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REVIEW

Nucleoside analogs: molecular mechanisms signaling cell death

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Nucleoside analogs are structurally similar antimetabolites that have a broad range of action and are clinically active in both solid tumors and hematological malignancies. Many of these agents are incorporated into DNA by polymerases during normal DNA synthesis, an action that blocks further extension of the nascent strand and causes stalling of replication forks. The molecular mechanisms that sense stalled replication forks activate cell cycle checkpoints and DNA repair processes, which may contribute to drug resistance. When replication forks are not stabilized by these molecules or when subsequent DNA repair processes are overwhelmed, apoptosis is initiated either by these same DNA damage sensors or by alternative mechanisms. Recently, strategies aimed at targeting DNA damage checkpoints or DNA repair processes have demonstrated effectiveness in sensitizing cells to nucleoside analogs, thus offering a means to elude drug resistance. In addition to their DNA synthesis-directed actions many nucleoside analogs trigger apoptosis by unique mechanisms, such as causing epigenetic modifications or by direct activation of the apoptosome. A review of the cellular and molecular responses to clinically relevant agents provides an understanding of the mechanisms that cause apoptosis and may provide rationale for the development of novel therapeutic strategies.
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Introduction

One of the most notable characteristics of nucleoside analogs is how drugs with similar structural features have different mechanisms of action and exert such diversity in their clinical activities (Plunkett and Gandhi, 2001). Many of these agents exert their cytotoxic effects by disrupting normal DNA synthesis through direct incorporation into extending DNA strands or by destabilizing the deoxynucleotide pool balance. Several nucleoside analogs can directly initiate apoptosis by activating the apoptosome and these have proven clinically active in indolent diseases, in which cells are not actively replicating. Others, which reverse epigenetic gene silencing caused by DNA methylation offer an additional use for these agents during cancer therapy. This article will address the actions of select nucleoside analogs that are established as effective cancer therapeutics and will briefly mention others that are being developed, focusing on mechanisms of action that induce apoptosis. Subsequently, the current state of knowledge regarding the cellular and molecular mechanisms that sense causes for nucleoside analog-induced toxicity is reviewed by proposing signaling models that lead to death signaling, or conversely, spare toxicity. In addition, we comment on new mechanism-based therapies that aim to combine nucleoside analogs with other active chemotherapeutic agents to overcome drug resistance.

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Targeting DNA replication

As anticancer drugs, many nucleoside analogs exert their cytotoxic effects after incorporation into DNA. The triphosphates contribute to cytotoxicity by competing with natural nucleotides for incorporation into DNA by DNA polymerases (Kufe *et al*., 1980; Huang *et al*., 1991). Incorporation of fraudulent nucleotides into actively replicating DNA causes steric hindrance of extending replication forks, leading to fork stalling. Incorporation into DNA is critical for toxicity; thus these agents show specificity for cells undergoing active DNA replication or excision repair synthesis (Kufe *et al*., 1980; Huang *et al*., 1990, 1991; Yamauchi *et al*., 2001). In turn, cells respond to blocked DNA synthesis by activating the S phase DNA damage checkpoint, which inhibits further firing of replication origins, halts DNA replication and causes cells to accumulate in the S phase of the cell cycle (Shi *et al*., 2001; Sampath *et al*., 2002; Zhang *et al*., 2006). This protective cascade is likely necessary for replication fork stabilization and may promote DNA repair (Lopes *et al*., 2001). Although these mechanisms are evolutionarily conserved to safeguard the genome, their functions can be exploited to enhance cell killing by nucleoside analogs and other DNA-targeting agents.

Pyrimidine nucleoside analogs

1-β-D-Arabinosylcytosine (ara-C, cytarabine) was the first nucleoside analog developed that contained an alteration in the carbohydrate moiety. It differs from the

parent nucleoside, deoxycytidine, only by the presence of a hydroxyl group in the β-configuration at the 2′ position of the sugar moiety (Figure 1). This agent is clinically active and is the major drug for the treatment of acute myelogenous leukemias (AML, Johnson, 2001). Upon cellular entry through nucleoside transport systems (Griffith and Jarvis, 1996), ara-C is metabolized to its triphosphate, ara-CTP, which competes with deoxycytidine triphosphate (dCTP) as a substrate for incorporation into DNA by DNA polymerases (Townsend and Cheng, 1987; Ohno *et al*., 1988). Once incorporated into extending DNA strands, the analog serves as a poor substrate for chain extension, which leads to the stalling of replication forks (Ross *et al*., 1990). Alternatively, incorporation of two or more residues in tandem most likely leads to chain termination. Although ara-CTP analogs can be excised from the 3′ terminus by the 3′→5′ proofreading exonuclease activities associated with DNA polymerases, this proceeds at a rate that is considerably less than a normal nucleotide (Huang *et al*., 1991). The discovery that ara-C had activity in hematological malignancies generated enthusiasm for other nucleoside analogs with similar modifications that might have a broader spectrum of activity (Grant, 1998).

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is a deoxycytidine analog with geminal fluorine atoms in the 2′-position of the sugar moiety (Figure 1). Although initially developed as an antiviral agent, it soon was recognized for its pronounced antitumor activity (Heinemann *et al*., 1988; Hertel *et al*., 1990). Like ara-C, gemcitabine requires intracellular phosphorylation by deoxycytidine kinase and accumulates in cells mainly as the triphosphate (dFdCTP), which competes with dCTP for incorporation into DNA (Huang *et al*., 1991). However, incorporation of a single gemcitabine nucleotide may be more efficiently extended than ara-C whereas tandem incorporation of gemcitabine nucleotides is likely more inhibitory to subsequent DNA chain extension than, causing chain termination (Plunkett *et al*., 1995, 1996). Unlike ara-C, gemcitabine has a second mechanism of action that contributes to cytotoxicity. The diphosphate of gemcitabine (dFdCDP) serves as an inhibitory alternative substrate for ribonucleotide reductase and inactivates this key enzyme in a mechanism-based manner, which leads to a decrease in deoxynucleotide pools (Baker *et al*., 1991; van der Donk *et al*., 1998; Wang *et al*., 2007). The change in the dFdCTP:CTP ratio likely leads to enhanced gemcitabine incorporation and further DNA synthesis inhibition, an action known as self-potentiation (Heinemann *et al*., 1990, 1992). These differences in drug metabolism and mechanism of action likely explain contrasts in clinical activity, compared with other nucleoside analogs with similar structures. Unlike ara-C, gemcitabine is active in a broad spectrum of solid tumors, including pancreatic, metastatic breast, ovarian and non-small cell lung cancer.

