Histone deacetylation and DNA methylation have a central role in the control of gene expression in tumours, including transcriptional repression of tumour suppressor genes and genes involved in sensitivity to chemotherapy. Treatment of cisplatin-resistant cell lines with an inhibitor of DNA methyltransferases, 2-deoxy-5′-azacytidine (decitabine), results in partial reversal of DNA methylation, re-expression of epigenetically silenced genes including hMLH1 and sensitisation to cisplatin both in vitro and in vivo. We have investigated whether the combination of decitabine and a clinically relevant inhibitor of histone deacetylase activity (belinostat, PXD101) can further increase the re-expression of genes epigenetically silenced by DNA methylation and enhance chemosensitisation in vivo at well-tolerated doses. The cisplatin-resistant human ovarian cell line A2780/cp70 has the hMLH1 gene methylated and is resistant to cisplatin both in vitro and when grown as a xenograft in mice. Treatment of A2780/cp70 with decitabine and belinostat results in a marked increase in expression of epigenetically silenced MLH1 and MAGE-A1 both in vitro and in vivo when compared with decitabine alone. The combination greatly enhanced the effects of decitabine alone on the cisplatin sensitivity of xenografts. As the dose of decitabine that can be given to patients and hence the maximum pharmacodynamic effect as a demethylating agent is limited by toxicity and eventual re-methylation of genes, we suggest that the combination of decitabine and belinostat could have a role in the efficacy of chemotherapy in tumours that have acquired drug resistance due to DNA methylation and gene silencing.

**Keywords:** DNA methylation; MLH1; drug sensitivity; decitabine; histone deacetylase inhibitor

There is considerable interest in the potential to use epigenetic therapies in combination with existing chemotherapeutic agents, both for improving initial tumour response and for overcoming acquired drug resistance. There have previously shown that treatment of ovarian and colon cell lines with the DNA hypomethylating agent 2-deoxy-5′-azacytidine (decitabine, DAC) results in partial reversal of DNA methylation, re-expression of methylated loci such as hMLH1 and sensitisation to cisplatin and carboplatin both in vitro and in vivo (Plumb et al, 2000). In studies of human tumour xenografts in mice we were able to demonstrate that decitabine treatment reduced the level of methylation of the hMLH1 gene promoter and that this was associated with re-expression of MLH1 in a small proportion of the tumour cells at doses that clearly conferred increased sensitisation and were well tolerated.

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Received 7 October 2008; revised 12 January 2009; accepted 15 January 2009

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British Journal of Cancer (2009) 100, 758–763. doi:10.1038/sj.bjc.6604932 www.bjcancer.com

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Keywords: DNA methylation; MLH1; drug sensitivity; decitabine; histone deacetylase inhibitor
tolerated. In a phase 1 clinical trial of decitabine in combination with carboplatin in advanced solid tumours, a reduction in methylcytosine content of PBMCs was observed that was comparable to that observed in mice where chemo-sensitisation of xenografts occurred (Appleton et al, 2007). However, limited demethylation of tumour was observed. The dose limiting toxicity of decitabine was identified as myelosuppression and this toxicity plus the limited demethylation in tumours and eventual re-methylation of genes may limit the clinical use of decitabine when used alone in solid tumours.

Baylin and co-workers have shown that the combination of the histone deacetylase (HDAC) inhibitor trichostatin A with decitabine is more effective in reactivating transcription of epigenetically silenced genes such as hMLH1 in tumour cell lines than either drug alone (Cameron et al, 1999). The combination of a demethylating agent and an HDAC inhibitor has now been examined in clinical trials of haematological malignancies. However, in solid tumours it may be that epigenetic therapies may be more effective when used in combination with cytotoxic agents. We have therefore investigated whether it is possible to use a low, non-toxic dose of decitabine in combination with an inhibitor of histone deacetylase activity, belinostat (PXD101), to enhance the level of re-expression of epigenetically silenced genes in drug-resistant A2780/cp70 both in vitro and in vivo and whether this combination enhances chemo-sensitisation of xenografts.

MATERIALS AND METHODS

Cell lines

Cell line A2780/cp70 is an in vitro derived cisplatin-resistant variant of the ovarian cancer cell line A2780 originally obtained from Dr RF Ozols (Fox Chase Cancer Centre, Philadelphia, PA, USA). Cells were grown in RPMI-1640 supplemented with glutamine (2 mM) and FCS (10%). A2780/cp70 is mismatch repair deficient and does not express MLH1 due to hypermethylation of the hMLH1 gene promoter (Strathdee et al, 1999) as well as having a number of other loci hypermethylated (Leu et al, 2003).

