

# DNA Polymerases as Therapeutic Targets<sup>†</sup>

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**ABSTRACT:** Numerous pathological states, including cancer, autoimmune diseases, and viral/bacterial infections, are often attributed to uncontrollable DNA replication. Inhibiting this essential biological process provides an obvious therapeutic target against these diseases. A logical target is the DNA polymerase, the enzyme responsible for catalyzing the addition of mononucleotides to a growing polymer using a DNA or RNA template as a guide for directing each incorporation event. This review provides a summary of therapeutic agents that target polymerase activity. A discussion of the biological function and mechanism of polymerases is first provided to illustrate the strategy for therapeutic intervention as well as the rational design of various nucleoside analogues that inhibit various polymerases associated with viral infections and cancer. The development of nucleoside and non-nucleoside inhibitors as antiviral agents is discussed with particular emphasis on their mechanism of action, structure–activity relationships, toxicity, and mechanism of resistance. In addition, commonly used anticancer agents are described to illustrate the similarities and differences associated with various nucleoside analogues as therapeutic agents. Finally, new therapeutic approaches that include the inhibition of selective polymerases involved in DNA repair and/or translesion DNA synthesis as anticancer agents are discussed.

**DNA Replication as a Therapeutic Target.** DNA replication is the process of duplicating DNA to generate two copies of an organism's genetic information (Figure 1A). This complex biological process is catalyzed by DNA polymerases that add mononucleotides to a growing primer using nucleic acid templates as a guide for directing each incorporation event (Figure 1A). DNA replication is absolutely essential for the proliferation and survival of all forms of life ranging from simple viruses and bacteria to more complex organisms, including humans. The importance of DNA replication is often highlighted by the variety of pathological states that result from dysfunctional and/or unregulated activity. For example, certain genetic diseases such as ataxia telangiectasia (1) are associated with the generation of mutations caused by decreased fidelity during replication. Hyperproliferative diseases such as cancer, autoimmune conditions, and viral/bacterial infections are associated with uncontrollable and, in some cases, promutagenic DNA replication. As such, a major goal in biomedical science is to develop therapeutic agents to combat these pathological states.

As outlined in Figure 1B, there are several strategies currently used to inhibit DNA replication. One approach is the use of DNA-damaging agents (step 1) that modify a nucleic acid so that it no longer acts as an effective substrate for the DNA polymerase. Examples include chemothera-

peutic agents such as Temozolomide and cisplatin that modify the composition and structure of DNA to inhibit DNA synthesis and prevent cellular proliferation (2). While effective, their utility is typically limited by the severity of side effects caused by the nonselective killing of cancerous and healthy cells in addition to the potential to induce mutagenic events that can actually accelerate disease development (3). Another strategy is to modulate the activity of individual enzymes involved in DNA replication (step 2). Agents such as etoposide inhibit the activity of topoisomerase, an enzyme that resolves the “knots” in DNA formed during replication (4). Inhibition of this enzyme

<sup>†</sup> Abbreviations: dNTP, deoxynucleoside triphosphate; RT, reverse transcriptase; AZT, 3-azidothymidine; d4T, 2',3'-didehydro-2',3'-dideoxythymidine; ddC, 2',3'-dideoxycytidine; (–)-3TC, 2',3'-dideoxy-3'-thiacytidine; CBV, 9-[4 $\alpha$ -(hydroxymethyl)cyclopent-2-ene-1 $\alpha$ -yl]guanine; acyclovir, 2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6H-purin-6-one; NNRTI, non-nucleoside reverse transcriptase inhibitor; efavirenz, 8-chloro-5-(2-cyclopropylethynyl)-5-(trifluoromethyl)-4-oxa-2-azabicyclo[4.4.0]deca-7,9,11-trien-3-one; nevirapine, 1-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido[3,2-*b*:2',3'-*e*][1,4]diazepin-6-one; delavirdine, *N*-(2-{4-[3-(1-methylethylamino)pyridin-2-yl]piperazin-1-yl}carbonyl-1H-indol-5-yl)methanesulfonamide; entravine, 4-{6-amino-5-bromo-2-[(4-cyanophenyl)amino]pyrimidin-4-yl}oxy-3,5-dimethylbenzonitrile; fludarabine, 2-fluoroarabinofuranosyladenine; cladribine, 2-chloroarabinofuranosyladenine; cytarabine, 1- $\beta$ -D-arabinofuranosylcytosine; gemcitabine, 2',2'-difluoro-2'-deoxycytidine; decitabine, 5-aza-2-deoxy-2'-cytidine; 5-NITP, 5-nitro indolyl-2'-deoxyribose triphosphate; 5-CE-ITP, 5-cyclohexene indolyl-2'-deoxyribose triphosphate; 5-PhITP, 5-phenyl indolyl-2'-deoxyribose triphosphate; 5-FITP, 5-fluoro indolyl-2'-deoxyribose triphosphate; 5-AITP, 5-amino indolyl-2'-deoxyribose triphosphate; 5-CH-ITP, 5-cyclohexyl indolyl-2'-deoxyribose triphosphate.

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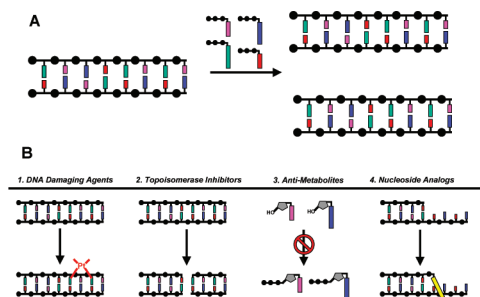


FIGURE 1: (A) DNA replication is catalyzed by a DNA polymerase. During this process, the polymerase incorporates 2'-deoxynucleoside triphosphates opposite a DNA template which guides each incorporation event. (B) Chemotherapeutic strategies used to inhibit DNA replication include (1) DNA-damaging agents, (2) inhibition of DNA-modifying enzymes, (3) inhibition of enzymes involved in nucleotide metabolism, and (4) nucleoside analogues that directly inhibit DNA polymerase activity.