The stereochemical form of natural nucleosides is the β-D-configuration. Therefore, cancer therapeutic nucleoside analogs were developed with this structure as the template. The assumption was that the proteins required for transport and metabolism of nucleic acids would be unable to recognize β-L-configuration nucleoside analogs. If these agents were unrecognizable, they would likely not be metabolized to their active form and would not be effective. However, it was later determined that the L-isomer of 2′,3′-dideoxythiacytidine had antiviral activity (Chang *et al*., 1992; Schinazi *et al*., 1992); thus, providing evidence that cells had the capability of transporting and metabolizing L-analogs to the active triphosphate form. Other L-nucleosides were subsequently synthesized and one such agent, troxacitabine (Figure 1; L-1,3-dioxolane-cytidine, L-OddC), was investigated as an anticancer therapy after it demonstrated considerable cytotoxic effects in cell lines and animal models (Grove *et al*., 1995; Gourdeau *et al*., 2001b). Although the mono-, di- and triphosphate forms of troxacitabine accumulate in cells, it is interesting that the diphosphate form predominates (Grove *et al*., 1995). This is likely because of the less efficient phosphorylation of troxacitabine diphosphate to the active metabolite, troxacitabine triphosphate, by 3-phosphoglycerate kinase rather than by nucleoside diphosphate kinase (Krishnan *et al*., 2002, 2003). Deficiencies in nucleoside transporters do not cause increases in drug resistance, suggesting that cellular uptake of troxacitabine is mainly by passive diffusion (Grove and Cheng, 1996; Gourdeau *et al*., 2001b). Interestingly, troxacitabine has mechanistic properties that differ from those of ara-C and gemcitabine. Troxacitabine does not inhibit ribonucleotide reductase, affect deoxynucleotide pools and is not readily deaminated (Grove *et al*., 1995; Grove and Cheng, 1996; Gourdeau *et al*., 2001a). Like other

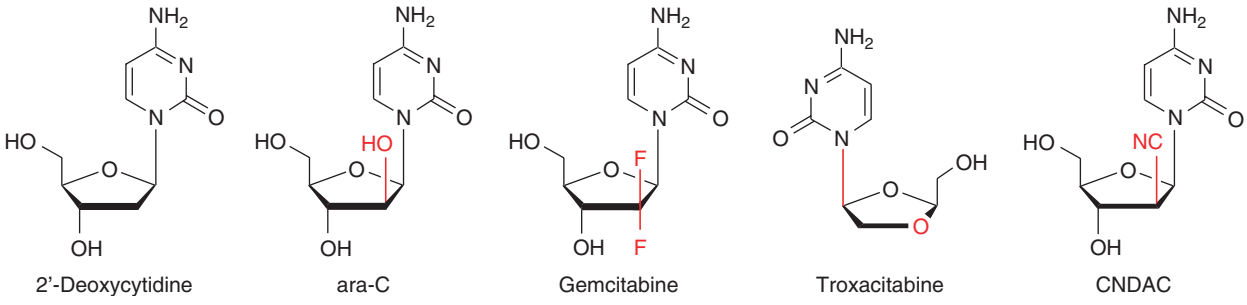

Figure 1 Structures of deoxycytidine, ara-C, gemcitabine, troxacitabine and CNDAC.

nucleoside analogs, the main mechanism causing apoptosis is believed to be incorporation of the triphosphate into DNA. However, as troxacitabine lacks a 3′-hydroxyl group, incorporation of a single troxacitabine molecule does not permit further extension (Kukhanova *et al*., 1995). Thus, once incorporated into DNA, this nucleotide acts as a *de facto* chain terminator.

Although cytosine nucleoside analogs generally inhibit DNA synthesis by stalling replication forks upon incorporation into DNA, 2′-*C*-cyano-2′-deoxy-1-β-D-arabino-pentofuranosylcytosine (Figure 1; CNDAC) has a novel mechanism. After being incorporated into DNA, ligation of the 3′hydroxyl of this analog initiates β-elimination, leading to rearrangement of CNDAC to 2′-*C*-cyano-2′,3′-didehydro-2′,3′-dideoxycytidine (CNddC). As CNddC lacks a 3′-hydroxyl group, this process leads to the formation of single-strand DNA nick (Matsuda *et al*., 1991; Azuma *et al*., 2001). As this lesion is not easily repaired by ligation, it is likely that this nick is processed into a double-strand break upon subsequent DNA replication (Liu *et al*., 2008; Wang *et al*., 2008). CNDAC gives rise to DNA damage that is different from that of other DNA-directed nucleoside analogs, and therefore the cellular responses to this molecule are qualitatively different. Unlike ara-C, gemcitabine, and troxacitabine which cause stalling of replication forks and arrest in S phase, the DNA breaks caused by CNDAC activate the G_2 checkpoint and cause an accumulation of cells in the G_2 phase of the cell cycle (Liu *et al*., 2005, 2008). An orally bioavailable prodrug of CNDAC, sapacitabine, is currently being studied for clinical activity in solid tumors and hematological malignancies (Kantarjian *et al*., 2007a).

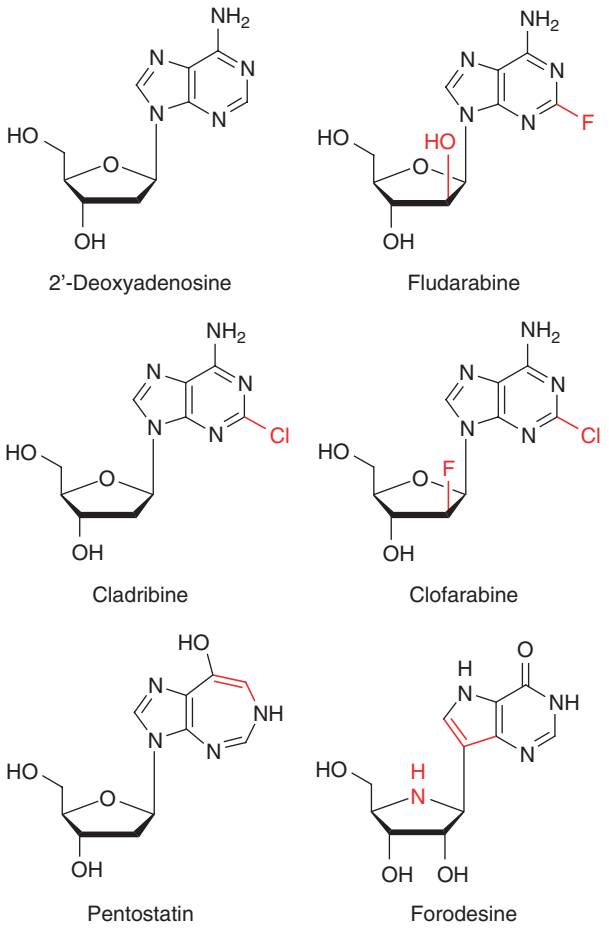

Figure 2 Structures of deoxyadenosine, fludarabine, cladribine, clofarabine, pentostatin and forodesine.

Purine nucleoside analogs

In parallel with the emergence of gemcitabine came purine nucleoside analogs, which were established as having major activity in indolent B-cell malignancies. The inhibitory actions against DNA replication of fludarabine (Figure 2; 9-β-D-arabinofuranosyl-2-fluoroadenine, F-ara-A), an arabinosyl nucleoside analog, are similar to those of ara-C (Huang *et al*., 1990; Huang and Plunkett, 1991). In addition, the triphosphate appears to act at a regulatory site of ribonucleotide reductase to inhibit the enzyme reversibly, lowering cellular dNTP pools by a different mechanism than gemcitabine (Tseng *et al*., 1982; Parker *et al*., 1988). Unlike ara-C and gemcitabine, once the analog triphosphate is incorporated in DNA, attempts to excise it result in inactivation of both the excision and polymerizing activities of DNA polymerase (Kamiya *et al*., 1996). In addition, when the fludarabine residue is at the 3′ terminus, the DNA cannot be ligated (Yang *et al*., 1992). Thus, it is likely that the signals for apoptosis in growing cells are largely because of the actions of fludarabine as a chain terminator. The cytotoxic actions of fludarabine are not so clear in quiescent cells, in which the incorporation of the nucleotide analog into DNA is barely detectable. Unlike other arabinosyl nucleosides, fludarabine is also incorporated into RNA, an action that can terminate transcription (Huang and Plunkett, 1991; Huang *et al*., 2000). This is associated with a decrease in antiapoptotic proteins with intrinsically short half-lives, such as Mcl-1 and XIAP, an action that may curtail the survival capacity of chronic lymphocytic leukemia (CLL) cells (Kitada *et al*., 1998; Chen *et al*., 2005).

A second purine nucleoside analog, cladribine (2-chloro-deoxyadenosine), is modified only on the nucleobase and contains a normal 2′-deoxyribose carbohydrate moiety (Figure 2). Accordingly, the triphosphate is readily incorporated into DNA, and thereafter is also a fair substrate for extension (Hentosh *et al*., 1990). As with fludarabine, this process may be facilitated by the inhibitory activity of the triphosphate against ribonucleotide reductase, which seems to be similar in mechanism, but more potent than that of fludarabine triphosphate (Parker *et al*., 1988). Cladribine was originally developed as a treatment for immunodeficient children deficient in adenosine deaminase (Carson and Carrera, 1990). However, studies demonstrated that cladribine was resistant to degradation by adenosine deaminase and was selectively toxic to lymphocytes.

Interestingly, it was determined that this agent is equally toxic to resting and proliferating T and B cells (Carson *et al*., 1983). It has since been determined that cladribine is a potent inhibitor of DNA repair in quiescent cells, which progressively accumulate DNA breaks when exposed to the drug (Seto *et al*., 1985; Robertson *et al*., 1993). These DNA strand breaks lead to a poly(ADP-ribose) polymerase response, which facilitates DNA break repair (Seto *et al*., 1985). Further investigations into the mechanism of action of this agent revealed that exposure to cladribine in quiescent cells causes a depletion in intracellular NAD and ATP that is associated with apoptosis (Carson *et al*., 1986). Cladribine was the first nucleoside analog to exhibit killing in resting cells, thus providing evidence for their possible usefulness in chronic leukemias. This activity of this agent has since been verified as a curative agent in a subset of indolent lymphocytic malignancies, such as hairy cell leukemia (Goodman *et al*., 2003) and has also demonstrated activity in pediatric acute myelogenous leukemia (Crews *et al*., 2002).

Based on experiences with fludarabine and cladribine, a new deoxyadenosine nucleoside analog, clofarabine (Figure 2; 2-chloro-2′-fluoro-arabinosyladenine), was synthesized with the intention of eliminating undesirable characteristics of the earlier analogs, whereas retaining therapeutic attributes (Montgomery *et al*., 1992). Substitution of a halogen atom for the hydrogen at the 2-position of the purine ring rendered clofarabine resistant to deamination (Montgomery *et al*., 1992), whereas the additional fluorine moiety at the 2′-carbon in the sugar ring increases the stability of clofarabine at acidic conditions, relative to deoxyadenosine and cladribine (Carson *et al*., 1992). Further, placement of the fluorine atom in the arabino-configuration stabilizes the glycosidic bond, rendering this analog relatively resistant to bacterial purine nucleoside phosphorylase; thus, stimulating the development of an orally administered drug (Montgomery *et al*., 1992). As with fludarabine and cladribine, the triphosphate of clofarabine is a good substrate for DNA polymerases for incorporation into DNA. It is likely that the arabino-configuration of the fluorine moiety is essential for inhibition of further chain elongation after DNA incorporation (Parker *et al*., 1991). Clofarabine triphosphate is retained for a long period of time in cell lines, which is an important attribute of this deoxyadenosine analog as compared with others (Xie and Plunkett, 1995, 1996). The triphosphate of all three deoxyadenosine analogs, fludarabine, cladribine and clofarabine, inhibit ribonucleotide reductase, thus decreasing the concentrations of cellular deoxynucleotides and further inhibiting DNA synthesis (Parker *et al*., 1991; Xie and Plunkett, 1996).

In addition, the deoxyadenosine nucleoside analogs have a DNA-independent mechanism of action that promotes apoptosis. Alterations in mitochondria membrane potential caused by these agents promote cell death by causing cytochrome *c* release, which is likely because of conformational changes and mitochondria translocation of the pro-apoptotic proteins Bax and Bak (Genini *et al*., 2000a; Bellosillo *et al*., 2002; Dewson *et al*., 2003). Cytochrome *c* binds with Apaf-1, pro-caspase-9 and dATP to form the apoptosome, which activates caspase-9 to initiate the intrinsic cell death program (Riedl and Salvesen, 2007). In addition, the triphosphates of the deoxyadenosine nucleoside analogs can substitute for dATP and thus further tip the balance toward apoptosis by promoting apoptosome formation (Leoni *et al*., 1998; Genini *et al*., 2000b). Caspase-9 activation leads to the activating cleavage of executioner caspases, such as caspase-3 and caspase-7, an irreversible event leading to DNA endonuclease activation, DNA fragmentation and eventual cell death by apoptosis. Conversely, high levels of the antiapoptotic proteins Bcl-2 and Bcl-2-related family members confer resistance to nucleoside analogs by preventing events that lead to cytochrome *c* release from the mitochondria (Miyashita and Reed, 1993; Konopleva *et al*., 2000). Except at higher concentrations of nucleoside analogs, these effects likely occur after DNA-directed actions (Genini *et al*., 2000a), thus further emphasizing the critical importance of DNA targeting.

Other purine nucleoside analogs, such as pentostatin (deoxycoformycin) and forodesine (Immucillin-H, BCX-1777), may indirectly affect DNA synthesis, which may contribute to cell toxicity (Figure 2). Pentostatin is a natural product active in indolent leukemias (Johnson, 2001), that is an extremely potent inhibitor of adenosine deaminase (Agarwal, 1982). This action blocks the metabolic clearance of deoxyadenosine that arises from the normal turnover of cells, particularly those of hematopoietic processes. As a result, deoxyadenosine triphosphate accumulates, particularly in cells with high activities of deoxycytidine kinase (Plunkett *et al*., 1982; Seto *et al*., 1986). This imbalance of dNTP pools can directly affect DNA replication and may also block the production of other dNTPs, as dATP is a strong negative allosteric inhibitor of ribonucleotide reductase (Bianchi *et al*., 1992). These actions may deplete dNTPs, stall replication forks and may also result in mis-inserted deoxynucleotides because of pool imbalances. Forodesine is a transition state guanosine analog that is a potent inhibitor of purine nucleoside phosphorylase (Kicska *et al*., 2001), a key enzyme in the purine salvage pathway (Krenitsky, 1967). The cytotoxicity of this agent requires deoxycytidine kinase activity and the presence of deoxyguanosine (dGuo), suggesting that dGuo and not forodesine acts as a drug that needs to be phosphorylated (Kicska *et al*., 2001). Although the exact mechanism of action of forodesine is unknown, accumulation of dGTP and deregulation of the pyrimidine deoxynucleotide pools leads to inhibition of DNA synthesis and cell death after p53 stabilization, caspase activation, changes in mitochondrial membrane potential and PARP cleavage (Kicska *et al*., 2001; Balakrishnan *et al*., 2006). It is postulated that this is caused by ribonucleotide reductase inhibition by increased dGTP levels (Bantia *et al*., 2003; Gandhi and Balakrishnan, 2007).

Cellular responses to DNA synthesis inhibition

The nucleoside analogs discussed above cause DNA damage in the form of stalled replication forks, frank termination of nascent DNA synthesis or DNA nicks. Although the exact nature of the DNA damage-induced by these agents is not always clear, many of the cellular and molecular responses to other agents that inhibit DNA synthesis by different mechanisms likely overlap, including signals for cell death. Replication blocks, in general, elicit activation of the S phase checkpoint and cause cells to accumulate in the S phase of the cell cycle. For instance, hydroxyurea and aphidicolin inhibit DNA replication by depleting deoxynucleotide pools and inhibiting DNA polymerases, respectively, and cause activation of the S phase checkpoint. Fludarabine, ara-C, gemcitabine (Shi *et al*., 2001; Zhao and Piwnica-Worms, 2001; Sampath *et al*., 2002), UV (Heffernan *et al*., 2002) and topoisomerase I poisons (Cliby *et al*., 2002) also activate an intra S phase checkpoint. Therefore, it is likely that a common complement of sensor molecules function to detect replication stress caused by a diversely acting set of replication-targeting agents, leading to apoptosis. However, it is now becoming clear that nucleoside analogs with unique mechanisms of action, such as CNDAC, may be recognized differently by causing a different type of DNA damage, to which cells respond by activating the G_2 checkpoint (Azuma *et al*., 2001; Liu *et al*., 2005).

Molecular sensing of DNA damage

Upon the induction of DNA lesions and stalled replication forks, molecular sensors recognize aberrant DNA structures by accumulating at sites of damage and elicit cellular responses, such as checkpoint activation, DNA repair or apoptosis. Ataxia-telangiectasia mutated (ATM), Ataxia-telangiectasia mutated and rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) are serine/threonine kinases that represent a class of molecular sensors central to the DNA damage response. ATR is an essential checkpoint protein kinase in eukaryotes that is activated in response to replication stress and functions as a central activation of downstream effectors for S phase checkpoint activation and apoptosis (Paulsen and Cimprich, 2007). This kinase is essential, as null mutations in mice are embryonic lethal and hypomorphic mutations in the ATR gene results in the human autosomal disorder, Seckel syndrome (Brown and Baltimore, 2000; de Klein *et al*., 2000; O'Driscoll *et al*., 2003). ATR is attracted to sites of stalled replication forks by single-stranded DNA that is coated by replication protein A (RPA) upon depletion in deoxynucleotide pools, inhibition of polymerases, or replication blocking by nucleoside analogs (Figure 3, Zou and Elledge, 2003). This coating of the DNA by RPA and an interaction between ATR and the ATR-interacting protein (ATRIP) likely serves as a platform for ATR activation (Cortez *et al*., 2001). ATR deficiency leads to a significant decrease in cellular recovery after exposure to nucleoside analogs (Karnitz *et al*., 2005), thus further suggesting its signaling function in response to replication stress. In another role, ATR may also be involved in signaling for apoptosis. ATR can directly or indirectly, through Chk1 kinase, activate p53 by phosphorylation (Tibbetts *et al*., 1999; Shieh *et al*., 2000), leading to protein stabilization and transcriptional attenuation (Gottifredi *et al*., 2001).

Although ATR is predominately responsible for activation of DNA damage checkpoints in response to replication stress, ATM has been classically identified as the primary mediator for the response to double-strand breaks (Shiloh, 2003). However, recent evidence from two independent groups suggests that ATR is activated by ATM in response to ionizing radiation-induced double-strand breaks (Jazayeri *et al*., 2006; Myers and Cortez, 2006). Thus, the theory of two parallel ATR and ATM checkpoint pathways may be evolving. The function that ATM kinase has in response to stalled replication forks is not clear. However, ATM becomes autophosphorylated on its activation site, Ser^{1981}, co-localizes at sites of replication forks induced by nucleoside analogs, and is required for survival under these conditions, suggesting its activation (Karnitz *et al*., 2005; Ewald *et al*., 2007). DNA damage-induced ATM activation requires many post-translational modifications, including acetylation, autophosphorylation and monomerization (Bakkenist and Kastan, 2003; Kozlov *et al*., 2006; Sun *et al*., 2007). These events are likely required before localization of active ATM monomers to sites of DNA damage, where it is involved in coordinating many cellular events including DNA damage recognition, checkpoint activation, chromatin relaxation and apoptosis (Shiloh, 2006; Matsuoka *et al*., 2007). In a manner similar to ATR, ATM can directly and indirectly phosphorylate many sites of p53, which can lead to apoptosis (Kurz and Lees-Miller, 2004). Exactly how ATM initiates the apoptotic response after DNA damage is not clear. However, ATM is associated with the regulation of other proteins that are closely involved with cell death signaling, such as noxa, puma, bax and Hdm2 (de Toledo *et al*., 2000; Villunger *et al*., 2003). The third related DNA damage sensor, DNA-PK, is known for its role in the nonhomologous end-joining (NHEJ) repair pathway. Although its function in response to nucleoside analog-induced stalled replication forks has not been extensively studied, DNA-PK may recognize such lesions and signal for apoptosis through p53 (Achanta *et al*., 2001).

Cell cycle checkpoint activation

When fork progression is halted, it is crucial that ongoing DNA replication stalls so that lesions or barriers can be repaired before the continuation of DNA replication. The ATR kinase and its downstream effector, Chk1, are central regulators of the S and G_2 checkpoint responses responsible for delays in the cell

