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Hydroxycarbamide in combination with azacitidine or decitabine is antagonistic on DNA methylation inhibition

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Summary

Azacitidine and decitabine are cytidine analogues that inhibit DNA methylation, and are used to treat myeloid haematological malignancies. Hydroxycarbamide (HC) (also known as hydroxyurea), a ribonucleotide reductase (RR) inhibitor, blocks the conversion of ribonucleotides to deoxyribonucleotides, and is also used to treat leukaemia and sickle-cell disease. Azacitidine is a ribonucleoside and decitabine is a deoxyribonucleoside; therefore, we hypothesized that inhibition of RR by HC would be antagonistic to azacitidine and synergistic to decitabine. HL-60 and T24 cancer cell lines were treated with azacitidine or decitabine in combination with HC and DNA methylation of *LRE1*, *MAGEA1* and *CDKN2A* was quantitatively measured by bisulphite-polymerase chain reaction pyrosequencing. Surprisingly, we found that HC blocked the ability of both azacitidine and decitabine to inhibit DNA methylation and this antagonistic effect was attributable to the arrest of the cell cycle induced by HC. However, this antagonism could be avoided with sequential treatment of HC followed by azacitidine or decitabine. This data suggest that concurrent combination of HC blocks the ability of azacitidine and decitabine to inhibit DNA methylation and therefore these drugs should be used sequentially.

Keywords: azacitidine, decitabine, hydroxycarbamide, myelodysplastic syndromes, DNA methylation.

Aberrant DNA methylation has been found in almost all tumour types, including leukaemia (Issa *et al*, 1996; Takai *et al*, 2001; Melki & Clark, 2002), and is associated with the aberrant silencing of tumour suppressor genes in cancer. Drugs that inhibit DNA methylation are currently used to treat myelodysplastic syndromes and are under clinical investigation for other types of cancer. 5-Azacytidine (azacitidine) and 5-aza-2′-deoxycytidine (decitabine) are pyrimidine analogues that inhibit DNA methylation. Both of these drugs are cytidine analogues with a nitrogen substitution in the 5-position of the pyrimidine ring. Incorporation of this azacytosine ring into DNA in place of cytosine leads to covalent trapping of DNA methyltransferase enzyme and subsequently suppresses DNA methylation (Christman, 2002). Treatment with azacitidine or decitabine has been shown to be effective in reversing DNA hypermethylation, reactivating silenced tumour suppressor genes, and suppressing tumour growth and leukaemic cell proliferation (Christman, 2002). Treatment with these drugs has also been clearly shown to inhibit DNA methylation in leukaemia patients, and this decrease in DNA methylation has been shown to correlate with both dose of the drug and response to the drug (Yang *et al*, 2006). There is a growing clinical interest in combining these DNA methylation inhibitors with epigenetic therapies, such as histone deacetylase inhibitors (Yoo & Jones, 2006) or standard cytotoxic chemotherapies. Hydroxycarbamide (HC) (also known as hydroxyurea) is used clinically in a variety of haematological disorders, including acute myeloid leukaemia (AML), chronic myeloid leukaemia, myeloproliferative diseases and sickle-cell disease (Donehower, 1992; Mehanna, 2001; Silver, 2003). In clinical trials using decitabine, HC is often used to control peripheral blast counts during the initial part of induction chemotherapy (Issa *et al*, 2005). In addition, both HC and DNA methylation inhibitors have been used in the treatment of sickle-cell disease

(De Simone *et al*, 1982; Saunthararajah *et al*, 2003). However, the effect of HC on the ability of these drugs to inhibit DNA methylation is not known. In the present study, we hypothesized that concurrent treatment of HC with DNA methylation inhibitors might generate synergism or antagonism because of differences between the biochemical processing pathways involved in the incorporation of decitabine and azacitidine.

Similar to other cytidine analogues, both azacitidine and decitabine are assumed to be taken up into the cell by the human equilibrative nucleoside transporter 1 (Mackey *et al*, 1998; Hubeek *et al*, 2005). 5-Aza-2′-deoxycytidine is a deoxyribonucleotide and therefore initially phosphorylated by deoxycytidine kinase (DCK) to 5-aza-2′deoxycytidine monophosphate and subsequently phosphorylated to 5-azadeoxycytidine triphosphate, which is incorporated into DNA by DNA polymerase (Yoo & Jones, 2006). In contrast, 5-azacytidine is a ribonucleoside and phosphorylated to 5-azacytidine monophosphate by uridine/cytidine kinase. 5-Azacytidine monophosphate will undergo further phosphorylation to its triphosphate form, and be incorporated into RNA. Alternatively, once azacitidine is converted to a diphosphate (aza-CDP), it can be reduced by ribonucleotide reductase (RR) to 5-aza-2′deoxyazacytidine diphosphate (aza-dCDP) and this deoxyribose form can be incorporated into DNA, leading to DNA methylation inhibition (Fig 1). As decitabine is in the form of a deoxyribonucleoside, it can be more readily incorporated into DNA and is thus believed to be a more potent inhibitor of DNA methylation than azacitidine (Jones *et al*, 1983).