Protein expression

For western blotting cells were grown in 25 cm² flasks and exposed to drugs as specified. Cells were harvested with trypsin/EDTA and washed two times with ice cold PBS. They were resuspended in 200 μl of lysis buffer (50 mM Hepes pH 7.0, 250 mM NaCl, 0.5% NP40) supplemented with protease inhibitors (Complete from Roche Diagnostics Ltd, Lewes, UK) and incubated on ice for 20 min. Samples were centrifuged at 12,000 g for 5 min at 4°C to remove debris. Supernatant proteins (20 μg) were separated by the NuPage electrophoresis system (Invitrogen, Paisley, UK) on 4–12% Bis-Tris gels with 4-morpholinepropanesulfonic acid SDS running buffer. The ‘Novex Xcell II’ blotting apparatus (Invitrogen) was used to transfer proteins onto Immobilon polyvinylidene fluoride membrane (Millipore, Watford, UK). The membrane was blocked for 1 h in Tris-buffered saline containing 0.02% Tween 20 and 5% powdered milk and then incubated overnight at 4°C with the primary antibody (MLH1 from Pharmingen, BD UK Ltd, Oxford, UK, MAGE-A1 from Neomarkers, Lab Vision Ltd, Cambridge, UK). The membrane was then washed and incubated for 1 h at room temperature with the secondary antibody (sheep anti-mouse HRP, Amersham, GE Healthcare, Amersham, UK). After washing, the membrane protein bands were visualised by enhanced chemiluminescence (ECL, Amersham). Band intensity was quantified by densitometry (GS-800, Bio-Rad, Hemel Hempstead, UK).

For immunohistochemistry, tumours were fixed in neutral-buffered formalin and processed as previously described (Plumb et al, 2000).

Human tumour xenografts

Animal studies were carried out under an appropriate United Kingdom Home Office Project Licence and all work conformed to the UKCCR Guidelines for the welfare of animals in experimental neoplasia. Monolayer cultures were harvested with trypsin/EDTA and resuspended in PBS. About 10⁶ cells were injected subcutaneously into the right flank of athymic nude mice (CD1 nu/nu mice from Charles River, Margate, UK). After 7–10 days when the mean tumour diameter was at ³0.5 cm, animals were randomised in groups of six for experiments. Standard sterile clinical formulations of cisplatin (Western Infirmary Pharmacy, Glasgow) decitabine (Supergen, Dublin, California, CA, UK) and belinostat (TopoTarget, Abingdon, UK) were used. Where specified, mice were pretreated with decitabine 6 days before cisplatin (6 mg kg⁻¹ intraperitoneally), when tumours were just visible. Decitabine (5 mg kg⁻¹) was administered intraperitoneally at 10:00, 13:00 and 16:00 hours (total dose 15 mg kg⁻¹ per mouse). Belinostat (40 mg kg⁻¹) was administered intraperitoneally 3 days before cisplatin where specified. Mice were weighed daily and tumour volumes were estimated by caliper measurements assuming spherical geometry (volume = d³ × π/6).

Pyrosequencing

The methylation status of specific cytosine residues in the MAGE-A1 gene promoter was determined following bisulphite modification of DNA extracted from tumours. Tumours were removed from mice and snap frozen in liquid nitrogen. Fractions were powdered in a ‘Mikro-Dismembrator’ homogeniser and DNA extracted with a BACC2 Nucleon extraction kit (Nucleon). Bisulphite modification was carried out with the CpGenome DNA modification kit (Chemicon International, Millipore, Watford, UK) according to the manufacturer’s instructions. The modified DNA was amplified by PCR with primers chosen to bracket the CpG island of the MAGE-A1 gene promoter (forward PCR: 5'-TTTTTATTTTTATTTAGGTAGG-3' and reverse PCR: Biotin-5'-TCTAAAAACACCCCAACACTAAAC-3'). The PCR was carried out in 50 μl volumes containing 2 U Faststart Taq polymerase, 10 × Faststart Buffer (Roche), 10 mM dNTPs (Applied Biosystems, Warrington, UK), 3.5 mM MgCl₂ (Roche), oligonucleotides (Biomers, www.biomers.net) at 1 μM and 2 μl of modified DNA template. A 40–μl PCR product was used for pyrosequencing according to the manufacturer’s instruction. Sixteen picomoles of the sequencing primers (5'-TGTTGTAGTTTGGTTTAT-3') were applied to detect the presence or absence of methylation.

