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REVIEW

DNA demethylation and invasive cancer: implications for therapeutics

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One of the hallmarks of cancer is aberrant DNA methylation, which is associated with abnormal gene expression. Both hypermethylation and silencing of tumour suppressor genes as well as hypomethylation and activation of prometastatic genes are characteristic of cancer cells. As DNA methylation is reversible, DNA methylation inhibitors were tested as anticancer drugs with the idea that such agents would demethylate and reactivate tumour suppressor genes. Two cytosine analogues, 5-azacytidine (Vidaza) and 5-aza-2′-deoxycytidine, were approved by the Food and Drug Administration as antitumour agents in 2004 and 2006 respectively. However, these agents might cause activation of a panel of prometastatic genes in addition to activating tumour suppressor genes, which might lead to increased metastasis. This poses the challenge of how to target tumour suppressor genes and block cancer growth with DNA-demethylating drugs while avoiding the activation of prometastatic genes and precluding the morbidity of cancer metastasis. This paper reviews current progress in using DNA methylation inhibitors in cancer therapy and the potential promise and challenges ahead.

LINKED ARTICLES

This article is part of a themed section on Epigenetics and Therapy. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-11

Abbreviations

5-azaC, 5-azacytidine; 5-azadC, 5-aza-2′-deoxycytidine; FDA, Food and Drug Administration; HDAC, histone deacetylase; SAH, S-adenosyl-homocysteine; SAHA, suberoylanilide hydroxamic acid; SAM, S-adenosyl-methionine

Tables of Links

TARGETS	
GPCRs[a]	HDAC1
CXCR4	MMP2
Enzymes[b]	MMP9
DNA methyltransferase 1 (DNMT1)	uPA (PLAU)
Histone deacetylases (HDACs)	

LIGANDS	
5-azacytidine (5-azaC)	Oxaliplatin
5-aza-2′-deoxycytidine	Paclitaxel
5-fluorouracil	SAHA (entinostat)
Adriamycin (doxorubicin)	SAM
ATP	Trichostatin A
Cisplatin	Valproic acid
Irinotecan (SN38)	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are bpermanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

DNA methylation overview

DNA methylation is an enzymatically catalysed covalent modification of DNA, occurring typically in the context of cytosine-phosphate-guanine (CpG) dinucleotides. In general, regions with high CpG content, named CpG islands, are demethylated in normal cells, whereas regions with an intermediate or low density of CpGs are differentially methylated in some tissues, but not in others (Bird *et al.*, 1985). Although rarely observed, non-CpG methylation is mainly found in embryonic stem cells (Lister *et al.*, 2009), but its function needs to be further explored. However, non-CpG methylation is abundant in adult brains, and recent data suggest that it plays a role in silencing promoter activity similar to CpG methylation (Guo *et al.*, 2013; Lister *et al.*, 2013).

DNA methyltransferases (DNMTs) catalyse the transfer of a methyl group from its donor S-adenosine-methionine (SAM) to the cytosine nucleotide of DNA. Three distinct phylogenic DNMTs were identified in mammals. DNMT1 is a maintenance DNMT, which shows preference for hemimethylated DNA *in vitro* and faithfully copies DNA methylation in a cytosine-guanine (CG) palindromic dinucleotide from the parental strand to the daughter strand during cell division (Zucker *et al.*, 1985; Flynn *et al.*, 1996; Pradhan *et al.*, 1999; Fatemi *et al.*, 2001). DNMT3a and DNMT3b methylate unmethylated and hemimethylated DNA at an equal rate, which is consistent with a *de novo* methylation function (Okano *et al.*, 1998). In addition, a demethylation activity was recently attributed to mammalian *de novo* DNMT3A and DNMT3B (Chen *et al.*, 2013).