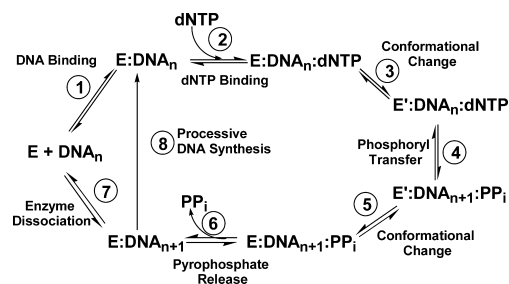


FIGURE 2: Kinetic mechanism for RNA- and DNA-dependent polymerases. Individual steps along the pathway for DNA polymerization are numbered and identified. Abbreviations: E, polymerase; DNA<sub>n</sub>, DNA substrate; E', conformational change in DNA polymerase; PP<sub>i</sub>, inorganic pyrophosphate; DNA<sub>n+1</sub>, DNA product (DNA extended by one nucleobase).

causes apoptosis by creating single- and double-stranded DNA breaks that interrupt the continuity of DNA synthesis. A third approach includes reducing the availability of dNTPs<sup>1</sup> that are the building blocks for DNA synthesis (step 3). By inhibiting enzymes involved in nucleotide metabolism, antimetabolites such as methotrexate and hydroxyurea reduce the size of nucleotide pools to indirectly hinder DNA synthesis (5). Perhaps the most direct approach, however, is the use of nucleoside analogues such as AZT (6) or fludarabine (7) that target the enzymatic activity of the DNA polymerase, the workhorse of the DNA replication apparatus (step 4). This last strategy represents the focus of this review. In this article, we shall discuss the design, mechanism of action, and pitfalls associated with various nucleoside analogues that are used as therapeutic agents against common hyperproliferative diseases, including viral infections and cancer.

**Mechanism and Dynamics of the DNA Polymerization Cycle.** Before I describe how nucleoside analogues act as therapeutic agents, it is first necessary to understand the mechanism of DNA polymerization and how perturbations in various kinetic steps can influence their effectiveness. A general mechanism that applies to both prokaryotic and eukaryotic DNA polymerases is provided in Figure 2. As illustrated, this intricate biological process occurs via a multiplicative mechanism in which the polymerase binds DNA prior to the binding of dNTP. The first point for generating high catalytic efficiency and fidelity occurs through the binding of dNTP to the polymerase:DNA

complex (step 2). The binding affinity ( $K_d$  value) for a nucleotide opposite its correct pairing partner is generally  $\sim 10 \mu\text{M}$ , while  $K_d$  values for incorrect nucleotides are usually 10-fold higher. After dNTP binding, an enzymatic conformational change (step 3) is proposed to align the incoming dNTP into a precise geometrical orientation that is necessary for phosphoryl transfer (step 4). This conformational change step reflects an "induced-fit" mechanism that also imposes discrimination against nucleotide misinsertion as misaligned intermediates perturb the geometry of the polymerase's active site to prevent the chemical step (8). Another point for error discrimination can occur during the phosphoryl transfer step (step 4) which may be rate-limiting for nucleotide incorporation with certain polymerases (9). Regardless, the maximal rate constant for polymerization (denoted as  $k_{\text{pol}}$ ) for the incorporation of a correct nucleotide opposite its cognate partner generally ranges from 20 to 200  $\text{s}^{-1}$ . Thus, the relatively low binding affinity for a correct nucleotide can be offset by a fast  $k_{\text{pol}}$  value such that the overall catalytic efficiency ( $k_{\text{pol}}/K_d$ ) for correct polymerization is very high. Indeed,  $k_{\text{pol}}/K_d$  values of  $\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$  are at the limits of diffusion control and place polymerases within the top tier of the most proficient enzymes (10). It is also important to emphasize that  $k_{\text{pol}}/K_d$  values for incorrect DNA synthesis, i.e., the misincorporation of dATP opposite 2-deoxycytidine, are typically several orders of magnitude lower due to decreases in  $k_{\text{pol}}$  coupled with increases in  $K_d$ . This reduction plays a large role in the maintenance of genomic fidelity.

Following phosphoryl transfer, there is a second conformational change (step 5) that is required for pyrophosphate release (step 6). After pyrophosphate (PP<sub>i</sub>) is released, the polymerase can either remain bound to the nucleic acid substrate to continue primer elongation (step 8) or dissociate from the elongated primer (step 7) to initiate DNA synthesis on another usable primer template. The ability to incorporate multiple nucleotides without dissociating from DNA defines the processivity of the polymerase.

Alterations in various kinetic steps can occur to influence the behavior of the polymerase and alter the effectiveness of certain chemotherapeutic agents. One example is the ability of a polymerase to reverse the polymerization step (Figure 2, step 4) via a process known as pyrophosphorolysis to remove inappropriately incorporated nucleotides (11). Other kinetic steps include the dNTP binding step (Figure 2, step 2) and the conformational change preceding chemistry (Figure 2, step 3).