RESULTS

Re-expression of MLH1 and MAGE-A1 in vitro by decitabine and belinostat

Treatment of MLH1 negative A2780/cp70 cells on days 1 and 2 with decitabine results in a dose-dependent re-expression of MLH1 as measured by western blot 3, 6 and 9 days after the start of treatment (Figure 1A). Belinostat treatment alone had no detectable effect on MLH1 levels. Treatment with decitabine on day 1 and with both decitabine and belinostat on day 2 results in a marked increase in MLH1 expression compared to treatment with decitabine alone on days 1 and 2. Re-expression of MLH1 was transient following treatment with decitabine at 0.1 μM (Figure 1B) but was more sustained at 0.2 μM (Figure 1C). Addition of belinostat increased the level of expression of MLH1 but did not alter the time course of re-expression or re-silencing. Decitabine treatment also induced re-expression of MAGE-A1 and again the expression was enhanced by the addition of belinostat. Re-expression of MAGE-A1 was transient at both concentrations.
of decitabine and belinostat and did not alter the time course of MAGE-A1 re-expression or re-silencing (Figure 1B and C).

Re-expression of MLH1 and MAGE-A1 in vivo by decitabine and belinostat

Treatment of mice with decitabine induces re-expression of MLH1 in A2780/cp70 xenografts and expression is maximal by about day 9 (Figure 2A and B). A similar time course is observed for MAGE-A1 expression (Figure 2C). Belinostat alone has no detectable effect on MLH1 and MAGE-A1 expression. The combination of decitabine and belinostat produces a marked increase in the level of re-expression of both MLH1 and MAGE-A1 to a greater extent than that achieved with decitabine alone (Figure 2). Gene re-expression is detectable by immunocytochemistry in only about 10% of cells and these cells appear in clusters following decitabine and belinostat treatment (Figure 2A, day 12).

Methylation of MAGE-A1

Cytosine methylation was examined at 3 CpG sites within the MAGE-A1 gene promoter. At each site the level of methylation was reduced by decitabine treatment but there was no further reduction following treatment with decitabine and belinostat in combination (Figure 3). Although only between 6 and 20% demethylation is observed at these sites it should be noted that this will be an average throughout the cell population and only those cells, which are proliferating will incorporate decitabine and become demethylated. Global 5-methyl-2'-deoxycytidine levels in DNA from the tumours taken on day 6 was measured by HPLC (Appleton et al, 2007). Decitabine treatment reduced cytosine methylation (5-methyl-2'-deoxycytidine as a percentage of total 2'-deoxycytidine) from 3.43 ± 0.16 in the control tumours to 2.78 ± 0.05 (n = 3, P<0.01) and this was not significantly different from the levels observed in the tumours taken from mice treated with decitabine in combination with belinostat (2.92 ± 0.12, n = 3). Belinostat treatment had no effect on 5-methyl-2'-deoxycytidine levels (3.70 ± 0.28, n = 3).

Effects of decitabine and belinostat pre-treatment on drug sensitivity

A2780/cp70 is resistant to the maximum-tolerated dose of cisplatin in vivo. Treatment with decitabine or belinostat either alone or in combination has no effect on tumour growth and belinostat did not sensitise tumours to cisplatin. Pre-treatment of the mice with decitabine 6 days before treatment with cisplatin results in a significant growth delay and this effect is enhanced by the
combination of decitabine and belinostat (Figure 4A). The treatments were well tolerated by the mice and there was no significant effect on body weight (Figure 4B).

DISCUSSION

We have shown clearly that the combination of low doses of decitabine and belinostat results in re-expression of epigenetically silenced genes and that when used in vivo in mice the combination can sensitise drug-resistant tumours to cisplatin more effectively than either drug alone.

We have already established that decitabine can be used to sensitise drug-resistant tumours to a number of clinically relevant cytotoxic drugs including cisplatin, carboplatin, epirubicin and temozolomide (Plumb et al., 2000). The inclusion of pharmaco-dynamic measurements in a phase 1 trial of decitabine and carboplatin has enabled us to show that decitabine can induce in patients the levels of demethylation in surrogate PBMCs seen in our mouse studies at doses that cause chemo-sensitisation (Appleton et al., 2007). However, it is also clear from the phase 1 trial that demethylation in PBMCs by decitabine is limited by the myelosuppressive activity of the drug. The level of demethylation observed in tumours was limited and heterogeneous between patients. In order to potentially increase the reversal of epigenetic silencing by decitabine, we have examined the addition of an HDAC inhibitor on re-expression of epigenetically silenced genes and chemo-sensitisation. Belinostat alone has no effect on MLH1 expression and this is consistent with the observation that histone deacetylase inhibitors cannot induce the expression of genes silenced due to promoter methylation (Suzuki et al., 2002). From the results with the cell line it is clear that belinostat can enhance the effects of decitabine on gene re-expression (Figure 1). Although belinostat increases the level of re-expression of both MLH1 and MAGE-A1 it does not appear to alter the kinetics of re-expression (Figure 1). Although belinostat increases the level of re-expression of both MLH1 and MAGE-A1 it does not appear to alter the kinetics of re-expression. This is consistent with the observation that the HDAC inhibitor 4-phenylbutyric acid does not inhibit re-silencing of p16 after decitabine treatment (Egger et al., 2007). Re-expression of MAGE-A1 is transient. It can be detected by 3 days after treatment but is lost after about 26 days. Re-expression of MLH1 is also transient at lower concentrations of decitabine (0.1 μM), but is more sustained at the higher concentration (0.2 μM) such that it remains detectable after 26 days. This may reflect the long half-life of the protein or...
may be due to a slower rate of gene re-methylation at the higher
dose of decitabine.