DNA methylation reversibility is one of the most controversial questions in the DNA methylation field. Over an extensive period of time, it was strongly believed that demethylation of DNA is a passive phenomenon. Methyl CpG binding domain 2 was the first protein suggested to have demethylase activity (Bhattacharya *et al.*, 1999a; Cervoni and Szyf, 2001b; Detich *et al.*, 2003a,b; Hamm *et al.*, 2008), although this activity is highly disputed. Moreover, several enzymes, acting together, are shown to be implicated in passive and active DNA demethylation (Kohli and Zhang, 2013), such as the ten–eleven translocation (TET) methylcytosine dioxygenases (Iyer *et al.*, 2009; Tahiliani *et al.*, 2009; Pastor *et al.*, 2013), activation-induced cytidine deaminase (AID) (Bhutani *et al.*, 2010; Santos *et al.*, 2013) and thymine DNA glycosylase (TDG) (Cortazar *et al.*, 2011; Cortellino *et al.*, 2011). First, TET was suggested to block maintenance of DNA methylation during replication, requiring DNMT1 and ubiquitin-like, containing PHD and RING finger domains, 1 (UHRF1) proteins (Bostick *et al.*, 2007), and lead to a passive demethylation. Experiments *in vitro* showed a reduction in UHRF1 binding (10-fold) and in recombinant DNMT1 activity at sites of hemi 5hmC in comparison with hemi 5mC (Hashimoto *et al.*, 2012). Alternatively, repair-based DNA demethylation could result from the combined action of TET proteins with AID (DNA cytosine) resulting in transformation of the methyl cytosine, triggering a repair mechanism that can involve glycosylases that remove the deaminated hydroxyl-methylated modified base such as TDG (Guo *et al.*, 2011; He *et al.*, 2011).

Not all CGs in the genome are methylated. A fraction of CGs remains unmethylated and the fraction of the genome that is unmethylated is different from tissue to tissue, creating a tissue-specific pattern of methylation. Razin and Riggs have proposed, three decades ago, that the pattern of methylation is responsible for tissue-specific gene expression (Razin and Riggs, 1980). Hypermethylated DNA is packaged in inactive chromatin associated with silent genes (Razin and Cedar, 1977), while transcriptionally active regions of the chromatin are associated with hypomethylated DNA. DNA methylation at 5′ regions of genes can silence transcription initiation (Blattler and Farnham, 2013; Baubec and Schubeler, 2014) in reporter assays in cell culture, and it is believed that it plays a similar role *in vivo* (Stein *et al.*, 1982; Cedar *et al.*, 1983). There are different mechanisms that are involved in silencing of gene expression by DNA methylation such as interference of methylation at cytosines with binding of transcription factors (Comb and Goodman, 1990; Inamdar *et al.*, 1991) or through attracting methylated DNA binding proteins, such as MeCP2 to methylated DNA (Nan *et al.*, 1997). MeCP2, in turn, recruits other proteins such as SIN3A and histone-modifying enzymes, which lead to the formation of a 'closed' chromatin configuration and silencing of gene expression (Nan *et al.*, 1997).

Although DNA methylation is described as a repressive epigenetic mark, its association with gene transcription depends on its location. Many methylated CG sites are found in intergenic and intragenic regions. As described earlier, DNA methylation in promoters or enhancers suppresses gene expression (Comb and Goodman, 1990; Inamdar *et al.*, 1991). However, DNA methylation in intragenic regions, within the body of coding genes, is positively correlated with gene expression (Ball *et al.*, 2009; Rauch *et al.*, 2009; Aran *et al.*, 2011), which is illustrated by the study on the active X chromosome (Hellman and Chess, 2007). The role of DNA methylation in gene bodies remains poorly understood; however, several ideas on its possible role are starting to emerge. A recent study showed that a small fraction (5–15%) of all methylated intronic regions (containing initiation transcription sites) could repress initiation of intragenic transcription suggesting a possibility for DNA methylation to suppress spurious transcriptional firing (Jjingo *et al.*, 2012). A different study linked gene body DNA methylation and reduction in transcriptional elongation (Lorincz *et al.*, 2004). Several studies postulated a role for DNA methylation in splicing, and a particular role for the methylated DNA binding protein MeCP2 (Young *et al.*, 2005; Cingolani *et al.*, 2013; Maunakea *et al.*, 2013). Gelfman *et al.* suggest a role for gene body DNA methylation during transcription-linked splicing (Gelfman *et al.*, 2013). The data suggest that these effects of DNA methylation are dependent on the CG distribution architecture at exon and intron boundaries.

DNA methylation is implicated in several gene regulatory pathways in addition to cellular differentiation during development such as suppression of retrotransposon expression, X chromosome inactivation and gene parental imprinting (Jones, 2012; Smith and Meissner, 2013).

DNA methylation in disease and its therapeutic implications

Although DNA methylation plays an important role in setting up cell type identity, it is not an exclusively static

mark after birth, but is dynamic to a certain extent during the life course. It can be responsive to experience and environmental exposures such as socio-economic positioning, stress and dietary supplements, etc. (McGowan *et al.*, 2009; Borghol *et al.*, 2012). The dynamic nature of DNA methylation pattern is a balance of DNMTs and putative demethylase activities (Bhattacharya *et al.*, 1999b; Chen *et al.*, 2013).