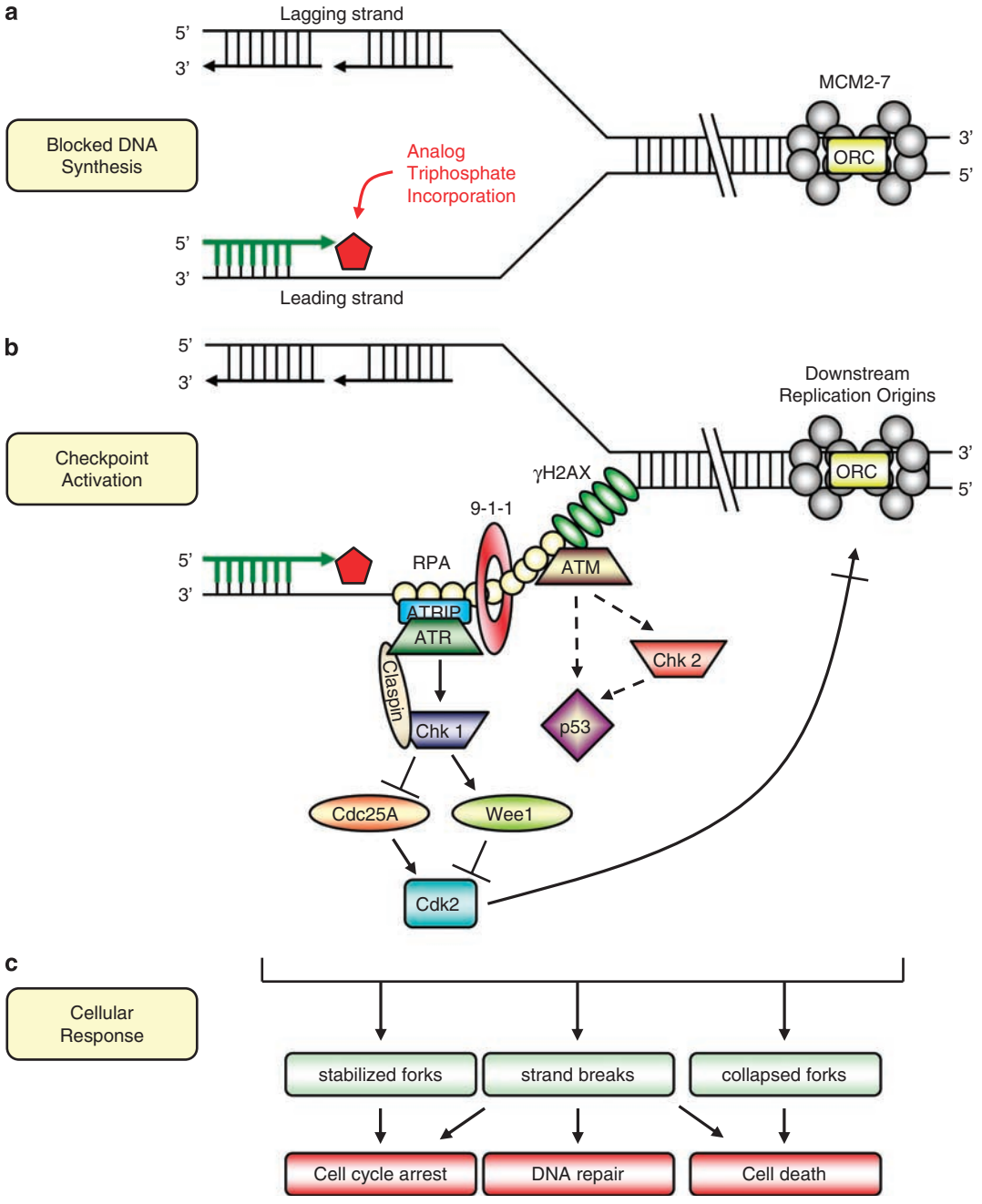

Figure 3 Proposed model of the molecular and cellular response to nucleoside analog-induced stalled replication forks. The actions of nucleoside analogs are most clearly envisioned when incorporated into the leading strand, as represented here. However, incorporation is also possible in the lagging strand. For clarity, the proteins of the DNA replication complex have been omitted. Incorporation of nucleoside analogs during DNA synthesis terminates DNA replication and leads to stalling of replication forks, causing an accumulation of single-stranded DNA (**a**), which is coated by RPA and attracts ATR-interacting protein (ATRIP), serving as a platform for ATR activation. ATR activates Chk1 by phosphorylation, which is facilitated by claspin and the Rad9-Rad1-Hus1 (9-1-1) clamp. Inhibition of Cdc25A phosphatase and activation of Wee1 kinase by Chk1 leads to subsequent phosphorylation and inactivation of cyclin-dependent kinase 2 (Cdk2), thus halting the firing of downstream replication origins. Checkpoint activation promotes fork stabilization, leading to cell cycle arrest. ATM may phosphorylate p53 directly or indirectly through Chk2, leading to apoptosis. Phosphorylation of the histone, H2AX (γ-H2AX), likely marks stalled replication forks and may be involved in molecule recruitment (**b**). Depending on the extent of damage and the molecular response, cells respond to stabilized forks, strand breaks, or collapsed forks by arresting the cell cycle, initiating DNA repair, or signaling for cell death (**c**). (→) activation; (⟞) inhibition; (⟞→) relief of activation; MCM2-7, mini-chromosome maintenance helicase; ORC, origin recognition complex.

cycle under these conditions (Chen and Sanchez, 2004). In a somewhat redundant fashion, ATM and DNA-PK may also contribute to checkpoint activation as these kinases are involved in DNA damage recognition and have many similar downstream substrates (Burma and Chen, 2004; Matsuoka *et al*., 2007). In response to

stalled replication forks, ATR activates Chk1 kinase by phosphorylation on Ser^{317} and Ser^{345} (Figure 3, Sampath *et al*., 2002; Karnitz *et al*., 2005; Robinson *et al*., 2006; Sampath *et al*., 2006; Ewald *et al*., 2007). In turn, Chk1 indirectly regulates cyclin-dependent kinases (Cdks) through inhibition of Cdc25 phosphatases and activation of wee1 kinase (Figure 3, Pines, 1999; Bartek and Lukas, 2003; Busino *et al*., 2004; Liu *et al*., 2005; Sampath *et al*., 2006; Ewald *et al*., 2007). The inactive Tyr^{15}-phosphorylated forms of these cyclin-dependent kinases that accumulate from checkpoint activation are unable to initiate replication origins (Figure 3, Mailand and Diffley, 2005), thus inducing cell cycle arrest (Shi *et al*., 2001; Cliby *et al*., 2002; Heffernan *et al*., 2002; Wang *et al*., 2002). Phosphorylation of Chk1 is facilitated by many molecular players, including claspin and the Rad9-Rad1-Hus1 (9-1-1) clamp, which are loaded onto chromatin in an ATR-independent manner. The binding of claspin to Chk1 and Rad9 suggests that it may serve a role in facilitating the efficient accumulation of required checkpoint substrates at sites of stalled replication (Chini and Chen, 2003; Jeong *et al*., 2003). Along with the 9-1-1 clamp and claspin, TopBP1 may serve a similar function as a direct activator of the ATR-ATRIP complex (Kumagai *et al*., 2006; Delacroix *et al*., 2007; Lee *et al*., 2007). Activation of the ATR-dependent S phase checkpoint (ATR-Chk1-Cdk2) in response to nucleoside analog-induced stalled replication forks promotes fork stabilization (Figure 3), likely allowing DNA repair mechanisms an opportunity to remove fraudulent nucleotides from DNA strands. However, when stabilization does not occur, replication fork collapse likely leads to a lethal accumulation of DNA breaks, which may activate an ATM-dependent response (ATM-Chk2-p53), leading to cell death (Figure 3; Karnitz *et al*., 2005; Ewald *et al*., 2007).

Checkpoint dysregulation

The systematic triggering of cell cycle checkpoints in response to DNA-damaging agents offers a potentially exploitable mechanism for maximizing drug sensitivity and increasing therapeutic use. In experimental systems, pharmacological inhibition of Chk1 in nucleoside analog-arrested cells results in rapid abrogation of the checkpoint, enhanced DNA damage and increased apoptosis (Shi *et al*., 2001; Liu *et al*., 2005; Xiao *et al*., 2005; Matthews *et al*., 2007; Ewald *et al*., 2007). A similar effect has been demonstrated with combinations of Chk1 inhibitors and other DNA-damaging agents, such as alkylating agents and topoisomerase poisons (Zhou and Bartek, 2004). The actions of Chk1 inhibitors in potentiating the toxicity of S phase DNA-damaging agents are likely independent of p53 status (Shao *et al*., 1997; Sugiyama *et al*., 2000; Eastman *et al*., 2002; Kohn *et al*., 2002), thus making them attractive for therapeutic uses.

The exact extent of DNA damage caused by such mechanism-based combinations is unknown, but evidence supports the postulation that increases in apoptosis after checkpoint abrogation is caused by collapsing of replication forks. For example, nucleoside analog exposure causes the phosphorylation of the DNA damage responsive histone, H2AX, which forms nuclear foci at sites of stalled replication forks (Figure 3). Upon checkpoint abrogation of gemcitabine-induced S phase arrested cells by inhibition of Chk1, H2AX phosphorylation further increases by 10-fold and is associated with a decrease in clonogenic survival (Matthews *et al*., 2007; Ewald *et al*., 2007), thus suggesting lethal increases in DNA damage. A similar effect was observed after only a 2-h exposure to gemcitabine. The brevity of which indicates such pharmacologic interaction with checkpoint function can rapidly generate such damage (Ewald *et al*., 2007). Interestingly, the fraction of cells with measurable H2AX phosphorylation does not increase upon checkpoint abrogation, suggesting that Chk1 inhibition specifically kills cells with an activated S phase checkpoint. As DNA damage accumulates, it is likely that a threshold for DNA repair is eventually overwhelmed, leading to apoptosis. However, the mechanism by which cell death signaling is triggered in response to checkpoint abrogation is unclear.

The first generation Chk1 inhibitor, UCN-01 (7-hydroxystaurosporine) is currently being investigated alone and in combination with DNA-damaging agents in phase I and II trials (Tse *et al*., 2007). Clinical studies of UCN-01 in combination with cisplatin (Lara *et al*., 2005; Perez *et al*., 2006), 5-fluorouracil (Kortmansky *et al*., 2005), topotecan (Hotte *et al*., 2006; Welch *et al*., 2007) and ara-C (Sampath *et al*., 2006) have been initiated in solid tumors and hematological malignancies. Second generation Chk1 inhibitors (Tse *et al*., 2007) and inhibitors of other kinases involved in checkpoint regulation are currently being developed (Hickson *et al*., 2004; Kawabe, 2004), which may offer increased clinical activity.

DNA repair and drug resistance

DNA damage is a serious threat to the stability and integrity of the genome. If not repaired, lesions may be cytotoxic or mutagenic. Therefore, organisms have developed complex molecular mechanisms to recognize and repair different types of DNA lesions within cells (Helleday *et al*., 2008). These mechanisms are likely severely challenged by exogenous sources of DNA damage, such as DNA-targeting cancer therapeutics, which cause many types of compromised DNA structures and DNA breaks. A discovery of the processes involved in the removal of nucleoside analogs and repair of stalled forks is necessary to better understand the mechanisms that spare toxicity to these agents.

A systematic approach to uncovering the exact mechanisms, which are responsible for DNA repair of nucleoside analog-induced DNA damage will likely have therapeutic value. Proofreading $3' \rightarrow 5'$ exonuclease activities associated with replicative DNA polymerases (Huang *et al*., 1991) and base excision repair processes (Chou *et al*., 2000) are capable of removing fraudulent nucleotides from DNA, providing a mechanism that

potentially causes drug resistance. However, a slow rate of drug removal and sustained cell cycle arrest after exposure to nucleoside analogs suggests that these mechanisms do not significantly promote survival (Shi *et al*., 2001). Other pathways have recently been explored for their involvement in drug removal. A non-functional nucleotide excision repair pathway caused by deletions in either CSB, XPB, XPF or ERCC1 leads to increased drug sensitivity to the DNA nick-causing nucleoside, CNDAC, but does not appear to be active in response to ara-C- or troxacitabine-induced stalled replication forks (Wang *et al*., 2008). Further, neither the base excision repair nor the mismatch repair pathways appear to be involved in the removal of CNDAC (Wang *et al*., 2008).

ATM and the Mre11-Rad50-Nbs1 (MRN) complex are DNA damage response molecules closely associated with the repair of double-strand breaks (D'Amours and Jackson, 2002; Stracker *et al*., 2004), although several lines of evidence suggest that these molecules may also be involved in the response to stalled replication forks. Dysfunction of ATM or the MRN complex subunits results in embryonic lethality in eukaryotes (Xiao and Weaver, 1997; Luo *et al*., 1999; Zhu *et al*., 2001a) and hypomorphic mutations are associated with a variety of human disorders, including ataxia-telangiectasia (AT), ataxia-telangiectasia-like disorder (ATLD) and Nijmegen breakage syndrome (NBS, Carney *et al*., 1998; Matsuura *et al*., 1998; Varon *et al*., 1998; Stewart *et al*., 1999; Shiloh, 2006), suggesting their involvement during normal DNA replication. At the molecular level, the MRN complex associates with chromatin in an S phase-specific manner (Mirzoeva and Petrini, 2001) and binds with RPA (Robison *et al*., 2004; Olson *et al*., 2007). A similar phenomenon is evident in response to stalled replication forks. Nuclear co-localization of Mre11, Rad50 and Nbs1 with other DNA damage response molecules, phosphorylated ATM and H2AX, increases in response to gemcitabine, ara-C, troxacitabine and hydroxyurea (Wang *et al*., 2000; Mirzoeva and Petrini, 2003; Robison *et al*., 2005; Ewald *et al*., 2008).

The function ATM and the MRN complex at sites of stalled replication forks is unknown, but they may prevent fork collapse, which could lead to double-strand breaks and chromosomal aberrations (Yamaguchi-Iwai *et al*., 1999; Costanzo *et al*., 2001; Trenz *et al*., 2006; Wen *et al*., 2008). By facilitating repair, it is likely that these molecules block death signaling and thus contribute to drug resistance (Ewald *et al*., 2008). It is possible that the MRN complex prevents fork collapse by tethering DNA strands together through the self-association of Rad50 coiled-coil domains, as occurs at double-strand break sites (van den Bosch *et al*., 2003; Moreno-Herrero *et al*., 2005). The termination of DNA synthesis on the leading strand presents a 3′ end for potential MRN-binding whereas the unannealed gaps between Okazaki fragments also presents DNA ends for potential binding on the lagging strand. Alternatively, the MRN complex may be capable of removing fraudulent nucleotides from the DNA as Mre11 has both $3' \rightarrow 5'$ exonuclease and single-strand endonuclease activities (Paull and Gellert, 1998; Trujillo *et al*., 1998), an action that may permit the re-start of DNA. This provides a novel mechanism for the removal of nucleoside analogs from DNA, which is poorly understood (Helleday *et al*., 2008). Simplified models utilizing purified enzymes/enzyme complexes, oligonucleotides and primer extension assays may be useful to answer these questions. Future investigations that seek to determine if Mre11 or other repair molecules are capable of excising nucleoside analogs from the DNA *in vitro* and *in vivo* are warranted.

Targeting DNA repair in quiescent cells

As DNA synthesis inhibitors, nucleoside analogs are effective in killing actively cycling populations. However, the requirement for incorporation into the DNA for most of these agents limits their action in indolent diseases, which do not have ongoing DNA synthesis. The quiescent nature of these malignancies reduces the opportunity for nucleoside analog incorporation into DNA and subsequent cytotoxicity. However, the induction of excision repair in non-cycling cells offers the opportunity for analog incorporation during re-synthesis steps, an action that leads to DNA-directed cell killing (Figure 4). Early investigations confirmed that nucleoside analogs, such as ara-C and fludarabine, are incorporated into UV-induced DNA repair patches of human quiescent cells, events which lead to apoptosis (Kufe *et al*., 1984; Snyder *et al*., 1984; Sandoval *et al*., 1996). Inhibition of the DNA repair patch leads to p53 stabilization, p53 phosphorylation and increased Fas expression (Rao and Plunkett, 2003). Blocking the incorporation of nucleoside analogs into the DNA repair patches of lymphocytes abrogates cell death, which confirms that the DNA damage response is insufficient to initiate cell death and that analog incorporation is a critical event (Rao and Plunkett, 2003).