**Strategic Interventions.** This mechanistic framework highlights the importance of kinetic steps that can be targeted to inhibit replication and prevent proliferation. One potential stage for therapeutic intervention is the use of competitive inhibitors to block dNTP binding to the polymerase's active site (Figure 2, step 2). Unfortunately, developing a reversible inhibitor that binds tightly and selectively to a DNA polymerase is an extraordinarily difficult task. Much of this challenge arises from the promiscuous and relatively weak binding affinity ( $K_d \sim 10 \mu\text{M}$ ) of natural nucleotides for the polymerase. Furthermore, "correct" polymerization occurs with an incredibly high commitment to catalysis ( $K_{\text{eq}} \sim 10^6$ ) since forward kinetic steps such as phosphoryl transfer ( $k_{\text{pol}} \sim 100 \text{ s}^{-1}$ ) are generally much faster than the corresponding reverse step of pyrophosphorolysis ( $k_{\text{pyro}} \sim 0.0001 \text{ s}^{-1}$ ) (12).

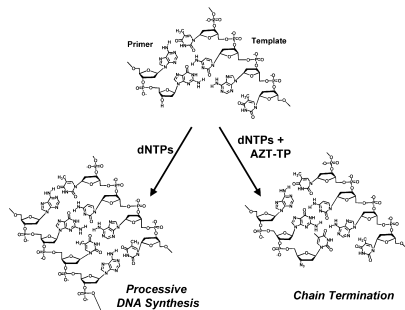


FIGURE 3: Mechanism by which chain-terminating nucleotides inhibit DNA synthesis. AZT-TP is an effective chain terminator due to the simple replacement of the hydroxyl group with an azide ( $N_3$ ) which prevents subsequent primer elongation.

These two features coupled with additional pharmacokinetic problems such as drug absorption (13) and metabolism (14) of nucleoside triphosphates have hindered this conventional approach.

An alternative strategy is to take advantage of the polymerase's high commitment to catalysis and trick the enzyme into using a potential suicide inhibitor. This Trojan Horse strategy outlined in Figure 3 represents the current paradigm in the design and implementation of many nucleoside analogues targeting prokaryotic and eukaryotic DNA polymerases. The polymerase is provided with a modified nucleotide such as AZT-TP that is incorporated into DNA as efficiently as its natural counterpart, dTTP. Once incorporated, however, the modified nucleotide creates a nucleic acid substrate that cannot be elongated and thus terminates DNA synthesis. In general, the development of an effective chain-terminating nucleotide depends upon several features, including (1) high catalytic efficiency for incorporation, (2) no potential for subsequent elongation, (3) poor excision after incorporation, (4) selective utilization by polymerases responsible for the pathogenic state, and (5) effective metabolism of the parental nucleoside to form the corresponding nucleoside triphosphate. The following sections describe successes and pitfalls associated with several therapeutically relevant nucleoside analogues. My discussion begins with an analysis of the development and application of several viral DNA polymerase inhibitors as this represents a major effort in modern pharmaceutical science.

**Agents That Target Retroviral Polymerases.** Viruses are responsible for a wide variety of human diseases ranging from simple ailments, including the common cold, chickenpox, and cold sores, to more serious health threats such as acquired immune deficiency syndrome (AIDS), severe acute respiratory syndrome (SARS), and cervical cancer (15). In addition, an alarming number of other diseases such as progressive multifocal leukoencephalopathy (PML) and multiple sclerosis may also be linked with viral infections (16). A universal feature for the virulence and propagation of any virus is the requirement of high levels of DNA synthesis. For example, reverse transcription of the viral single-stranded (+) RNA genome of the human immunodeficiency virus (HIV) into double-stranded DNA is catalyzed by the retroviral polymerase, reverse transcriptase (RT). This RNA- and DNA-dependent polymerase is a prime target for antiviral drug development since it plays an essential role in the HIV life cycle. To date, two distinct types of RT inhibitors with unique pharmacodynamic behavior have been

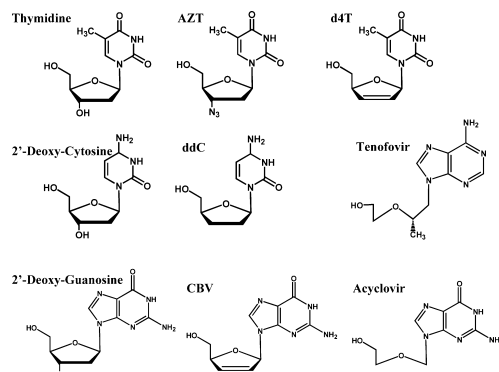


FIGURE 4: Structures of several nucleoside analogues that are commonly used as antiviral agents. See the text for details.

developed for clinical use. These include nucleoside RT inhibitors and non-nucleoside RT inhibitors (NNRTIs).

(i) **Nucleoside Reverse Transcriptase Inhibitors.** Figure 4 compares the structures of natural nucleosides with several nucleoside analogues approved as antiviral agents against HIV or herpes simplex virus (HSV), another important viral target. All of these nucleoside analogues share a common feature that distinguishes them from their natural counterparts, the absence of the 3'-OH group that is required for subsequent primer elongation. As previously illustrated in Figure 3, AZT-TP is an effective chain-terminator due to the simple replacement of the hydroxyl group with an azide ( $N_3$ ). Since the coding potential of the nucleobase remains identical to that of the natural nucleoside, the binding affinities for chain terminators such as AZT as well as d4T (Stavudine) and ddC (Zalcitabine) are nearly identical to those of their natural counterparts (17–19). However, the maximal rate for the incorporation of AZT-TP is significantly slower than the rate of dTTP incorporation (compare  $k_{pol}$  values of 0.7 and  $\sim 20 \text{ s}^{-1}$  for AZT-TP and dTTP, respectively) (17). Thus, alterations to the ribose moiety appear to influence the phosphoryl transfer step and/or the ability of the polymerase to mediate the conformational change preceding this kinetic step. Regardless, the  $k_{pol}/K_d$  values for these modified nucleotides are comparable to those of their natural counterparts, and the inhibition of viral replication via chain termination corroborates clinical data showing viral load reductions upon treatment with these analogues.