Aberrant methylation in cancer is characterized by hypermethylation of CG islands in tumour suppressor genes, as well as non-CG island CGs in other promoters and hypomethylation of unique genes and repetitive elements (Issa *et al.*, 1993; Baylin *et al.*, 2001; Ehrlich, 2002). These changes in DNA methylation may be partially explained by aberrant expression of methyltransferases (Issa *et al.*, 1993) and putative demethylases (Patra *et al.*, 2002). Interestingly, several nodal oncogenic pathways up-regulate DNMT1 and demethylase activity (MacLeod *et al.*, 1995a; Rouleau *et al.*, 1995; Szyf *et al.*, 1995), and tumour suppressor Rb negatively regulates DNMT1 (Slack *et al.*, 1999). Hypermethylation of tumour suppressor genes leads to their inactivation and is highly implicated in cancer growth. In contrast, the up-regulation of prometastatic genes, induced by DNA hypomethylation, promotes invasion and metastasis pathways, one of the most morbid aspects of cancer (Pakneshan *et al.*, 2004; Shukeir *et al.*, 2006). Hence, DNA hyper- and hypomethylation trigger different cellular mechanisms involved in cancer (Stefanska *et al.*, 2011).

DNA methylation steady state can be modulated by pharmacological reagents, dietary supplements and other chemicals (Day *et al.*, 2002; Szyf, 2005). This fact makes DNA methylation much more attractive as a therapeutic target than fixed and irreversible genetic mutations. Cancer was one of the first diseases where DNA methylation was proposed as a therapeutic target (Szyf, 1994). The main focus of drug development in cancer therapy has been on developing DNMT inhibitors with the goal of demethylating and activating tumour suppressor genes silenced by DNA methylation. Accordingly, two drugs, 5-azacytidine (5-azaC), and its deoxy analogue, 5-aza-2′-deoxycytidine (5-azadC), are currently used in clinical practice as a general inhibitor of all DNMTs. These drugs were the first epigenetic drugs approved by the FDA for anticancer treatment. In addition to activation of tumour suppressors, 5-azaC and 5-azadC were reported to up-regulate a panel of prometastatic genes that stimulate cancer cell invasiveness and metastasis formation (Ateeq *et al.*, 2008; Yu *et al.*, 2010; Chik and Szyf, 2011a).

Therefore, the challenge in the field of DNA methylation drugs is to identify specific inhibitors of DNA methylation that target the growth-silencing functions without triggering activation of prometastatic genes. This review focuses on the current state of DNA methylation drug discovery and provides a perspective for the future directions in anticancer therapy.

Mechanisms of action of 5-azaC and 5-azadC

5-azaC (Vidaza) (Kuendgen and Lubbert, 2008) is an analogue of cytidine ribose nucleoside and its deoxy derivative is the 5-aza-2′-deoxycytidine nucleoside (5-azadC, Decitabine or Dacogen). They were first synthesized in the early 1960s (Sorm *et al.*, 1964) and were later demonstrated to inhibit DNMT activity (Jones and Taylor, 1980).

5-azaC and 5-azadC are prodrugs that require activation *via* phosphorylation to be incorporated into the DNA during replication (Stresemann and Lyko, 2008). Once incorporated, both, non-modified cytosine CG dinucleotides and modified azacytosine-guanine are recognized by DNMTs during replication and DNA methylation initiation reaction, thereby leading to a widespread genomic hypomethylation (Esteller, 2005; Momparler, 2005). In contrast to the intact cytosine, DNMTs form an irreversible covalent bond with the carbon at position 6 of the azacytosine ring trapping the DNMT on the nascent strand of DNA during DNA synthesis, preventing the regeneration of the catalytic cysteine and passive loss of DNA methylation in the extending nascent strand (Wu and Santi, 1985). Interestingly, 5-azaC can cause demethylation in non-dividing neurons (Miller and Sweatt, 2007). It stands to reason, therefore, that 5-azaC can act through an additional mechanism that does not require its incorporation into replicating DNA, for example by triggering proteasomal degradation of DNMTs (Ghoshal *et al.*, 2005).