To study the combination in human tumour xenografts we used
the same schedule for decitabine that was shown to sensitis
the tumours to cisplatin (Plumb et al, 2000) and attempted to improve
on this response. Initial studies investigated the effects on gene re-
expression and we have shown that a single dose of belinostat
administered 3 days after decitabine treatment results in an increase
in expression of both MLH1 and MAGE-A1 to a level greater that is
seen with decitabine alone (Figure 2). MLH1 and MAGE-A1 gene re-
expression is detected in about 6% of cells following treatment with
decitabine and increases to about 10 – 12% when mice are treated with
the combination of decitabine and belinostat. The apparent clustering
of cells that re-express MLH1 and MAGE-A1 in the xenografts could
represent areas of active proliferation within the tumours, which
would be consistent with decitabine being incorporated into DNA
during S-phase only and cell proliferation being required for
demethylation. For the MAGE-A1 gene promoter decitabine treat-
ment results in a decrease in the methylation of all three CpG sites
studied (Figure 3). However, there is no further reduction in
methylation following addition of belinostat to decitabine, which
suggests that the enhanced gene expression observed with the
combination is not due to direct effects on gene methylation. A study
of the combination of decitabine and trichostatin A on MLH1
expression also concluded that the effect of the HDAC inhibitor was
not due to a further reduction in DNA methylation (Cameron et al,
1999). It is possible that the HDAC inhibitor allows increased access
of the transcription factors to the demethylated gene as a result of
increased levels of histone acetylation and the resulting chromatin
remodelling (Egger et al, 2007).

Re-expression of MLH1 is clearly apparent by 6 days
after treatment and is maximal by about 9 days. As A2780/cp70
is a rapidly growing tumour, we treated with the cytotoxic drug as
early as possible after decitabine treatment (day 6). This is the
schedule used previously for decitabine alone (Plumb et al, 2000).
A2780/cp70 xenografts are resistant to cisplatin. However,
treatment with decitabine sensitises the tumours to cisplatin and
the growth delay is further enhanced by the addition of belinostat
(Figure 4). These results give clear support to the proposal to
use decitabine in combination with a histone deacetylase inhibitor
to enhance the chemo-sensitisation observed with decitabine
alone.

Neither decitabine, belinostat nor the combination had any
effect on tumour growth. This is not surprising as we have not
attempted to use these drugs in the optimal schedule for
antitumour activity. We have already shown that A2780/cp70 is
sensitive to belinostat when mice are treated daily for 7 days
(Plumb et al, 2003). As the aim was to combine the epigenetic
therapies with a cytotoxic drug we have intentionally used low,
non-toxic doses of the agents. Although decitabine treatment
results in a reduced MAGE-A1 methylation in PBMCs the gene is
not re-expressed and this may be due to a lack of the necessary
transcriptional activators (Karpf et al, 2004). Few studies have
examined the effects of demethylating agents on normal cells;
however, there is some evidence that fewer genes become
demethylated than in tumour cells (Liang et al, 2002). This
suggests that epigenetic therapies will not necessarily be associated
with genome-wide effects in normal tissues. Furthermore, the
combination of a low dose of decitabine and the HDAC inhibitor
phenylbutyrate has been shown to inhibit carcinogen-induced lung
tumours in mice (Belinsky et al, 2004). This raises the possibility
that in addition to sensitising drug-resistant tumours to
chemotherapy the epigenetic therapy might also protect the
normal tissues from some of the damage caused by the cytotoxic
agent.

As the dose of decitabine that can be given to patients and hence
the maximum pharmacodynamic effect as a demethylating agent is
limited by toxicity and eventual re-methylation of genes, we
suggest that the combination of decitabine and belinostat could
have a role in increasing the efficacy of chemotherapy in tumours
that have acquired drug resistance due to DNA methylation and
gene silencing.

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