Further investigations have supported moving such rationales into the clinic, which exploit DNA repair capacities of quiescent cells by combining agents with complementary mechanisms of action. Alkylating agents have long been the mainstay in the conventional treatment of the indolent disease, CLL. However, remissions are often incomplete, which leads to progressive disease and drug resistance. DNA adducts caused by alkylating agents or platinum derivatives cause DNA intrastrand and interstrand crosslinks, which initiate base excision repair and nucleotide excision repair (NER, Chaney and Sancar, 1996). The NER repair process and the proteins involved can be summarized in five steps: damage recognition (XPC), introduction of lesions on the damaged strand on each side of the adduct (TFIIH complex containing XPB and XPD), excision of 24–32 residues on the damaged strand (XPG, ERCC1-XPF), DNA polymerase gap filling, and DNA ligase sealing (Figure 4, de Laat *et al*., 1999). Therefore, NER is an integral part of crosslink repair and is likely a mechanism underlying drug resistance in CLL, as lymphocytes resistant to alkylating agents have

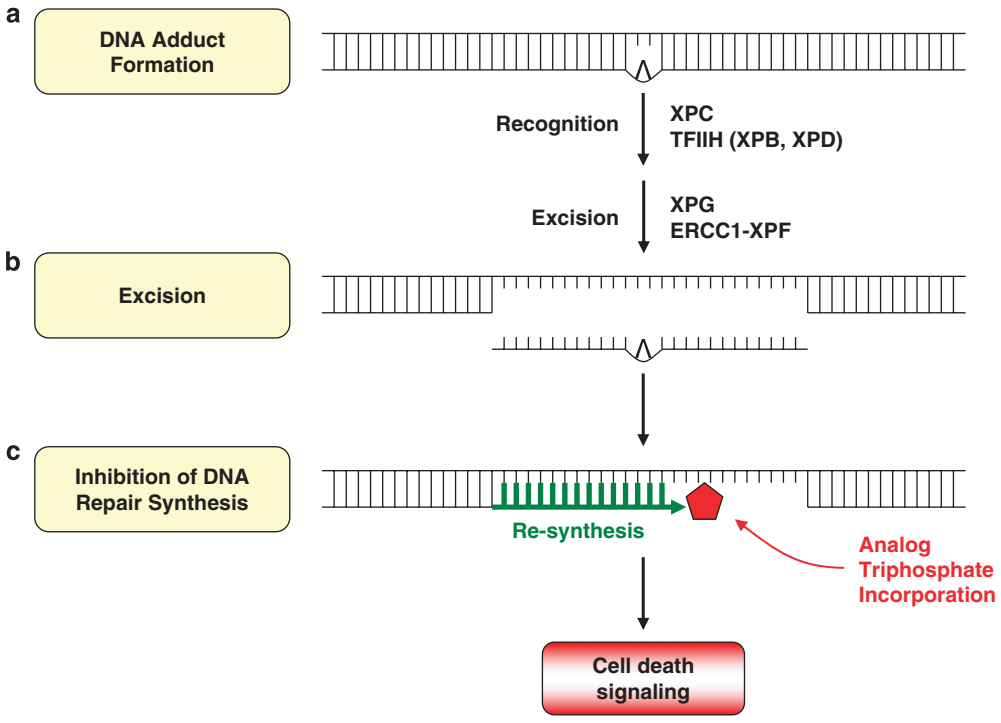

Figure 4 Targeting nucleotide excision repair with nucleoside analogs. Induction of DNA damage, such as thymine dimers by UV or crosslinking by alkylating agents, leads to activation of nucleotide excision repair processes (**a**). Upon recognition of DNA adducts by XPC and the TFIIH complex containing XPB and XPD, a 24–32 nucleotide strand containing the lesion is excised on the 5′ end by XPG and on the 3′ end by ERCC1-XPF (**b**). In the presence of nucleoside analog triphosphate, gap-filling DNA synthesis is inhibited by analog incorporation into the DNA repair patch, leading to cell death signaling (**c**).

increased NER activity (Geleziunas *et al*., 1991; Buschfort *et al*., 1997). This presents an opportunity to exploit DNA repair in CLL.

The use of alkylating agents offers an opportune chance to induce NER; thus potentially allowing analog incorporation into DNA repair patches (Figure 4). In primary CLL cells, fludarabine and clofarabine successfully inhibit DNA repair induced by cyclophosphamide, leading to significant increases in cell death, as compared with conditions in which alkylating agents were used alone (Yamauchi *et al*., 2001; Moufarij *et al*., 2006). This principle has been clinically validated, as relapse-free survival is extended with combinations of cyclophosphamide and fludarabine (Eichhorst *et al*., 2006; Catovsky *et al*., 2007; Flinn *et al*., 2007). New combinations of nucleoside analogs and platinum derivatives with complementary mechanisms of action and non-overlapping side effect profiles may further increase activity (Tsimberidou *et al*., 2008). These studies validate strategies that target DNA repair mechanisms and suggest that this may be an important step in the development of novel approaches for overcoming drug resistance to DNA-targeting chemotherapeutics.

Targeting DNA methylation

Azanucleosides

The ribo- and deoxyribonucleosides, azacytidine (5-azacytidine) and decitabine (2′-deoxy-5-azacytidine), were initially developed as classical cytostatic agents (Figure 5, Vesely and Cihak, 1977). They demonstrated a wide range of antitumor activity against cells *in vitro* and in AML. However, in addition to their DNA-directed actions, these compounds, when incorporated into DNA, potently inhibited DNA methylation. This led to the successful development of these agents as targeted drugs aimed at reversing epigenetic silencing in cancer cells.

Epigenetic silencing in cancer

Epigenetic changes usually result in the alteration of gene function without any change in the DNA sequence of genes. In cancer, epigenetic silencing often occurs by multiple processes, such as the action of non-coding RNA, methylation of cytosines on DNA, specific modifications to the histones on the chromatin and nucleosome positioning (Jones and Baylin, 2007). Until recently, epigenetic studies in cancer focused on the aberrant methylation of stretches of cytosine–guanine residues that formed CpG islands within gene promoters (Takai and Jones, 2002). DNA methyltransferases are a family of enzymes that catalyse the addition of a methyl group to the 5 carbon of a cytosine that is immediately 5′ to a guanine (CG dinucleotide). Surprisingly, many tumors are characterized by a global hypomethylation with localized regions of hypermethylation on CpG islands, which leads to transcriptional inactivation when it occurs within a promoter region. The expression of

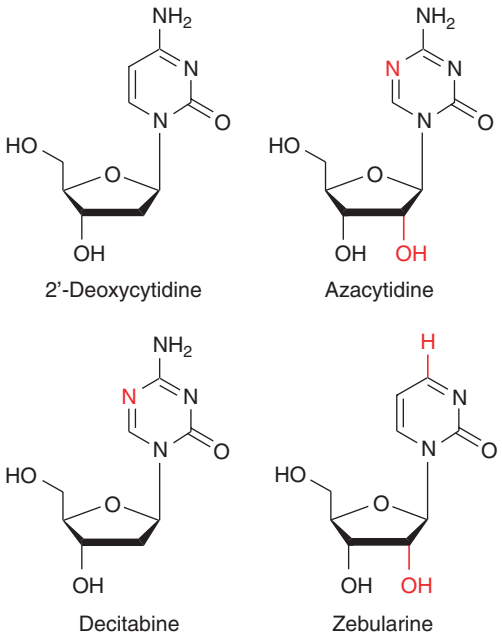

Figure 5 Structures of deoxycytidine, azacytidine, decitabine and zebularine.

certain tumor suppressor genes containing CpG-rich islands can be downregulated by *de novo* methylation in primary tumors *in vivo* (Herman *et al*., 1996, 1997), which links promoter methylation and tumor initiation. For instance, hypermethylation-mediated silencing of *p15* and *p16* represents some of the most common and earliest epigenetically mediated losses of tumor suppressor gene function that occur in hematological, breast, colon and lung cancers (Herman *et al*., 1997; Belinsky *et al*., 1998; McDermott *et al*., 2006). Other genes that are abnormally methylated in cancer include *APC* and the *GATA-4,-5* transcription factors, which are linked to the pathogenesis of colon cancer (Jones and Baylin, 2007), the death-associated protein kinase in hematological and lung cancers (Nakatsuka *et al*., 2003; Toyooka *et al*., 2003), and p53 in hepatomas (Pogribny and James, 2002). Similarly, methylation of multiple genes within a regulatory pathway composed of *p73, p15* and *p57KIP2* occurred in Philadelphia chromosome (Ph)-negative patients with acute lymphocytic leukemia, such that inactivation of these genes predicts for a poor prognosis (Shen *et al*., 2003). In general, it has been estimated that, on average, 10% of CpG islands in DNA are abnormally methylated in tumors (Ahluwalia *et al*., 2001; Yan *et al*., 2001), offering a novel target for cancer therapy.

Role of nucleoside analogs in reversing epigenetic silencing in cancer

The azanucleosides, azacytidine and decitabine, are phosphorylated by uridine–cytidine kinase and deoxycytidine kinase, respectively (Stresemann and Lyko, 2008) and accumulate in cells as their active triphosphates. Azacytidine is a ribonucleoside analog that preferentially becomes incorporated into RNA and thereby interferes with protein synthesis. However, a minor portion (10%) is incorporated into DNA in place of deoxycytidine (Li *et al*., 1970). Decitabine is generally assumed to be more specific since it is more directly incorporated into DNA (Brueckner *et al*., 2007). Once incorporated, both aza-analogs covalently trap the DNA methyltransferases and mediate their degradation, leading to a passive loss in DNA methylation in the cell (Stresemann and Lyko, 2008). Both azacytidine and decitabine were initially administered at their maximum tolerated doses and were associated with substantial toxicity. However, newer regimens that administered low doses of azacytidine or decitabine produced significant therapeutic effects in Phase II and III randomized trials for myelodysplastic syndromes (MDS, Oki and Issa, 2006). Maintenance of *p15* hypermethylation and lack of gene expression correlated with poor or no response to treatment of AML and myelodysplastic syndrome patients (Oki and Issa, 2006). Conversely, therapeutic response in these trials were associated with a demethylation of initially hypermethylated CpG islands of the *p15* gene and re-expression of p15 protein (Kantarjian *et al*., 2007b). Although such correlations between p15 and treatment response emphasize the potential importance of p15 re-expression to disease treatment, it remains unclear whether remission in patients treated with broad range demethylating agents is a direct result of re-expressing p15 (Raj *et al*., 2007).

Regarding their mechanism of action, the efficacy of azacytidine or decitabine as antineoplastic agents appears to result from two distinct mechanisms: cytotoxicity when administered at high doses and inhibition of DNA methyltransferases when given at low doses. At higher doses, decitabine induces a classical DNA damage response characterized by the activation of strand break repair proteins, cell cycle checkpoint proteins, phosphorylation of H2AX, activation of the ATM-p53-p21 pathway, leading to cell cycle arrest and apoptosis (Hsi *et al*., 2005; Jiemjit *et al*., 2008; Palii *et al*., 2008). At lower doses, hypomethylation associated reactivation of genes appears to mediate its antileukemic action. A thorough evaluation of the downstream consequences of hypomethylation-induced gene reactivation, such as apoptosis, differentiation or senescence would provide a mechanistic basis for the observed clinical efficacy of 5-azacytidine and decitabine.

Owing to the chemical decomposition that results in short plasma half-lives for azacytidine (1.6 h; Zhao *et al*., 2004) and decitabine (2.5 h; Liu *et al*., 2006), efforts have been focused on the development of chemically stable cytosine analogs for epigenetic therapy. The cytosine analog, zebularine [Figure 5; 1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one], has been shown to mediate epigenetic reactivation of the *p16* tumor suppressor gene efficiently in human cancer cell lines and bladder carcinoma xenografts (Cheng *et al*., 2003). Further, zebularine is stable in aqueous solutions. Oral drug delivery in mice results in detectable plasma concentrations up to 16 h after administration

(Brueckner *et al*., 2007). Further investigations are required in humans to determine the stability of zebularine in human plasma.

In addition to being evaluated as single agents, DNA methylation inhibitors show promise when used in combination with another class of epigenetically acting drugs, the histone deacetylase inhibitors. The coordinate expression of genes is regulated by the methylation status of promoter-associated CpG islands in conjunction with modifications in the biochemical composition of nucleosome-associated histone tails (Jones and Wolffe, 1999). For instance, acetylation of specific residues in histone H3 and H4 is associated with an open chromatin configuration and gene transcription. In contrast, deacetylation of these residues is associated with a repressive state (Rice and Allis, 2001). Consequently, combinations of histone deacetylase inhibitors with hypomethylating agents results in reactivation of gene expression (Richon and O'Brien, 2002), cell cycle arrest and apoptosis (Zhu *et al*., 2001b; Tang *et al*., 2004; Schmelz *et al*., 2005; Walton *et al*., 2008). The azanucleosides analogs have also shown synergistic activity with conventional nucleoside analog chemotherapeutic agents such as 5-fluorouracil, which is based on their ability to reactivate previously silenced pro-apoptotic genes (Kanda *et al*., 2005; Morita *et al*., 2006).

Nucleoside analogs and microRNA

MicroRNAs (miRNA, miR) are a newly recognized class of small non-coding RNAs that negatively regulate gene expression by inducing RNA degradation or by interfering with translation. Aberrant expression of miRNA has been linked to the pathogenesis of several tumors (Calin and Croce, 2006). In general, miRNA are downregulated in cancer (Tili *et al*., 2007). Depending on the cellular context in which they are expressed, miRNA can function as tumor suppressors or oncogenes (Doench and Sharp, 2004). A recent report has demonstrated that up to 10% of all miRNA may be regulated by methylation (Han *et al*., 2007). Aberrant methylation of miRNA promoters has been mechanistically linked to silencing of miRNA in several instances. For instance, CpG island methylation leads to the silencing of miR-127 expression, leading to the enhanced expression of Bcl-6, a proto-oncogene linked to non-Hodgkin's lymphoma (Saito *et al*., 2006). Epigenetic silencing of miR15a and 16-1, miRs that target Bcl-2, were found in B-CLL (Mertens *et al*., 2006). Aberrant hypermethylation of miR-9-1, miR-124a, miR-148, miR-152 and miR-663 is an early event in breast cancer (de Klein *et al*., 2000). Other reports have demonstrated hypermethylation-induced silencing of miR-203 leading to overexpression of oncogenic Bcr-Abl in chronic myelogenous leukemia (Bueno *et al*., 2008). Consequently, exposure to decitabine, alone or in combination with histone deacetylase inhibitors restores miRNA expression with corresponding declines in target oncogene expression and apoptosis of neoplastic cells (Calin and Croce, 2006; Mertens *et al*., 2006; Saito *et al*., 2006; Zhang *et al*., 2008). Therefore, as methylation patterns that affect miRNA expression become better understood, opportunities may arise that support the use of select nucleoside analogs to target specific gene expression.

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