Although these two drugs are incorporated into DNA (Li *et al.*, 1970), only 5-azaC can be incorporated into RNA and cause the inhibition of RNA and protein synthesis (Jiří Veselý, 1978). Cihak *et al.* demonstrated that 5-azaC administration induces a rapid degradation of polyribosomes and subsequent inhibition of protein synthesis (Cihak *et al.*, 1968). It was reported, that 5-azaC changes the structure of the ribosomal precursor RNAs and inhibits the processing of ribosomal RNA (Jiří Veselý, 1978).

5-azadC is more potent and more cytotoxic than 5-azaC (at least 10-fold) (Flatau *et al.*, 1984; Momparler *et al.*, 1984). Moreover, toxic DNMT-5-azadC complexes might be triggering long-term side effects such as mutations and chromosomal re-arrangements (Juttermann *et al.*, 1994). Hypomethylation, mediated by 5-azadC, was found to be directly involved in mismatch repair deficiency leading to gains and losses of chromosomes (Lengauer *et al.*, 1997). Demethylation is also associated with mitotic dysfunction and translocation (Schmid *et al.*, 1984; Thomas, 1995). In addition to activation of gene expression through promoter demethylation, hypomethylation causes genomic instability (Chen *et al.*, 1998) and unleashes the expression of repetitive sequences disrupting gene expression programming (Howard *et al.*, 2007).

5-azaC and 5-azadC mediate inactivation of all DNMT isoforms, cause demethylation and reactivation of a wide panel of tumour suppressor and other genes, and are able to block cancer growth (Ghoshal and Bai, 2007). These effects made these two drugs promising in cancer therapy. However, in addition to affecting growth, 5-azaC and 5-azadC can activate metastatic genes such as HEPARANASE (Shteper *et al.*, 2003) and uPA, which play an important role in metastasis (Pakneshan *et al.*, 2004; Shukeir *et al.*, 2006). This raises a critical challenge in using DNA methylation inhibitors: How to dissociate the growth inhibitory activities of DNMT inhibitors from the prometastatic activity?

Contradictory effects of 5-azaC and 5-azadC on cell invasion and apoptosis

While there is agreement regarding 5-azaC and 5-azadC anti-proliferative effects in cancer (Thakur *et al.*, 2012; Jeschke *et al.*, 2013; Zhang *et al.*, 2013; Chik *et al.*, 2014), their effects on cell migration and invasion are highly controversial. It was reported that 0.2 μM 5-azaC inhibits invasiveness of human breast carcinoma MDA-MB-468 cells (Bandyopadhyay *et al.*, 2004); 5 μM 5-azaC reduces migration of a human non-small cell lung carcinoma cell line H1299 (Mateen *et al.*, 2013); 10 μM 5-azaC reduces migration and invasiveness in ovarian tumour cells BeWo (Rahnama *et al.*, 2006), and 5-azadC inhibits migration and invasion of bladder cancer T24 cell line (Zhang *et al.*, 2013). However, other studies that span several decades have demonstrated an increase in cell invasion and metastasis in animal tumour models (Olsson and Forchhammer, 1984; Habets *et al.*, 1990). One and 5 μM 5-azadC convert non-metastatic breast cancer cell lines (MCF-7 and ZR-75-1) to invasive cells *in vitro* and *in vivo* (Ateeq *et al.*, 2008; Chik *et al.*, 2014); 5 μM 5-azadC increases human fibrosarcoma HT1080 cells invasion through induction of matrix metalloproteinase-1 (MMP-1), which was up-regulated 44.6-fold through recruitment of RNA Pol II and Sp1 to its promoter compared with non-treated cells. 5-azadC also up-regulated MMP2 (1.9-fold), and MMP9 (8.6-fold) (Poplineau *et al.*, 2013). In another study, 1 μM 5-azadC increased the invasiveness of several pancreatic cancer cell lines (Sato *et al.*, 2003).