Acyclovir (Figure 4) is a unique guanine analogue that is widely used in the treatment of herpes simplex and herpes zoster (shingles) infections (reviewed in ref 20). Acyclovir differs significantly from the other aforementioned antiviral agents as the 2'-deoxyribose sugar is replaced with an open chain structure which accounts for its chain termination capabilities. However, this modification confers two additional pharmacodynamic properties that make it a highly effective antiviral agent. The first is an unusually high selectivity for conversion into acycloguanosine monophosphate (acylo-GMP) by the virally encoded thymidine kinase as opposed to the host kinase. The 3000-fold higher activity of the viral enzyme leads to higher concentrations of acylo-GMP in infected cells as opposed to healthy, uninfected cells. Second, the triphosphate form of acyclovir (acyclo-GTP) is incorporated 100-fold more efficiently by the viral polymerase compared to the host enzyme. These two features make acyclovir one of the safest antiviral agents currently on the market. The success of acyclovir has spawned the



development of a number of acyclonucleosides as antiviral agents. Page limitations prohibit a thorough discussion of these analogues. However, an excellent overview of these unique antiviral agents is provided by Hewlett et al. (21).

(ii) *Pitfalls Associated with Chain-Terminating Nucleoside Analogues.* Despite their widespread use, the therapeutic utility of most nucleoside analogues is often restricted by major complications, including the development of resistance and the induction of adverse side effects. Resistance to all HIV-related nucleoside analogues invariably develops in clinical settings through one of several mechanisms. First, conversion of the nucleoside into the required triphosphate form is limited by the action of the host thymidine kinase and results in lower plasma concentrations that may limit their effectiveness (22). A second mechanism occurs via the generation of selective point mutations in RT that hinder incorporation of the chain terminator but have no effect on the utilization of natural nucleotides. One example is the M184V mutant that causes resistance to 3TC by simple steric hindrance between the  $\beta$ -branched amino acid side chain (valine) with the  $\beta$ -L-oxathialone ring of the nucleoside triphosphate (23). This simple mutation decreases the binding affinity of the chain terminator but has no effect on the binding of the natural substrate, dTTP. The generation of point mutations also accounts for the third most common mechanism of resistance, enhanced enzymatic removal of the chain terminator from the viral genome via pyrophosphorolysis, the reversal of polymerization (11).

Adverse side effects arise from the inherently low selectivity of these nucleoside analogues as they can be effectively utilized by either viral or host DNA polymerases to inhibit DNA synthesis. In the case of HIV, however, it was hypothesized that viral replication would be selectively inhibited since HIV RT does not possess exonuclease activity to remove the chain terminators from the viral genome. In contrast, most eukaryotic polymerases have robust exonuclease activities that can rapidly excise the analogue and allow for replication of the host's genome. In hindsight, these assumptions proved inaccurate as many antiviral agents are slowly excised by host enzymes and/or are rapidly removed from the viral genome. In the former case, chronic administration of certain nucleoside analogues can cause side effects resembling heritable mitochondrial diseases (24). Patients treated with one or more nucleoside analogues generally display phenotypes such as peripheral neuropathy, cardiac and skeletal muscle myopathy, pancreatitis, and bone marrow suppression (24). The molecular basis for most of these side effects appears to be linked with the inhibition of mitochondrial DNA replication catalyzed by pol  $\gamma$  (25). In fact, a recent review by Lee et al. (26) provides an excellent description of a predictive index correlating the clinical toxicity of a nucleoside analogue with the probability of the chain-terminating nucleotide being incorporated and subsequently excised from DNA by pol  $\gamma$ .

It is clear that a diminution in the level of exonuclease proofreading by human polymerases can cause an increased risk in toxicity. However, a different complication arises if the viral polymerase efficiently excises the chain terminator from the viral genome via pyrophosphorolysis (11). As described previously, pyrophosphorolysis plays an important role in drug resistance as the efficient removal of these chain terminators allows RT to reinitiate viral DNA synthesis.

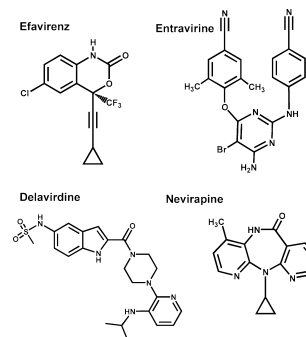


FIGURE 5: Structures of various non-nucleoside reverse transcriptase inhibitors (NNRTIs) that target HIV reverse transcriptase. See the text for details.

During long-term treatment with AZT, a set of mutations, including M41L, D67N, K70R, T215F/Y, and K219Q, develop within the active site of HIV RT that confer resistance (27). The structures of these mutants complexed with chain-terminated DNA (reviewed in ref 28) have been instrumental in understanding the mechanism of this resistance. In this model, RT can position the 3'-OH group of the primer into two distinct locations denoted as the priming site (P-site) or the nucleotide-binding site (N-site). During normal replication, RT binds the 3'-OH group in the P-site while dNTP binding occurs in the N-site. After phosphoryl transfer, the newly extended primer is transferred from the N-site to the P-site and the catalytic cycle continues. However, the dynamics of this cycle are altered if a chain terminator is incorporated as the absence of the 3'-OH group on a chain-terminated primer prevents elongation. Thus, the binding of the next correct dNTP in the N-site typically causes the formation of a dead-end ternary complex to prevent DNA synthesis. However, the aforementioned mutations allow RT to partition the chain-terminated primer from the P-site back into the N-site. This movement allows for pyrophosphate ( $PP_i$ ) binding and subsequent pyrophosphorolysis to excise the chain terminator so that DNA synthesis can resume. Although  $PP_i$  is the natural substrate for this excision reaction, ATP can also act as the acceptor substrate (29). ATP appears to interact directly within the active site through stacking interactions between the purine moiety and the aromatic ring of Y215 (30).