Hydroxycarbamide is an antimetabolite drug that inhibits RR, the rate-limiting enzyme in DNA synthesis (Colly *et al*, 1992). Biologically, the inhibition of RR leads to a depletion of nucleotide pools and arrests of the cell cycle in S-phase. Clearly, with azacitidine, HC will block RR and prevent the conversion of 5-aza-CDP to 5-aza-dCDP. Thus, HC will probably be antagonistic to the ability of azacitidine to inhibit DNA methylation. However, decitabine in combination with HC could have two possible effects. Firstly, arrest of the cell cycle in S-phase could theoretically inhibit the incorporation of deoxynucleotides into DNA and thus block its ability to inhibit DNA methylation. Alternatively, it is also possible that the depletion of nucleotides could in fact increase the incorporation of decitabine into DNA and thus potentiate its demethylating ability (Colly *et al*, 1992). This study examined whether there is synergism or antagonism between HC and the DNA methylation inhibitors, azacitidine and decitabine.

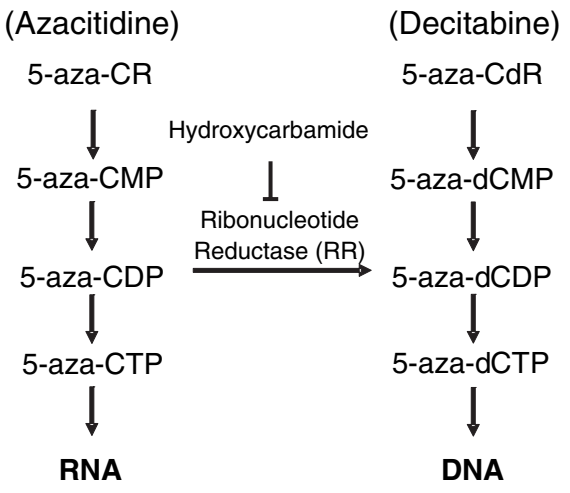

Fig 1. Metabolism of azacitidine (5-aza-CR, VIDAZA) and decitabine (5-aza-CdR, DACOGEN). Azacitidine, a ribose cytidine analogue is phosphorylated and incorporated into RNA. However, 5-aza-CDP can be converted to 5-aza-dCDP by ribonucleotide reductase (RR), which will allow azacitidine to be incorporated into DNA and inhibit DNA methylation. Hydroxycarbamide inhibits RR. In contrast to azacitidine, decitabine, a deoxycytidine analogue, is phosphorylated to 5-aza-dCTP and directly incorporated into DNA.

Materials and methods

Cell culture and drug treatments

HCT116, PC-3, T24, HL-60, K-562, MCF7, Hep G2 and SK Hep cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured using standard methods. Azacitidine (MP biomedicals, Aurora, OH, USA), decitabine (Sigma, St Louis, MO, USA) and HC (TCI America, Tokyo, Japan) were dissolved in sterilized water, aliquoted and frozen at −70°C until use. Aphidicolin (Novagen, La Jolla, CA, USA) was dissolved in 100% ethanol. Cells (2×10^4 cells/ml) were plated and cultured for 24 h prior to treatment with azacitidine or decitabine daily for 3 days. Media was changed daily and new drug was added. Finally, cells were cultured 1 day without treatment prior to being harvested. Increasing concentrations of HC or aphidicolin were used in combination with azacitidine or decitabine. For cell cycle analysis, T24 cells were treated daily for 3 days with increasing concentrations of HC or aphidicolin and followed by flow cytometry analysis (fluorescence-activated cell sorting, FACS). For sequential treatment of HC with azacitidine or decitabine, T24 cells were treated with 0·1 mmol/l HC for 8 h; media was changed and treated with 5 μmol/l azacitidine or 0·5 μmol/l decitabine for 24 h.

DNA extraction and bisulphite modification

DNA from cell lines was extracted by using the ZR-96 well genomic DNA kit™ (Zymo Research, Orange, CA, USA) as recommended by the manufacturer. Bisulphite modification of genomic DNA was performed by using the EZ-96 DNA Methylation-Gold Kit™ (Zymo Research, Orange, CA, USA), according to the manufacturer's recommended protocol.

Quantification of DNA methylation by pyrosequencing

Methylation analysis of Line Retrotransposable Element 1 (*LRE1*, also known as *LINE1*), *CDKN2A* (also known as *P16*)

promoter and *MAGEA1* was performed by bisulphite-polymerase chain reaction (PCR) pyrosequencing assay using a previously described assay (see Appendix S1 for details in Supplementary material) (De Smet *et al*, 1999; Kawamoto *et al*, 2006; Yang *et al*, 2006). *LRE1* is a DNA repetitive element that is abundant in the human genome and serves as a surrogate marker for global DNA methylation (Yang *et al*, 2004), *CDKN2A* is a tumour suppressor gene frequently found methylated in cancer (Kawamoto *et al*, 2006), and *MAGEA1* is a gene that is heavily methylated in cancer and normal tissue and serves as a excellent marker for DNA methylation decrease.

Measurement of CDKN2A expression

Total RNA was extracted from T24 cells using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed by using the first strand cDNA synthesis kit (NEB, Beverly, MA, USA). The primers and conditions for *CDKN2A* and *GAPDH* are previously described (Schmutte *et al*, 1996; Fang *et al*, 2003). Negative controls for PCR were run under the same conditions without reverse transcriptase (data not shown).

Measurement of cell cycle distribution using flow cytometry

Cells were pelleted, resuspended in 0·2-ml phosphate-buffered saline (PBS), and fixed by the addition of 1 ml of ice-cold 70% ethanol/30% PBS. Fixed cells were pelleted, vigorously resuspended in PBS, and incubated for 30 min at 37°C with 100 μg/ml RNase A (Novagen, La Jolla, CA, USA) and 40-μg propidium iodide (Calbiochem, La Jolla, CA, USA). The fluorescence of the stained cells was analysed using a FACScan flow cytometer. Data were analysed with CELL QUEST software.

Statistical analysis

All values are reported as mean ± SD. Data were analysed by using the Student's *t*-test using STATVIEW software (Abacus, Berkeley, CA, USA). The significance level was set at $P < 0{\cdot}05$.

Results

Hydroxycarbamide is antagonistic to the effects of azacitidine and decitabine on DNA methylation

To study the effects of HC on DNA methylation inhibitors, we first tested the ability of decitabine to inhibit DNA methylation on a variety of different cell lines. Eight cell lines were treated with low-dose decitabine for 3 days, and *LRE1* methylation was used as a surrogate for global DNA methylation changes. As expected, DNA methylation decreased in all cell lines treated; however, the response to decitabine varied (Fig 2). T24 cells showed the greatest relative change in DNA methylation and therefore were chosen for further studies. HL-60 only showed a 12% decrease, but was chosen for further studies because it is a myeloid leukaemia cell line and drugs that induce hypomethylation are known to be active in myeloid leukaemia (Issa *et al*, 2004, 2005).

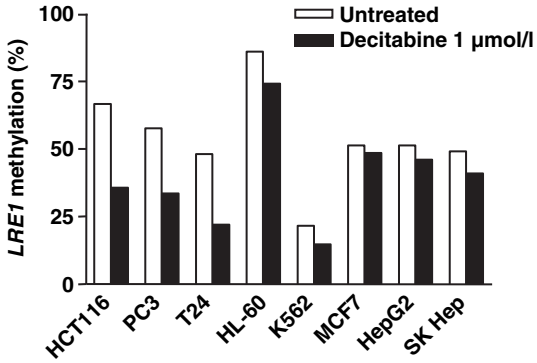

Fig 2. Effect of decitabine on *LRE1* methylation on cancer cell lines. HCT116, PC-3, T24, HL-60, K-562, MCF7, Hep G2 and SK Hep cell lines were treated with 1 μmol/l decitabine daily for 3 days and DNA methylation was quantitated by bisulphite-polymerase chain reaction pyrosequencing of the *LRE1* repetitive DNA elements.

HL-60 cells were treated for 3 days with 0·5 μmol/l or 5 μmol/l azacitidine or decitabine in combination with HC at increasing concentrations (0, 0·01, 0·1, 0·5 and 1 mmol/l). DNA methylation was quantitatively assessed by using bisulphite-PCR pyrosequencing of the *LRE1* repetitive elements (Fig 3A) and *MAGEA1* (Fig 3B). As expected, the higher concentration of azacitidine was more effective in inhibiting DNA methylation. In HL-60 cells treated with 0·5 or 5 μmol/l azacitidine and HC in combination, the effect of HC clearly blocked the ability of azacitidine to inhibit DNA methylation in a dose-dependent manner. Doses of HC >0·1 mmol/l completely inhibited the ability of 0·5 μmol/l azacitidine to inhibit methylation of *LRE1* ($P = 0{\cdot}067$). Hydroxycarbamide at 0·1 mmol/l decreased the ability of 5 μmol/l azacitidine to inhibit DNA methylation ($P = 0{\cdot}001$), and doses of HC above 0·5 mmol/l completely inhibited 5 μmol/l azacitidine ($P < 0{\cdot}001$).

The lower dose of decitabine was more effective in decreasing both *LRE1* and *MAGEA1* methylation in HL-60 cells. This is consistent with previous work, which showed lower doses of the drug to be more effective in inhibiting DNA methylation (Jones & Taylor, 1980). Overall, decitabine was less effective at inhibiting DNA methylation in HL-60 cells compared with azacitidine, which was unexpected as decitabine, being a deoxyribonucleotide, is thought to be more effective at inhibiting DNA methylation. Surprisingly, HC also inhibited the ability of decitabine to inhibit DNA methylation in HL-60 cell lines. Baseline *LRE1* methylation was 86% ± 0·9% and was decreased to 74% ± 1·3% or 77% ± 1·6% with decitabine at 0·5 or 5 μmol/l, respectively (Fig 3A). However, HC significantly blocked DNA methylation inhibition at doses of 0·1 mmol/l or greater with statistical significance (from $P = 0{\cdot}02$ to $P < 0{\cdot}001$ depending on HC dose).

These data examining global DNA methylation were confirmed by examining DNA methylation of a specific gene, *MAGEA1*. Increasing doses of HC were able to inhibit the ability

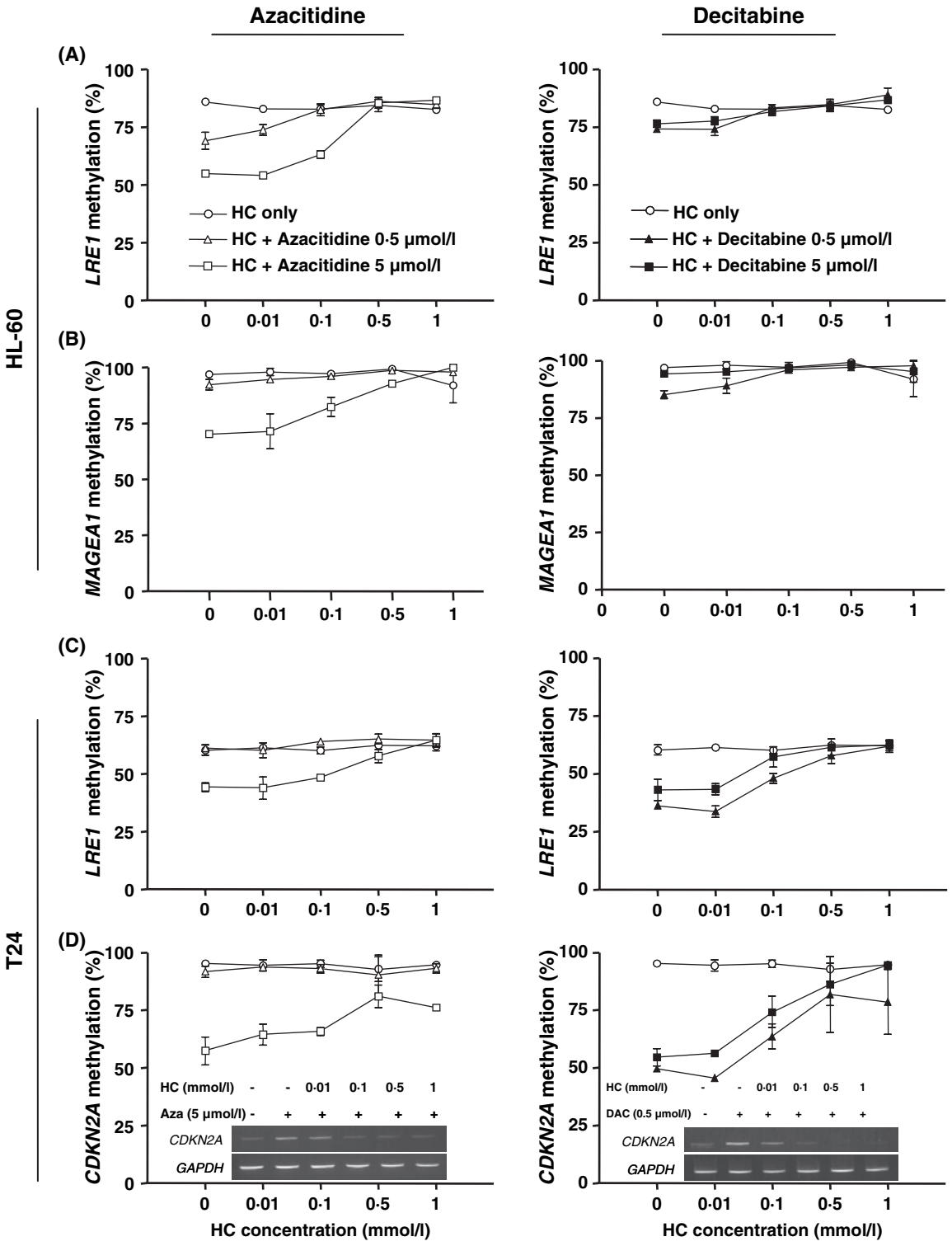

Fig 3. Hydroxycarbamide (HC) blocked the ability of azacitidine or decitabine to inhibit DNA methylation in HL-60 and T24 cells. Both HL-60 and T24 cells were treated with 0·5 µmol/l (triangle) or 5 µmol/l (square) azacitidine (open symbols) or decitabine (solid symbols) daily for 3 days. HC (open circle) was used in combination with azacitidine or decitabine at increasing doses (0, 0·01, 0·1, 0·5 and 1 mmol/l). DNA methylation of *LRE1* (A) and the *MAGEA1* promoter (B) in HL-60 cells, and DNA methylation of *LRE1* (C) and the *CDKN2A* promoter (D) in T24 cells were quantitated by bisulphite-polymerase chain reaction pyrosequencing. Reverse transcription polymerase chain reaction analysis shows that HC has an antagonistic effect on reactivation of *CDKN2A* expression after concurrent treatment of HC with azacitidine (5 µmol/l) or decitabine (0·5 µmol/l) (D).

of both azacitidine and decitabine to induce hypomethylation of *MAGEA1* (Fig 3B). Doses of HC as low as 0·1 mmol/l could significantly inhibit the ability of azacitidine and decitabine to inhibit *MAGEA1* methylation at increasing doses of HC (from $P = 0{\cdot}013$ to $P < 0{\cdot}001$ depending on HC dose).

As the DNA methylation changes induced by decitabine in HL-60 were small, we tested the T24 cell line, which showed larger effects by decitabine (Fig 2) in *LRE1* and *CDKN2A* promoter (Fig 3C and D). Both azacitidine and decitabine inhibited DNA methylation in T24 cells, and HC was found to inhibit this effect in T24 cells for both drugs. Hydroxycarbamide in combination with either azacitidine or decitabine was shown to be strongly antagonistic in inhibiting DNA methylation. In addition, both azacitidine and decitabine reactivated *CDKN2A* expression, but this reactivation was decreased when given with increasing doses of HC (Fig 3D).

S-phase arrest of cell cycle by hydroxycarbamide and aphidicolin

We hypothesized that the inhibitory effect of HC on DNA methylation inhibitors was attributable to inhibition of the cell cycle. Hydroxycarbamide is a known inhibitor of the cell cycle and both decitabine and azacitidine are known to be S-phase specific drugs to inhibit DNA methylation. To examine the effects of HC on the cell cycle, T24 cells were treated with 0·01 to 1 mmol/l HC for 3 days. FACS analysis showed that 0·01, 0·1 and 0·5 mmol/l HC caused 15%, 18% and 45% respectively of T24 cells to be arrested in S-phase (Fig 4). This level of cell cycle inhibition was consistent with the level of DNA methylation observed.

To determine the effect of cell cycle arrest on DNA methylation inhibitors, we investigated the effect of another cell cycle inhibitor that works through a different mechanism than HC. As a control of S-phase arrest, we also treated cells with aphidicolin, a direct α polymerase inhibitor, which is also known to arrest cells in S-phase. A quantity of 0·01, 0·1, 1 μmol/l of aphidicolin treatment arrested 20%, 23% and 57% respectively of cells in S-phase (Fig 4). Clearly, both HC and aphidicolin could block the cell cycle. It should be noted that at higher doses (1 mmol/l HC and 10 μmol/l aphidicolin) only 33% and 11% of cells were arrested in S-phase, but corresponding increases of cells were found in G_1-phase.

Aphidicolin also blocks the ability of methylation inhibitors

We extended our investigation to determine if S-phase arrest alone could inhibit the ability of azacitidine and decitabine to

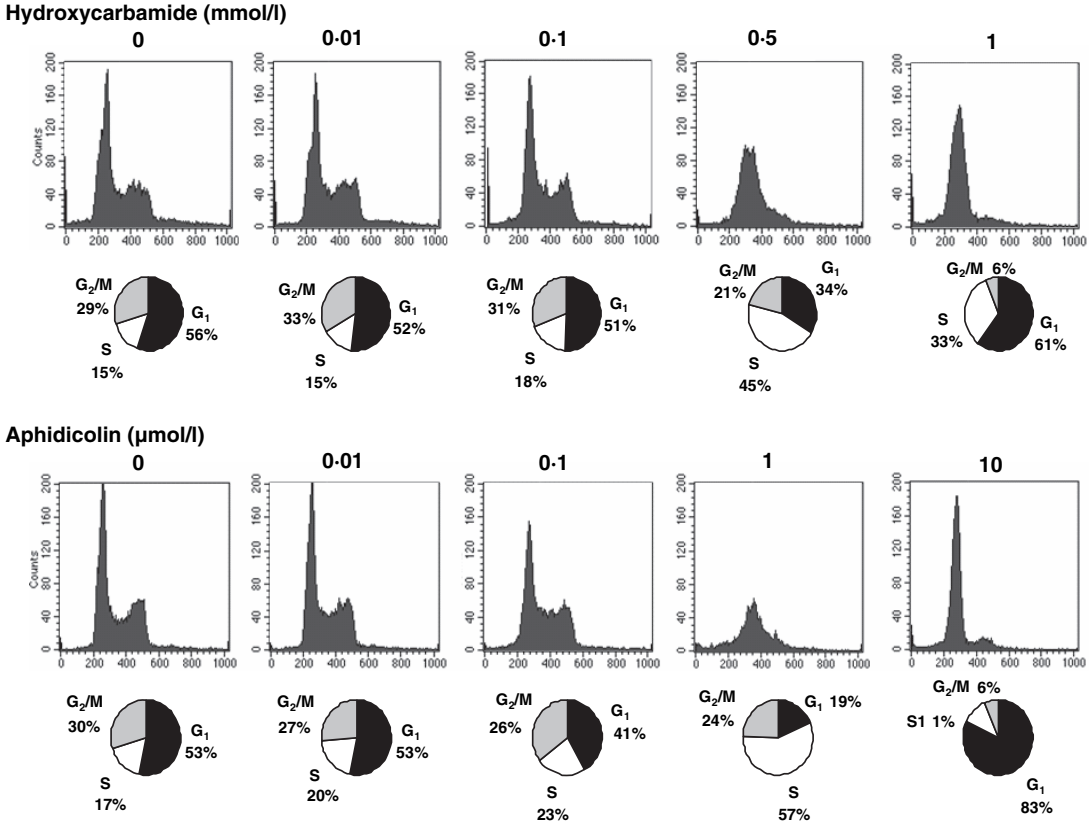

Fig 4. Effect of hydroxycarbamide (HC) and aphidicolin on the cell cycle. T24 cells were treated with HC or aphidicolin at increasing doses daily for 3 days, stained with propidium iodide, and analysed by fluorescence-activated cell sorting analysis. Both HC and aphidicolin induced S-phase arrest of the cell cycle.

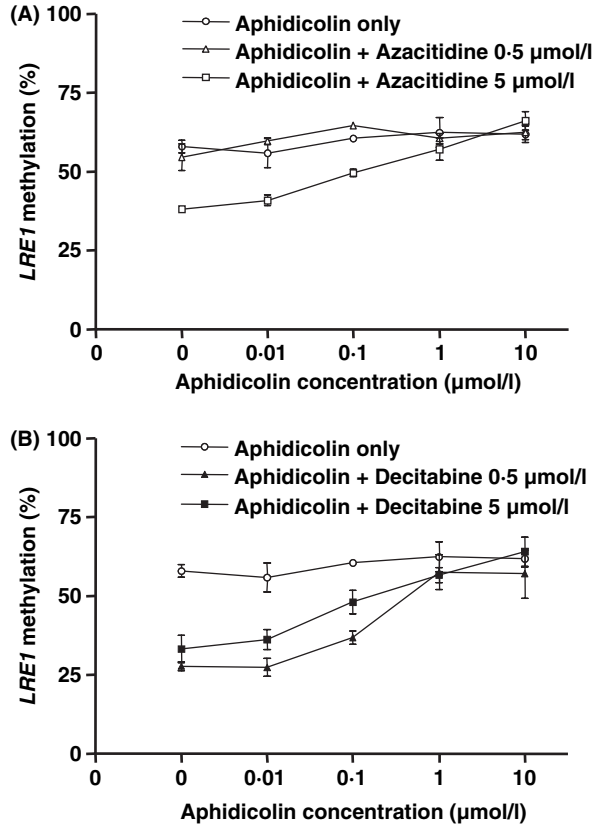

Fig 5. Aphidicolin, a DNA polymerase inhibitor, blocked the ability of azacitidine (A) or decitabine (B) to inhibit DNA methylation. T24 cells were treated with azacitidine or decitabine in combination with aphidicolin at increasing doses (0, 0·01, 0·1, 1 and 10 μmol/l). DNA methylation of *LRE1* was quantitated by bisulphite-polymerase chain reaction pyrosequencing.

inhibit DNA methylation. Aphidicolin was used in combination with both azacitidine and decitabine to determine the affect of S-phase arrest on the ability of these drugs to inhibit DNA methylation. T24 cells were treated with azacitidine and decitabine in combination with aphidicolin and assessed the changes in *LRE1* methylation using bisulphite-PCR pyrosequencing (Fig 5). Aphidicolin alone had no effect on DNA methylation. A quantity of 5 μmol/l azacitidine decreased DNA methylation from 58% ± 2·0% to 38% ± 0·8% ($P < 0·001$), but this inhibition of DNA methylation was blocked by increasing doses of aphidicolin. For cells treated with 0·01 mmol/l vs. 0·5 mmol/l HC, there was a threefold increase in S-Phase arrest and a 13·1% and 24·5% inhibition of DNA methylation by azacitidine 5 μmol/l and decitabine 5 μmol/l, respectively. For cells treated with 0·01 μmol/l vs. 1 μmol/l aphidicolin, there was an approximate threefold increase in S-phase arrest and a 16·3% and 30·2% inhibition of DNA methylation by azacitidine 5 μmol/l and decitabine 5 μmol/l, respectively. This showed that the inhibition of azacitidine and decitabine by HC and aphidicolin was proportional to the effect seen on cell cycle arrest.

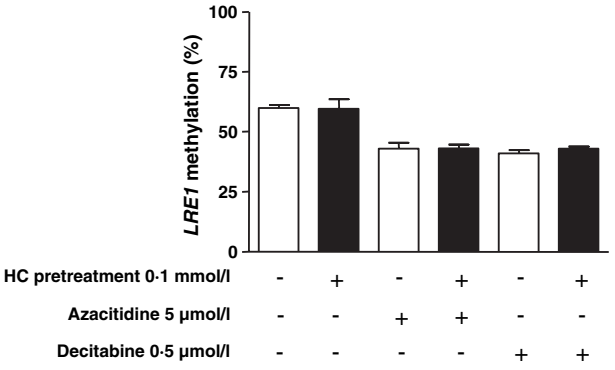

Fig 6. Sequential treatment of hydroxycarbamide (HC) with azacitidine or decitabine. T24 cells were treated with 0·1 mmol/l HC for 8 h and then treated with 5 μmol/l azacitidine or 0·5 μmol/l decitabine for 24 h. DNA methylation of *LRE1* was quantitated by bisulphite-polymerase chain reaction pyrosequencing.

Sequential treatment avoids the antagonistic effect of hydroxycarbamide in combination with methylation inhibitors

As the HC-induced S-phase arrest resulted in a strong antagonistic effect in combination with DNA methylation inhibitors, we investigated if the sequential treatment of HC with DNA methylation inhibitors avoided the antagonistic effect on DNA methylation. T24 cells were pretreated with 0·1 mmol/l HC for 8 h as previously reported (Zhou *et al*, 2002), and then treated with 5 μmol/l azacitidine or 0·5 μmol/l decitabine for 24 h, and *LRE1* methylation was subsequently quantitated by bisulphite-PCR pyrosequencing (Fig 6). Sequential treatment of HC first, followed by 5 μmol/l azacitidine and 0·5 μmol/l decitabine, reduced *LRE1* methylation from 60% ± 1·2% to 43% ± 2·4% ($P = 0·012$) and 43% ± 1·5% ($P = 0·012$) respectively and avoided the previously observed antagonism. However, the decrease in DNA methylation of sequential treatment was not significantly different from 5 μmol/l azacitidine ($P = 0·956$) or 0·5 μmol/l decitabine ($P = 0·157$) alone, and the synergy of sequential treatment was not observed, as previously reported with other pyrimidine analogues (Zhou *et al*, 2002).

Discussion

The successful combination of HC with either azacitidine or decitabine will be clinically important. Decitabine and azacitidine both have activity in AML. However, because of the delayed therapeutic action of hypomethylating agents, HC will be required to transiently control white blood cell counts until control of the leukaemia is achieved.

This study showed that increasing doses of HC in combination with DNA methylation inhibitors *in vitro* had a strong antagonistic effect on inhibition of DNA methylation. We speculated that this antagonistic effect was due to depletion of nucleotide pools and arrest of the cell cycle. Aphidicolin, which

also causes S-phase arrest by a different mechanism, was used to confirm this. Aphidicolin had an identical antagonistic effect on azacitidine and decitabine that was demonstrated by HC. In a previous study (Nyce *et al*, 1986), HC and aphidicolin were shown to inhibit DNA synthesis and increased DNA methylation, but we did not observe this in our study.

Traditionally, decitabine is thought to be a more potent inhibitor of DNA methylation, because it is a deoxyribose and is phosphorylated by DCK. In contrast, azacitidine, which is phosphorylated by uridine/cytidine kinase, also requires reduction by RR before incorporation into DNA and inhibition of DNA methylation (Fig 1). However, we showed that the cell line HL-60 is more sensitive to azacitidine than decitabine. The reason for this is unknown, but may be related to the different phosphorylation enzymes used by each drug. DCK has been shown to be mutated in some leukaemia samples, which would lead to decreased activity of DCK and decitabine. This may be clinically relevant in the treatment of some leukaemia as cytarabine (Ara-C) is also phosphorylated by DCK, and therefore azacitidine may be a better choice in some Ara-C resistant patients.

Our study underscores the importance of the cell cycle on cytidine analogues that are dependent on the S-phase for incorporation into DNA and activity. This must be considered in future studies that look to combine azacitidine or decitabine with other chemotherapeutic agents. Ara-C, which also inhibits the cell cycle and is also active against myeloid leukaemias, must be used carefully, as there are two potential mechanisms by which it can antagonize DNA methylation inhibitors. Firstly, it can cause antagonism by inhibiting the cell cycle and preventing DNA incorporation. Secondly, it can cause competitive inhibition for DCK or other kinases.

In addition, HC is used in the treatment of sickle-cell disease. There are several studies that have shown that azacitidine and decitabine can also be used to treat sickle-cell disease (De Simone *et al*, 1982; Saunthararajah *et al*, 2003). Epigenetic agents, such as azacitidine and decitabine, are particularly attractive in sickle-cell disease because of their ability to upregulate gamma-globin and therefore are used to treat haemaglobinopathies (Saunthararajah *et al*, 2003). In combining HC with DNA methylation inhibitors in treatment, timing is important. When HC was administered 30 min prior to azacitidine, stimulation of fetal haemoglobin production was decreased by at least 70%, compared with azacitidine alone (De Simone *et al*, 1982). This result was consistent with our finding that combining HC with DNA methylation inhibitors had an antagonistic to the ability of methylation inhibitors. It would be clinically beneficial if the antagonistic effect could be circumvented.

In our study, although combination of 0·1 mmol/l HC with DNA methylation inhibitors showed an antagonistic effect on DNA methylation inhibition, pretreatment of 0·1 mmol/l HC followed by DNA methylation inhibitors had no significant effect on DNA methylation changes. This result was consistent with the sequential treatment of HC followed by gemcitabine or azacitidine (De Simone *et al*, 1982; Zhou *et al*, 2002). Zhou *et al* (2002) showed that pretreatment of HC inhibited RR M2 subunit activity and depleted dCTP pools, which led to increased incorporation of gemcitabine, another deoxycytidine analogue. We were unable to produce similar results with decitabine or azacitidine (data not shown). A Phase I clinical study of HC in combination with gemcitabine showed that treatment with HC 24 h prior to gemcitabine administration is safe and has activity (Yen *et al*, 2002). In addition, sequential combinational treatment of HC and azacitidine appeared to have a greater effect on the onset of myelosuppression than azacitidine alone (Lomen *et al*, 1980).

We clearly showed that sequential treatment of HC, followed by DNA methylation inhibitors, could avoid an antagonistic effect on the ability of DNA methylation inhibitors. Pharmacodynamic studies have shown that HC is absorbed through the gastrointestinal tract and reaches a peak in plasma concentration within 1 h. Hydroxycarbamide was detected in higher concentrations in leucocytes 2 h after oral administration and was completely cleared from the circulation within 6 h (Gwilt & Tracewell, 1998; Navarra & Preziosi, 1999). Following oral administration of 40 mg to 80 mg/kg, the plasma concentration of HC reached 0·5 to 2·0 mmol/l (Donehower, 1992). Thus, doses of HC used clinically are equivalent to the doses used *in vitro* to inhibit azacitidine and decitabine in our studies. We predict a single 500-mg tablet of HC would achieve a plasma concentration of approximately 0·09 mmol/l in a 70-kg individual, which would inhibit azacitidine and decitabine. Taken together, it is likely that the antagonistic effects of HC with azacitidine or decitabine can be avoided by sequencing treatment. However, HC can be completely cleared from circulation within 6 h (Navarra & Preziosi, 1999); therefore, it could be discontinued the day before initiating treatment with a DNA methylation inhibitor. Clinical studies combining HC with azacitidine or decitabine in combination or sequentially would be necessary to confirm these data and are being planned.

Acknowledgements

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Supplementary material

The following supplementary material is available for this article:

Appendix SI. Bisulfite-PCR pyrosequencing.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/i.1365-2141.2007.06707x

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