5-azaC and 5-azadC treatment in leukaemia

5-azaC (Vidaza) and 5-azadC (Decitabine) were approved by the FDA for treatment of childhood acute myeloid leukaemia (AML). Vidaza demonstrated activity in 15–25% of AML (Cashen *et al.*, 2010; Fenaux *et al.*, 2010; Kantarjian *et al.*, 2012). In elderly patients, AML is associated with poor prognosis with 10% 2 year overall survival (OS) and only 2% 5 year survival (Menzin *et al.*, 2002). In a phase II study, during which 55 elderly AML patients were treated with decitabine, complete remission was observed in 24% of patients (Cashen *et al.*, 2010). Moreover, it was demonstrated (Fenaux *et al.*, 2010) that 5-azaC treatment, in a phase III trial, significantly increased the OS of elderly patients with low marrow blast count World Health Organization-defined AML. Half of the treated patients were still alive after 2 years in comparison with only 16% in the conventional care regimen group (Fenaux *et al.*, 2010). These results were recently confirmed by Pleyer *et al.*, who showed that 62% of a group of 48 elderly patients (mean 71 years old) survived for 2 years after 5-azaC treatment (Pleyer *et al.*, 2014).

Decitabine is clinically effective for the treatment of MDS, and was shown to improve time to AML transformation or death. Indeed, patients treated with Decitabine had a greater median time to AML transformation or death (4.3 months) than patients receiving supportive care alone (Kantarjian *et al.*, 2012).

These results emphasize the high efficacy of 5-azaC and 5-azadC as drugs for treating leukaemia. Currently, clinical efforts are focused on the possibility that these drugs could be effective in treating solid tumours as well (For the list of recent clinical trials with 5-azaC and 5-azadC see Supporting Information Table S1.).

5-azaC and 5-azadC effects on solid tumours

Solid tumour size reduction upon 5-azaC treatment was observed in several studies using mice models of breast (Thakur *et al.*, 2012) and spleen tumours (Mikyskova *et al.*, 2014). Lindner *et al.* demonstrated that 5-azadC treatment reduces tumour vascularization as well as tumour volume (Lindner *et al.*, 2013). A phase I study, on invasive urothelial cancer in a canine model with 5-azaC, showed an antitumour activity with 22% of partial remission and 56% of tumour size reduction (Hahn *et al.*, 2012).

Several clinical trials on patients with solid tumours were performed in the past. However, few of them focused only on 5-azaC and 5-azadC effects as a monotherapy; most used combinations of 5-azaC/dC and other chemotherapeutic agents (discussed later). The first clinical trials using 5-azaC alone in phase I and II studies in solid tumours were performed during the 1970s. An antitumour effect was demonstrated in 17 and 21% of patients with breast carcinoma and malignant lymphomas, respectively, who were treated with a dose of 1.6 $mg \cdot kg^{-1}day^{-1}$ of 5-azaC for 10 days (Weiss *et al.*, 1977). In another study, with doses of 5-azaC ranging from 1.0 to 24.0 $mg \cdot kg^{-1}$ given over a minimal period of 8 days, remission was observed in 11 out of 30 patients with a variety of solid tumours (Weiss *et al.*, 1972; 1977). However, responses to 5-azaC were transient with a minimal effect on other solid tumours (Weiss *et al.*, 1977). One of the major problems in all these studies was toxicity. Because of the toxic effects (myelosuppressive, gastrointestinal, sepsis and cerebral haemorrhage), leucopoenia and thrombocytopenia in another study (Weiss *et al.*, 1972) the initial dose of 225 $mg \cdot m^{-2}$ (i.v., on days 1–5 every 3 weeks) was reduced to 150 $mg \cdot m^{-2}$ in one study (Quagliana *et al.*, 1977). It also became clear that these drugs should not be used as a single agent in solid tumours.

Other phase I studies analysed the effects of 5-azadC in patients with solid tumours, such as non-small cell lung carcinoma, thoracic malignancies, renal cell carcinoma, malignant pleural mesothelioma, and lung and oesophageal cancers (Momparler and Ayoub, 2001; Aparicio *et al.*, 2003; Samlowski *et al.*, 2005; Schrump *et al.*, 2006). Although these studies show that 5-azadC treatment induces a significant DNA hypomethylation (Aparicio *et al.*, 2003; Samlowski *et al.*, 2005), causes gene induction (Schrump *et al.*, 2006) and increases the survival duration of the patients (Momparler *et al.*, 1997, Momparler and Ayoub, 2001), these studies did not report a reduction in tumour size.