Since pyrophosphorolysis plays an important role in drug resistance, significant effort has been spent in developing molecules to inhibit this process. One example is foscarnet, a structural mimic of  $PP_i$ , that selectively inhibits the binding of pyrophosphate to various viral DNA polymerases (31). Foscarnet is widely used as an antiviral agent against cytomegalovirus retinitis and HSV type 1 and 2 infections. However, its use in HIV therapy is limited as chronic administration causes electrolyte imbalance and nephrotoxicity due to alterations in calcium, magnesium, potassium, and phosphate levels.

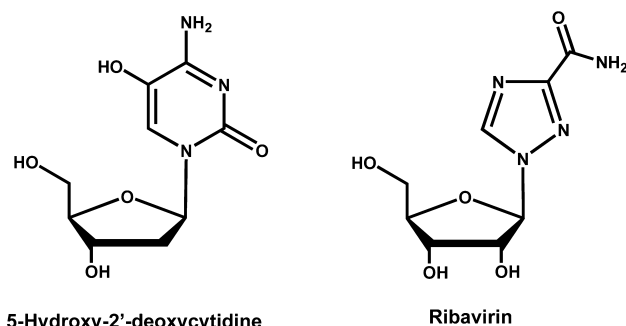
(iii) *Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs).* In addition to nucleoside analogues, another important class of RT inhibitors widely used in the treatment of HIV infection consists of non-nucleoside reverse transcriptase inhibitors (NNRTIs). It is clear from the structures provided in Figure 5 that these analogues do not bear any resemblance to nucleoside analogues such as AZT and ddC. In fact, these agents were initially identified as potent

inhibitors of HIV RT through high-throughput screening efforts rather than through rational drug design targeting the active site of the polymerase (32). Steady-state inhibition studies determined that NNRTIs such as efavirenz act as uncompetitive inhibitors at low drug concentrations ( $<50$  nM) (33). This result is consistent with a mechanism in which efavirenz binds to the ternary RT–DNA–dNTP complex with a higher affinity than to either free RT or the RT–DNA complex (33). Transient kinetic studies monitoring polymerization revealed that NNRTIs block the phosphoryl transfer reaction without interfering with nucleotide binding (Figure 2, step 2) or the nucleotide-induced conformational change (Figure 2, step 3) (33). Instead, NNRTI binding allows the incoming nucleoside triphosphate to bind tightly ( $K_d \sim 100$  nM) but in a nonproductive manner (34). This represents one of the first examples of a small molecule that binds to an allosteric site to cause potent inhibition. Indeed, structural analysis reveals that the NNRTI binds to a hydrophobic pocket on the polymerase lined with large and hydrophobic amino acids, including Y181, Y188, F227, and L234, which is located  $\sim 10$  Å from the enzyme's polymerase active site (35).

Not surprisingly, resistance to NNRTIs develops rapidly through the generation of point mutations within this hydrophobic binding site. For example, a recent retrospective cohort study of HIV-infected patients treated with nevirapine revealed that resistant isolates developed in 92% of treated patients within 24 weeks (36). Furthermore, cross resistance to other NNRTIs such as efavirenz and delavirdine was detected in  $\sim 70\%$  of these patients, suggesting that a common set of mutations lead to resistance (36). In general, drug resistance arises due to the replacement of a large amino acid with a smaller one that weakens the molecular interactions of the inhibitor with RT. For example, nevirapine binds 500-fold more weakly to the Y181C mutant than to wild-type RT, and the reduced affinity reflects an increase in the rate of dissociation of the inhibitor from the RT–DNA complex rather than simple steric hindrance (37). Surprisingly, etravirine is a new NNRTI that remains active as an allosteric inhibitor against mutant RTs that are resistant to other currently used NNRTIs such as nevirapine (38).

Combination therapy, the co-administration of multiple drugs targeting various pathological pathways, is a strategy that is widely used in the treatment of hyperproliferative diseases such as HIV. In fact, nucleoside inhibitors are often combined with NNRTIs such as nevirapine to synergistically reduce viral loads in vivo. This particular drug combination is beneficial as it delays the onset of resistance to nucleoside analogues that occurs through pyrophosphorolysis (39). This occurs since the binding of an NNRTI to the allosteric site inhibits the pyrophosphorylytic activity of RT by reducing the binding affinities for the phosphoryl acceptor ( $PP_i$  or ATP) as well as reducing the rate of the chemical step. While this combination of drugs reduces the rate of drug resistance, it does not completely eliminate it as the intrinsically low fidelity of RT eventually generates drug-resistant mutants.

(iv) *Nucleotide Analogues That Induce Lethal Mutagenesis.* Viral DNA synthesis is viewed as an error-prone process in which at least one mutation is introduced per round of replication. The lack of fidelity causes low mutation rates that are considered beneficial for certain viruses as it generates selective point mutations in critical proteins that



**5-Hydroxy-2'-deoxycytidine**

**Ribavirin**

FIGURE 6: Structure of promutagenic nucleoside analogues that are proposed to induce lethal mutagenesis in various viral systems. See the text for details.

confer resistance. However, higher rates of mutagenesis should reduce viral fitness and viability. Thus, a provocative therapeutic strategy is to use promutagenic nucleoside analogues to increase the mutation frequency of a virus beyond a critical threshold capable of supporting its viability (40). These nucleoside analogues would have little effect on the integrity of the host's genome since eukaryotic polymerases exhibit higher intrinsic fidelity and would prevent these analogues from being stably incorporated into DNA. The proof of concept for this strategy was first demonstrated by the Loeb laboratory using 5-hydroxy-2'-deoxycytidine (Figure 6) as a promutagenic nucleoside that pairs with either guanine or adenine (41). Treatment of HIV-infected cells with this promutagenic nucleoside showed an 80% reduction in the level of viral replication that correlated with a disproportionate increase in the number of G to A substitutions in isolated viral DNA. Another example is ribavirin, the ribose form of 1,2,4-triazole-3-carboxamide (Figure 6), that is used to treat viral infections such as SARS and hepatitis C. This promutagenic nucleoside reduced infective poliovirus production to as little as 0.00001% that coincides with the induction of mutations in the viral genome caused by the misincorporation of ribavirin opposite C or U (42). These studies provide an exciting and novel therapeutic strategy for converting a perceived strength of a virus (low polymerization fidelity) into a target for therapeutic intervention.

*Nucleoside Analogues as Anticancer Agents.* Despite advances in the detection and prevention of cancer, this disease still remains the second-leading cause of death in industrialized nations. Cancer is best defined as a hyperproliferative disease since the hallmark of this pathological malignancy is uncontrolled DNA synthesis. Treatment modalities vary depending upon whether the cancer is a solid tumor (brain, lung, pancreatic, colon, etc.) or a hematological malignancy (leukemia and lymphomas). However, combination therapy is typically employed in any case in which various chemotherapeutic agents are used to target the different biological pathways that are responsible for sustaining the uncontrollable growth of the cancer. One important class of chemotherapeutic agents consists of nucleoside analogues such as fludarabine,<sup>2</sup> cladribine, gemcitabine, and cytarabine (Figure 7). Their mechanism of action is similar to those of previously discussed antiviral agents such as AZT

<sup>2</sup> The monophosphate form of this analogue is used clinically as the addition of the monophosphate increases its solubility. The monophosphate is rapidly hydrolyzed in plasma so that the free nucleoside enters cells via passive diffusion.

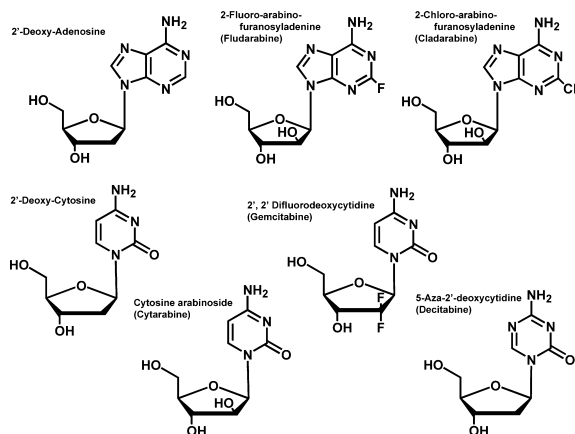


FIGURE 7: Structures of several nucleoside analogues that are widely used as anticancer agents. See the text for details.

as the ribose moiety that is essential for primer elongation is modified compared to the natural nucleoside. However, a major difference is that the deoxyribose sugar is replaced with arabinose or another modified sugar moiety. This simple replacement causes a number of pharmacodynamic effects that make these analogues effective anticancer agents by inhibiting DNA synthesis via multiple mechanisms (see Figure 1B). For example, fludarabine behaves as a classic chain terminator of DNA synthesis (43) since the alteration in sugar composition makes it refractory to elongation after incorporation into DNA. However, fludarabine exhibits additional chemotherapeutic properties by acting as an antimetabolite by inhibiting the activity of ribonucleotide reductase and adenosine deaminase (44), two key enzymes involved in purine metabolism. Since this agent hits multiple targets, it is highly effective as a single agent in the treatment of chronic lymphocytic leukemia. However, it is also used in combinations with other chemotherapeutic agents such as DNA-damaging agents (cyclophosphamide and mitoxantrone) and glucocorticoid steroids (dexamethasone).

Cytarabine (Figure 7) is a pyrimidine analogue that acts as a substrate for both eukaryotic DNA and RNA polymerases and exerts its cytotoxic effects by terminating replication and transcription (45). This pyrimidine analogue is similar to fludarabine as it also indirectly inhibits DNA synthesis by disrupting the proper balance of nucleotide pools by inhibiting ribonucleotide reductase activity. 5-Aza-2'-deoxycytidine (decitabine) is another pyrimidine analogue that is a near-perfect mimetic of 2-deoxycytidine as only C5 is replaced with nitrogen (Figure 7). This simple substitution does not influence the ability of 5-aza-2'-deoxycytidine to properly base pair with a guanine so there is no overt inhibitory effect on DNA synthesis. Instead, this subtle modification prevents the nucleobase from being modified by a class of DNA-modifying enzymes known as DNA methyltransferases which influence transcription rather than replication (46). DNA methylation is an epigenetic modification that reversibly regulates gene expression without changing the original DNA sequence of the gene promoter. In eukaryotes, the expression of many genes is inversely correlated with the methylation status of its promoter region. In certain forms of cancer, the promoter region of critical genes that regulate important cellular functions can become hypermethylated (47). These inappropriate epigenetic modifications lower the level of gene expression to reduce the

intracellular level of critical proteins. For example, many sporadic colon cancers show hypermethylation of the hMLH1 gene promoter which correlates with a lower level of expression of the gene product, hMLH1, an essential DNA repair protein (48). Reduced levels of this repair enzyme play important roles in the development of carcinogenesis in addition to drug resistance to various chemotherapeutic agents such as Temozolomide that damage DNA. However, cells treated with 5-aza-2'-deoxycytidine as a "demethylating agent" regain expression of the hMLH1 protein which then sensitizes cells to the cytotoxic effects of certain chemotherapeutic agents. Although 5-aza-2'-deoxycytidine is used as a chemotherapeutic agent, it possesses a relatively narrow therapeutic window as it affects the methylation patterns of normal and cancer cells (49).

(i) *Pitfalls Associated with Nucleoside Analogues in Cancer Chemotherapy.* While nucleoside analogues are important in treating cancer, their utility is often limited by dose-limiting toxicity that arises due to their nonselective nature. As expected, a number of the body's normal cells also divide rapidly and are therefore killed by this class of chemotherapeutic agent. This nonselective killing contributes to the development of debilitating side effects that include alopecia (temporary hair loss), mucositis (mouth sores), anemia (reduced red blood cell number), leukopenia (reduced white blood cell number), thrombocytopenia (decreased numbers of platelets), and gastrointestinal symptoms such as nausea, vomiting, and diarrhea. The intensity of these side effects depends heavily upon the dose and duration of exposure to the chemotherapeutic agent. For example, patients treated with large doses of fludarabine often develop leukopenia and thrombocytopenia which leads to opportunistic infections and bleeding disorders. As a result, patients receiving fludarabine are sometimes also treated with antibiotics as prophylactic measures against opportunistic infections.

*New Frontiers in the Development of Nucleoside Analogues.* A major disadvantage with the current arsenal of nucleoside analogues is the dose-limiting toxicity that arises from the lack of selectivity to inhibit one DNA polymerase versus another. This is not unexpected since all current analogues bear structural identity to natural nucleosides and are thus incorporated opposite templating nucleobases with high catalytic efficiency. An additional complication is that all DNA polymerases characterized to date share common structural features that prohibit the use of rational drug design in selectively targeting the active site. The overall structure of DNA polymerases resembles a "right hand" containing fingers, palm, and thumb subdomains (50). The palm domain is the most closely conserved structural feature as it contains the amino acids required for phosphoryl transfer. The fingers domain interacts with the incoming dNTP as well as the templating base and thus plays an important role in nucleotide selection. The thumb domain plays dual roles by positioning duplex DNA for the incoming dNTP as well as in the processivity and translocation of the polymerase.

Rather than directly target the active site of the polymerase, an alternative approach is to develop nucleoside analogues that can be selectively incorporated opposite modified or damaged DNA. This approach would inhibit promutagenic DNA synthesis and could be advantageous for several reasons. First, this approach represents a chemopreventive



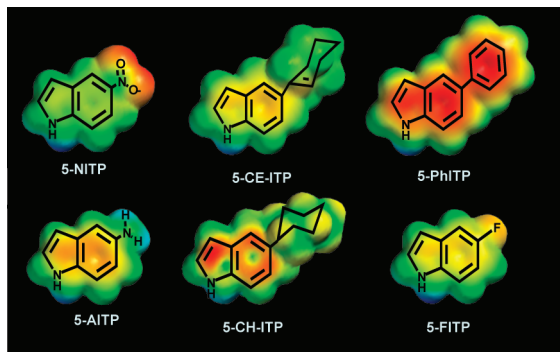


FIGURE 8: Structures of a new series of 5-substituted indolyl-2'-deoxyribosides that exhibit selectivity for incorporation opposite damaged vs undamaged DNA.

strategy as the generation of disease-causing mutations caused by the misreplication of damaged DNA would be inhibited. Second, this approach could be used as an adjunctive therapy to sensitize cancer cells to the effects of other chemotherapeutic agents that damage DNA (refer to Figure 1B, step 1). The *in vitro* applications of this strategy have been demonstrated in our laboratory using the abasic site as a model form of DNA damage. Abasic sites are commonly formed DNA lesions that result from the hydrolysis of the C–N bond between the 2'-deoxyribose and a purine/pyrimidine. Hydrolysis occurs spontaneously but is significantly enhanced by DNA-damaging agents such as Temozolomide (51). The net effect of this hydrolysis reaction is that templating information originally present in the DNA is lost which causes the abasic site to be highly promutagenic.

Despite the nontemplating nature of an abasic site, most DNA polymerases preferentially incorporate dATP opposite the lesion (52). We applied this information in rationally designing a series of 5-substituted indolyl-2'-deoxyriboside triphosphates that mimic the core structure of dATP (Figure 8) (reviewed in refs 53 and 54). *In vitro* kinetic analyses reveal that analogues containing significant  $\pi$ -electron density such as 5-NITP, 5-Ph-ITP, and 5-CE-ITP are incorporated opposite the abasic site with high catalytic efficiencies of  $10^7 \text{ M}^{-1} \text{ s}^{-1}$ . These values are  $\sim 1000$ -fold higher than that for the preferred natural substrate, dATP. In contrast, non-natural nucleotides that lack extensive  $\pi$ -electron density such as 5-FITP, 5-AITP, and 5-CH-ITP are poorly inserted opposite the abasic site. These kinetic studies demonstrate that simple alterations to the  $\pi$ -electron surface area of a non-natural nucleotide can modulate its efficiency for incorporation opposite an abasic site. In addition, there is evidence that certain non-natural nucleotides are utilized by various DNA polymerases more effectively than other analogues in the same class (55). One possibility is that while the functions of the fingers and thumb subdomains are conserved among DNA polymerases, there are subtle differences with respect to sequence and structural characteristics such that selective inhibitors that exploit these nuances could be developed. The development and application of this class of non-natural nucleoside opens new avenues toward understanding the mechanism and biological consequences of translesion DNA synthesis which can then be exploited as adjunctive chemotherapeutic agents.

**Conclusions and Prospectus.** The inhibition of DNA replication remains an important therapeutic strategy against numerous diseases, including viral infections and cancer. The

current arsenal of agents targeting polymerase activity are generally based upon modifications to the 2'-deoxyribose moiety of a nucleotide which are designed to prevent elongation of nucleic acid. While effective, the vast majority of these nucleoside analogues exhibit pharmacodynamic complications that include an intrinsic lack of selectivity and the development of drug resistance. In addition, pharmacokinetic problems such as conversion to the triphosphate form and degradation through salvage pathways can limit their effectiveness. However, new therapeutic approaches are currently being developed to circumvent these complications that include developing selective inhibitors for polymerases involved in DNA repair and/or translesion DNA synthesis.

## REFERENCES

- Kulkarni, A., and Wilson, D. M., III (2008) The involvement of DNA-damage and -repair defects in neurological dysfunction. *Am. J. Hum. Genet.* 82, 539–566.
- Havelka, A. M., Berndtsson, M., Olofsson, M. H., Shoshan, M. C., and Linder, S. (2007) Mechanisms of action of DNA-damaging anticancer drugs in treatment of carcinomas: Is acute apoptosis an “off-target” effect? *Mini-Rev. Med. Chem.* 7, 1035–1039.
- Guillem, V., and Tormo, M. (2008) Influence of DNA damage and repair upon the risk of treatment related leukemia. *Leuk. Lymphoma* 49, 204–217.
- Baldwin, E. L., and Osheroff, N. (2005) Etoposide, topoisomerase II and cancer. *Curr. Med. Chem. Anticancer Agents* 5, 363–372.
- McGuire, J. J. (2003) Anticancer antifolates: Current status and future directions. *Curr. Pharm. Des.* 9, 2593–2613.
- Yarchoan, R., Pluda, J. M., Perno, C. F., Mitsuya, H., Thomas, R. V., Wyvill, K. M., and Broder, S. (1990) Initial clinical experience with dideoxynucleosides as single agents and in combination therapy. *Ann. N.Y. Acad. Sci.* 616, 328–343.
- Anderson, V. R., and Perry, C. M. (2007) Fludarabine: A review of its use in non-Hodgkin's lymphoma. *Drugs* 67, 1633–1655.
- Johnson, K. A. (1993) Conformational coupling in DNA polymerase fidelity. *Annu. Rev. Biochem.* 62, 685–713.
- Showalter, A. K., and Tsai, M. D. (2002) A reexamination of the nucleotide incorporation fidelity of DNA polymerases. *Biochemistry* 41, 10571–10576.
- Wolfenden, R., and Snider, M. J. (2001) The depth of chemical time and the power of enzymes as catalysts. *Acc. Chem. Res.* 34, 938–945.
- Meyer, P. R., Matsuura, S. E., Zonarich, D., Chopra, R. R., Pendarvis, E., Bazmi, H. Z., Mellors, J. W., and Scott, W. A. (2003) Relationship between 3'-azido-3'-deoxythymidine resistance and primer unblocking activity in foscarnet-resistant mutants of human immunodeficiency virus type 1 reverse transcriptase. *J. Virol.* 77, 6127–6137.
- Capson, T. L., Peliska, J. A., Kaborod, B. F., Frey, M. W., Lively, C., Dahlberg, M., and Benkovic, S. J. (1992) Kinetic characterization of the polymerase and exonuclease activities of the gene 43 protein of bacteriophage T4. *Biochemistry* 31, 10984–10994.
- Diab, R., Degobert, G., Hamoudeh, M., Dumontet, C., and Fessi, H. (2007) Nucleoside analogue delivery systems in cancer therapy. *Expert Opin. Drug Delivery* 4, 513–531.
- Piliero, P. J. (2004) Pharmacokinetic properties of nucleoside/nucleotide reverse transcriptase inhibitors. *JAIDS, J. Acquired Immune Defic. Syndr.* 37, S2–S12.
- Bermejo, J. F., and Muñoz-Fernandez, M. A. (2004) Severe acute respiratory syndrome, a pathological immune response to the new coronavirus: Implications for understanding of pathogenesis, therapy, design of vaccines, and epidemiology. *Viral Immunol.* 17, 535–544.
- Becker, L. E. (1977) Slow infections of the central nervous system. *Can. J. Neurol. Sci.* 4, 81–88.
- Kerr, S. G., and Anderson, K. S. (1997) Pre-steady-state kinetic characterization of wild type and 3'-azido-3'-deoxythymidine (AZT) resistant human immunodeficiency virus type 1 reverse transcriptase: Implication of RNA directed DNA polymerization in the mechanism of AZT resistance. *Biochemistry* 36, 14064–14070.
- Yang, G., Wang, J., Cheng, Y., Dutschman, G. E., Tanaka, H., Baba, M., and Cheng, Y. C. (2008) Mechanism of inhibition of

- HIV-1 reverse transcriptase by a stavudine analogue 4'-ethynyl-D4TTP. *Antimicrob. