at a density of 0.2×10^6 in 10 ml of medium. Cells were harvested at 24 h. Cell viability was assessed using 0.4% trypan-blue staining immediately after culture. Black staining cells were considered as non-viable cells, and unstained bright cells as viable. All experiments were repeated 3 times with averages displayed graphically.

Semi-quantatitive RT-PCR and real-time PCR. RT-PCR was performed on total RNA extracted from HL-60 cells. The primers used for p15[INK4b], p21[WAF1/CIP1], caspase-3, MMP-9 and the human GAPDH gene were prevously described (14). All PCR products were sequenced to confirm the identities and repeated 3 times.

Scanning densitometry analyses were performed on RT-PCR products using the GeneSnap imaging software (Synoptics, UK). Band intensity values for p15[INK4b], p21[WAF1/CIP1], caspase-3 and MMP-9 mRNA were normalized to those for GAPDH mRNA and the resultant p15[INK4b], p21[WAF1/CIP1], caspase-3 and MMP-9: GAPDH ratios were plotted to illustrate variations in gene expression.

Real-time PCR was performed as previously described (14). The primers for ß-actin: 5'-GAC AGG ATG CAG AAG GAG ATT ACT-3' and 5'-TGA TCC ACA TCT GCT GGA AGG T-3'. Forward and reverse primers for Nur-77: 5'-GCT GCA GAA TGA CTC CAC C-3' and 5'-ACA GCA GCA CTG GGC TTA-3'. Data were normalized to ß-actin and presented as the mean fold change compared with control.

SDS-PAGE and zymography studies. An aliquot of conditioned medium from HL-60 cells treated with AZA or/and MCT-3 or MG, together with untreated control cells, was electro-phoresed through a 10% acrylamide SDS-PAGE gel containing 0.15% gelatin under non-denaturing conditions. Gels were washed and incubated in developing buffer at 37°C overnight. The gels were stained with Coomassie blue R250 and destained with water. A SDS-PAGE gel (without gelatin) was used as a loading control. All experiments were repeated 3 times.
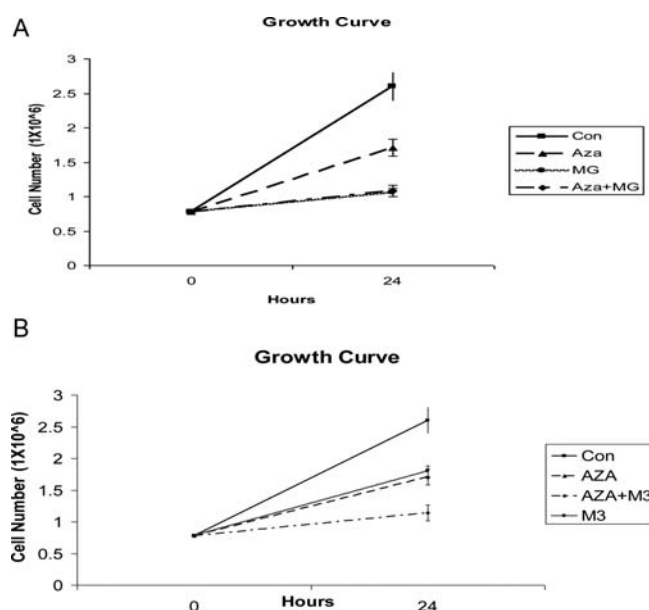

Figure 1. Effect of MG (A) or MCT-3 (B) alone and in combination with AZA on HL-60 cell growth. Numbers on the Y-axis represent the total number of viable cells at 24 h. Con, HL-60 cells without treatment; AZA, cells treated with 1.0 μM 5-aza-cytidine; M3, cells treated with 0.5 μM MCT-3; MA, cells treated with 0.5 μM MCT-3 + 1.0 μM AZA; MG, cells treated with 0.5 μM MGCD0103; MGA, cells treated with 0.5 μM MGCD0103 + 1.0 μM AZA.

Statistical methods. The effects of AZA, MCT-3, and MG alone and in combination on HL-60 cell growth, p15[INK4b], p21[WAF1/CIP1], caspase-3 and MMP-9 expression were assessed by analysis of variance (ANOVA). Data were expressed as means ± SEM and $^*P < 0.05$ was considered statistically significant.

Results

MG, MCT-3 and AZA inhibit HL-60 cell growth. We examined the effects of MG (Fig. 1A) and MCT-3 (Fig. 1B) alone and in combination with AZA on the growth of the human leukemia cell line HL-60. Cells were exposed to MG (0.5 μM), MCT-3 (0.5 μM) and/or AZA (1.0 μM) for up to 24 h. These doses were selected as they induced growth inhibition in previous *in vitro* studies (14,21). MG, MCT-3 and AZA treatment significantly inhibited HL-60 cell growth *in vitro* with MG being the most potent agent. MG in combination with AZA demonstrated no significant increase in inhibition of cell growth over MG treatment alone whilst MCT-3 in combination with AZA inhibited cell growth more effectively than either agent alone although no more effectively than MG alone (Fig. 1).

Effect of MG, MCT-3 and/or AZA on the expression of p15[INK4b], Caspase-3, Nur-77 and p21[WAF1/CIP1] mRNA in HL-60 cells. Cameron *et al* previously identified a combination of 5-AZa-CdR and Trichostatin A (TSA) as producing a synergistic reactivation of p15[INK4b] in human leukemic cells (15). We aimed to determine if similar results could be obtained using MG and MCT-3. MG alone significantly increased p15[INK4b] and caspase-3 mRNA expression (Fig. 2). The combination
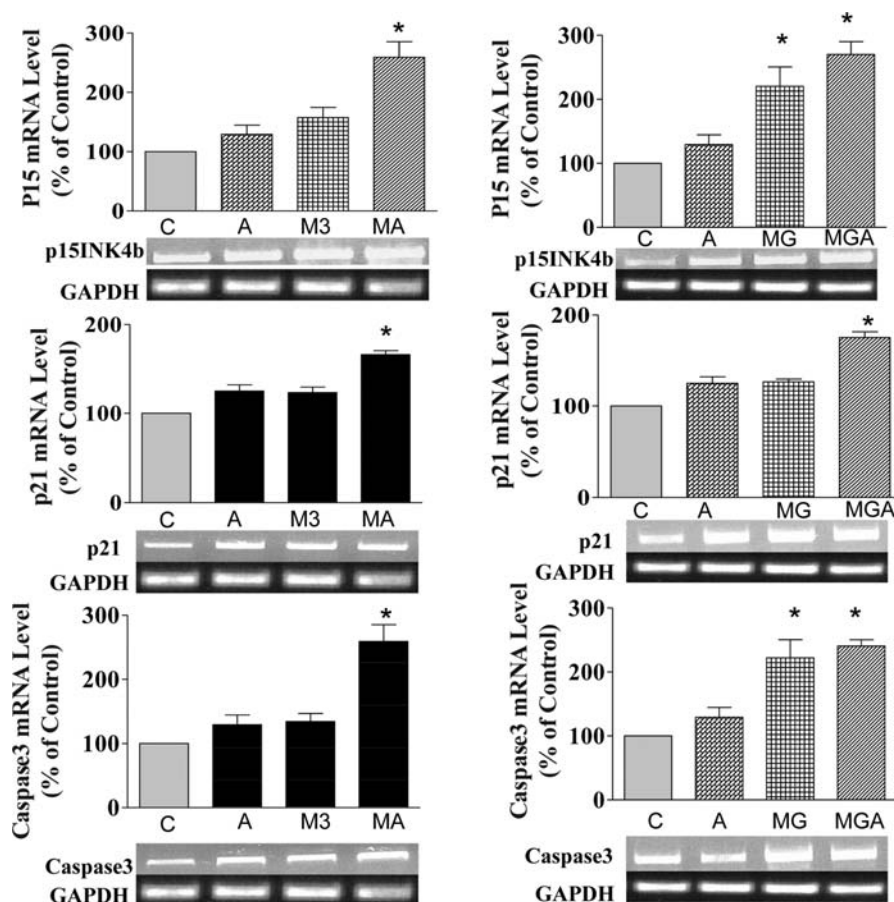
Figure 2. Effect of MG or MCT-3 alone and in combination with AZA on p15^{INK4b}, p21$^{WAF1/CIP1}$ and caspase-3 mRNA expression in HL-60 cells. Con, HL-60 cells without treatment; AZA, cells treated with 1.0 μM 5-aza-cytidine; M3, cells treated with 0.5 μM MCT-3; MA, cells treated with 0.5 μM MCT-3 + 1.0 μM AZA; MG, cells treated with 0.5 μM MGCD0103; MGA, cells treated with 0.5 μM MGCD0103+ 1.0 μM AZA. *P<0.001 vs con, n=3.
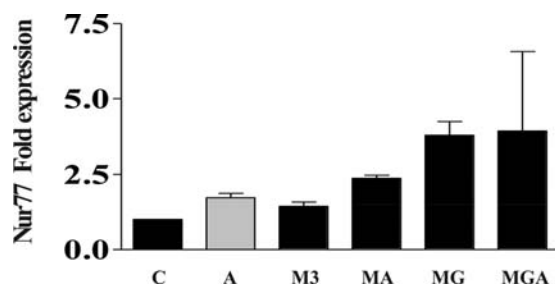

Figure 3. Effect of MG or MCT-3 alone and in combination with AZA on Nur-77 mRNA expression in HL-60 cells. Con, HL-60 cells without treatment; AZA, cells treated with 1.0 μM 5-aza-cytidine; M3, cells treated with 0.5 μM MCT-3; MA, cells treated with 0.5 μM MCT-3 + 1.0 μM AZA; MG, cells treated with 0.5 μM MGCD0103; MGA, cells treated with 0.5 μM MGCD0103+ 1.0 μM AZA.

of AZA with either MCT-3 or MG significantly increased p15^{INK4b} (MCT-3 only), p21 and caspase-3 (MCT-3 only) mRNA expression more effectively than either agent alone (Fig. 2).

Real-time PCR analysis demonstrated MG, AZA and MCT-3 increased expression of the novel tumour suppressor gene Nur77 with MG the most potent inducer as a single agent (Fig. 3). The combination of MG and AZA increased Nur77 expression nominally over single agent treatment akin to inhibitory effects on cell growth (Fig. 3).

Effect of MG, MCT-3 and/or AZA on MMP-9 mRNA and proteolytic activity in HL-60 cells. AZA mediated induction of MMP-9 expression has been previously reported (24-26) together with HDACi-mediated inhibition of this inductive effect (14). In our system AZA treatment also resulted in a significant increase in MMP-9 mRNA expression and proteolytic activity (Fig. 4). MG and MCT-3 significantly attenuated AZA-induced MMP-9 mRNA expression and proteolytic activity (Fig. 4).

Discussion

To investigate the anti-leukemic effects and molecular mechanism of action of MG and MCT-3 alone and in combination with AZA we tested the effects of these agents in growth inhibition studies using the HL-60 cell line and on expression of several cell cycle and apoptosis associated genes implicated in the pathogenesis of MDS and AML.

Growth inhibition studies clearly identify MG as the most potent inhibitor of cell growth, of any agent either alone or in combination, with no benefit gained, unlike with MCT-3,
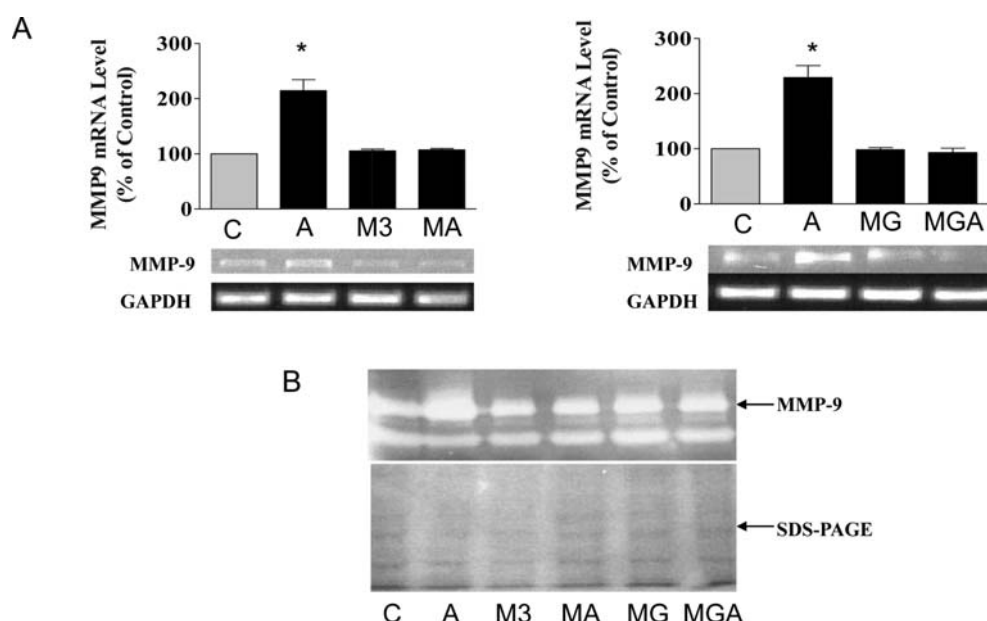
Figure 4. Effect of MG or MCT-3 alone and in combination with AZA on (A) MMP-9 mRNA expression and (B) AZA-induced MMP-9 proteolytic activity in HL-60 cells. Con, HL-60 cells without treatment; AZA, cells treated with 1.0 μM 5-aza-cytidine; M3, cells treated with 0.5 μM MCT-3; MA, cells treated with 0.5 μM MCT-3 + 1.0 μM AZA; MG, cells treated with 0.5 μM MGCD0103; MGA, cells treated with 0.5 μM MGCD0103+ 1.0 μM AZA. *P<0.01 AZA vs con, M3, MA, MG, MGA, n=3.

by adding AZA to MG (Fig. 1). Additional studies were performed to gain an understanding of the possible molecular mechanisms responsible for this effect.

The effect of AZA on p15^{INK4b} expression has been well documented and is thought pivotal in the therapeutic activity of this agent in MDS (19,27,28). Results of our studies demonstrated p15^{INK4b} expression was, as expected, reactivated by AZA (Fig. 2). Whilst MCT-3 alone increased p15^{INK4b} expression, consistent with the effects of hydroxamates, short-chain fatty acid and cyclic tetrapeptide HDACi (6,14,29), MG was more potent at induction of p15^{INK4b}. The combination of AZA and MCT-3 resulted in a significant increase in expression of p15^{INK4b} over single agent treatment although this was not identified when using MG in combination with AZA. MG as a single agent was as potent as all other agents alone or in combination at induction of p15^{INK4b} (Fig. 2).

It has been suggested that epigenetic silencing of the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ is also implicated in the pathogenesis of human cancers (7). We identified a minor increase in p21$^{WAF1/CIP1}$ expression with AZA or MCT-3 or MG alone, whilst addition of a combination of MG or MCT-3 to AZA significantly increased expression of p21$^{WAF1/CIP1}$ over single agent treatment (Fig. 2).

Recent reports suggest demethylating agents and/or HDACi induce apoptosis reliant on activation of the effector caspase-3 (8). Our results concur with these observations and indicate that MG alone was able to significantly induce caspase-3 expression and was the most potent single agent. Whilst MCT-3 as a single agent was less effective than MG at induction of caspase-3 MCT-3 in combination with AZA was more effective than either compound alone at significantly inducing caspase-3 expression (Fig. 2).

Abrogation of the expression of the tumour suppressor gene Nur-77 results in development of acute myeloid leukemia

in mice and is a common feature in leukemic blasts from human AML patients (13). We have previously observed up regulation of Nur77 expression by an HDACi alone and in combination with AZA (14). Our observations suggest that MG, AZA and MCT-3 single agent treatment results in an increase in expression of Nur-77 with MG being the more potent agent (Fig. 3). The combination of MCT-3 to AZA treatment slightly increased expression of Nur-77 over single agent treatment (Fig. 3). MG+AZA treatment was no more potent than MG alone which was more potent than any agent alone or in combination. These results identify for the first time induction of Nur77 with a novel benzamide HDACi.

The differences in gene expression profiles for the cell cycle and apoptosis genes examined in response to MG alone or combination with AZA treatment may in part provide a molecular explanation for the observed increase in single agent potency and relative lack of a combined effect of MG and AZA on inhibition of cell growth. The relatively profound induction of p15^{INK4b}, caspase-3 and Nur77 expression by MG as a single agent over AZA suggests a significant and potent targeting by MG of these critical cell cycle and apoptosis mediating genes. The lack of effect of the MG+AZA combination over MG alone also suggests that HDACi activity may be of significance in regulating expression of these genes, an effect previously described (30,31).

Our previous results identify attenuation of AZA-induced MMP-9 expression and proteolytic activity by concomitant administration of MCT-1 (14). These observations are now extended to confirm this effect using the novel hydroxamate (MCT-3) and benzamide (MG) HDACi and re-iterate that the anti-leukemia benefit postulated using the combination of AZA and HDACi may also afford protection from potential adverse effects associated with AZA treatment.

Potency of the effects on cell growth of the novel hydroxamate (MCT-3) and benzamide (MG) HDACi, alone and in combination with AZA, correlate with modulation in expression of genes implicated in cell cycle arrest, apoptosis and the recently identified tumour suppressor Nur77. Interestingly, both classes of HDACi were able to inhibit AZA-mediated induction of MMP-9 expression. These observations suggest that the enhanced anti-leukemia activity of the combination of AZA and HDACi may reside with only certain HDACi classes and may be explained by regulation of genes associated with cell cycle arrest, apoptosis and tumour suppression.

Acknowledgments

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