What is the mechanism of action of the antitumour effect of 5-azaC in solid tumours? A large body of data has

established that 5-azaC has a broad effect on the expression of tumour suppressor genes, cell cycle regulatory genes and antiapoptotic genes in many solid cancer cell lines. For example, a recent study performed an integrated expression and methylation analysis on 63 different cancer cell lines (breast, ovarian and colorectal) treated with 5-azaC (Li *et al.*, 2014). In this study, 5-azaC was shown to affect key biological pathways involved in tumourigenesis such as, *inter alia*, cell cycle and mitotic pathways, and transcription and DNA replication. Additionally, 5-azaC induced an up-regulation of immunomodulatory genes, which might suggest an immunomodulatory function for 5-azaC in multiple solid tumour cancers, which can be classified as immune low or immune enriched (Li *et al.*, 2014). However, it is unclear whether similar mechanisms are at work in patients treated with 5-azaC/dC. For information regarding current ongoing clinical studies on colorectal and ovarian cancers, see Supporting Information Table S1.

Using 5-azaC and 5-azadC in combination with other agents

Both 5-azadC and 5-azaC exhibited rather disappointing results against solid tumours (Aparicio and Weber, 2002). Perhaps, this might be due to the relative instability of 5-azaC/dC (Lin *et al.*, 1981) and its potential deamination and inactivation *in vivo* (Momparler *et al.*, 1984). This prompted the idea of using 5-azaC/dC in combination with other chemotherapeutic agents. The rationale being that 5-azaC can possibly reduce resistance to chemotherapy that is caused by DNA methylation and silencing of genes involved in chemotherapeutic response. These ideas were tested in cell culture. For example, cell culture experiments demonstrated antiproliferative effects in MDA-MB-231 when 5-azadC was used in combination with anticancer agents: paclitaxel (PTX), adriamycin or 5-fluorouracil (5-FU), while in MCF-7 semi-additive antiproliferative effects were observed only with a combination of 5-azadC and 5-FU in comparison with the treatme nt with each of the drugs on its own (Mirza *et al.*, 2010).

In another study, 5-azadC had an antiproliferative effect and increased apoptosis in combination with anticancer drugs 5-FU, PTX, oxaliplatin, SN38 (irinotecan), and gemcitabine in human gastric cancer cell lines when treated simultaneously as compared with monotherapy (Zhang *et al.*, 2006).

The clinical results are less conclusive. In a clinical study on 29 ovarian cancer patients the effect of carboplatin, a chemopreventive reagent, was compared with combined effect of carboplatin with 5-azaC (Glasspool *et al.*, 2014) (For additional information regarding ongoing clinical trial on ovarian cancer using carboplatin and decitabine refer to Supporting Information Table S1.). In the combination group, the use of 5-azaC reduced the efficacy of carboplatin in partially platinum-sensitive ovarian cancer. Moreover, hypersensitivity reactions reduced deliverability of the combination of 5-azaC with carboplatin on a 28 day schedule (Glasspool *et al.*, 2014).

Combination of 5-azaC and 5-azadC and histone deacetylase (HDAC) inhibitors

There is a crosstalk between DNA methylation and histone acetylation (Cervoni and Szyf, 2001b; Cervoni *et al.*, 2001a; D'Alessio and Szyf, 2006). Methylated DNA binding proteins recruit HDACs to genes resulting in histone deacetylation, and HDAC inhibitors induce DNA demethylation (Detich *et al.*, 2003a). This is supported by the *in vitro* interaction of DNMT1 with HDAC1 (Fuks *et al.*, 2000; Ou *et al.*, 2007; Palii and Robertson, 2007). Therefore, several studies examined the combinatory effects of 5-azaC and 5-azadC with other HDAC inhibitors *in vitro*. Cameron *et al.* were the first to show synergistic effects in activation of tumour suppressor genes of the HDAC inhibitor trichostatin A (TSA) and 5-azadC (Cameron *et al.*, 1999). Particularly, they demonstrated that several genes, silenced by methylation, were induced with a combination treatment of 5-azadC and TSA, but not when treated with only 5-azadC. Numerous papers have replicated this observation in the last decade and a half (Griffiths and Gore, 2008)

Moreover, it was found that TSA, 5-azadC and cisplatin, a DNA cross-linking agent, commonly used to treat ovarian cancer, alone or in combination, significantly suppressed spheroid formation and growth of ovarian cancer cells *in vitro*, and sequential treatment of epigenetic modifiers and low-dose cisplatin reduced tumourigenesis more effectively than either drug alone in xenograft mouse models (Meng *et al.*, 2013).

Another combination of 5-azaC with valproate (valproic acid, VPA) attracted attention. VPA initially used for epilepsy treatment was demonstrated to also act as a HDAC inhibitor (Gottlicher *et al.*, 2001; Phiel *et al.*, 2001). Although, the combination of VPA and decitabine reactivates hypermethylated genes as demonstrated by re-expressing fetal haemoglobin, this combination is limited by toxicity, specifically by neurological symptoms in patients with non-small cell lung cancer (Chu *et al.*, 2013). Another HDAC inhibitor, sodium butyrate, when combined with 5-azadC, inhibited mammary tumourigenesis in a murine model (Elangovan *et al.*, 2013). Similarly, 5-azadC, in combination with suberoylanilide hydroxamic acid (SAHA), a different HDAC inhibitor, inhibited hepatoma cell growth in a human hepatoma xenograft model (Venturelli *et al.*, 2007). In another study, a combination of 5-azadC and SAHA activated tumour suppressor genes *ARHI* and *PEG3*, and inhibited tumour growth in a xenograft model (Chen *et al.*, 2011). The effect of combined treatment of two HDAC inhibitors VPA and SAHA with 5-azadC was also shown in a murine model of mesothelioma to kill malignant pleural mesothelioma cells and induce tumour antigen expression in the remaining living tumour cells (Leclercq *et al.*, 2011).

Overall, *in vivo* studies show that combination of reagents targeting different epigenetic levels of gene regulation machinery (DNA methylation and histone modifications), is more efficient than monotherapy, and this is reflected in augmentation and overall activation of silenced genes in cancer. However, it is clear that 5-azaC-based therapeutics encounter serious challenges in treatment of solid tumours.

Other approaches to DNA methylation-based therapy need to be developed in order to realize the potential of DNA methylation-based therapeutics.

Inhibition of DNMT1 as anticancer therapy

As discussed previously, the main challenge in cancer therapy is how to target tumour suppressor genes and stop cancer growth with DNMT inhibitors while avoiding the activation of prometastatic genes. Is it possible, by selective targeting of these mechanisms, to enhance the specificity and reduce the toxicity of DNMT inhibitors in cancer?

There is an urgent necessity to develop new, potent and selective DNMT inhibitors that possess good pharmacokinetic profiles with minimal toxicity. Based on several studies, isoform-specific inhibitors of DNMT1 might be a reasonable strategy for anticancer therapeutics. It was demonstrated, that overexpression of DNMT1 in non-transformed cells leads to cellular transformation (Wu *et al.*, 1993). The anticancer effects of DNMT1 inhibition were demonstrated both pharmacologically and genetically using antisense oligonucleotide inhibitors (MacLeod and Szyf, 1995b; Ramchandani *et al.*, 1997) and dnmt1–/– mice (Laird *et al.*, 1995) respectively. Although depletion of *DNMT1* had the strongest effect on colony growth suppression in cellular transformation assays, it did not result in demethylation and activation of *uPA*, *S100A4*, *MMP2* and *CXCR4* in *MCF-7* cells. It was demonstrated, that depletion of *DNMT1* did not induce cellular invasion in either MCF-7 or ZR-75-1 non-invasive breast cancer cell lines and did not lead to activation of prometastic genes (*uPA*, *HEPARANAZE*) (Chik and Szyf, 2011b). Taken together, these data support the idea that selective inhibition of DNMT1 rather than pan inhibition of DNMTs should be a reasonable strategy for anticancer therapeutics. New approaches that are more selective than Vidaza might also enhance the overall antitumour activity of DNA methylation inhibitors.

Prospect of using SAM in anticancer therapeutics

The landscape of DNA methylation in cancer involves both loss and gain of DNA methylation (Stefanska *et al.*, 2011). Importantly, DNA demethylation is associated with an activation of genes involved in migration and movement, functions required for metastasis formation (Nakamura and Takenaga, 1998; Guo *et al.*, 2002; Rosty *et al.*, 2002; Szyf, 2005; Ateeq *et al.*, 2008). Therefore, it stands to reason that blocking these demethylation events might provide some therapeutic effect against cancer metastasis.

SAM (AdoMet) is the methyl donor in cellular methylation reactions. SAM is a natural product that is synthesized from ATP and methionine by methionine adenosyltransferase (Cantoni, 1953). The methyl group CH3, of SAM, is transferred to cell components such as DNA, proteins and lipids (Lu and Mato, 2005; Roje, 2006). There are several

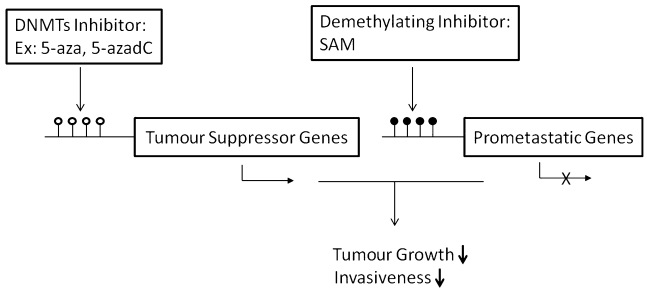

Figure 1

Combinatory effect of demethylating drugs and demethylating inhibitor. DNMTs inhibitors (5-azaC and 5-azadC) target tumour suppressor and prometastatic genes by demethylating promoters and inducing their expression. This effect can be compensated by combining DNMT inhibitors with a demethylating inhibitor (SAM), which causes prometastatic gene-specific methylation and subsequent down-regulation in gene expression without targeting tumour suppressor genes (Chik *et al.*, 2014). Filled (black) circles correspond to methylated Cs; unfilled (white) circles correspond to demethylated Cs.

mechanisms by which SAM alters DNA methylation. The balance of SAM and S-adenosyl-homocysteine (SAH) is critical for DNMT activity, as SAH inhibits DNMT. SAM also inhibits demethylase activity (Detich *et al.*, 2003b).

Clinical efficacy of SAM in depression, osteoarthritis and liver diseases was demonstrated in dozens of studies summarized in the report, which searched through 25 biomedical databases (Hardy *et al.*, 2003; Lu and Mato, 2012). There is extensive *in vitro* evidence that SAM suppress both growth and invasion in highly invasive cell lines (Pakneshan *et al.*, 2004; Shukeir *et al.*, 2006). *In vivo*, SAM was demonstrated to inhibit invasiveness and metastasis of human breast (Pakneshan *et al.*, 2004), prostate and colorectal cancer cell lines (Hussain *et al.*, 2013). Several studies demonstrated that SAM treatment had a chemopreventive effect in liver cancer in rat (Pascale *et al.*, 2002). A possible mechanism for SAM action is silencing the expression of prometastatic genes through DNA methylation (van der Westhuyzen, 1985; Fuso *et al.*, 2001; Ross, 2003; Pakneshan *et al.*, 2004; Shukeir *et al.*, 2006; Chik *et al.*, 2014).

Using several cytotoxic assays, it was demonstrated that SAM specifically enhances the anticancer effect of 5-FU, but not that of cisplatin (Ham *et al.*, 2013). We have recently demonstrated that SAM antagonizes the effects of 5-azadC on cell invasiveness and increases the antigrowth effects of 5-azadC, more than this, SAM inhibited the global hypomethylation induced by 5-azadC (Figure 1) (Chik *et al.*, 2014). However, SAM has broad pleoitropic effects and there is a need to develop more target-specific inhibitors that target DNA demethylation events that are critical for cancer by focusing on proteins that are involved in these demethylation reactions.

Concluding remarks

Although 5-azaC and 5-azadC have served a very important role in deciphering the role of DNA hypermethylation in

cancer and in providing the proof of principle for DNA demethylation therapy in clinical practice, several important obstacles remain. Firstly, the mechanism of action of 5-azaC in clinical response has to be clarified, as this is essential for dosing, scheduling and patient stratification. We need to identify the patients who will most benefit from 5-azaC/dC therapy. Secondly, the poor response in solid tumours to 5-azaC and 5-azadC requires examining different combinations as well as careful testing of dosing and scheduling. Moreover, testing new DNA methylation inhibitors might provide more effective ways for inhibiting DNA methylation of tumour suppressor genes in solid tumours in clinical practice. Thirdly, cell line data suggest that therapy could be improved by focusing on DNMT1. This calls for investing effort in developing DNMT1-specific inhibitors rather than using 5-azaC/dC, which inhibits all DNMTs resulting in possible activation of genes that could promote cancer. Fourthly, more attention needs to be given to the critical role of hypomethylation in driving cancer and cancer metastasis. There is a need to develop agents that target this process such as SAM, as well as developing more specific DNA demethylation inhibitors. Combining such agents with DNMT1 inhibitors might have synergistic effects on cancer inhibiting both tumour growth and tumour metastasis.

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Conflict of interest

Authors declare no conflict of interest.

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Supporting information

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Table S1 Recent clinical studies with epigenetic drugs.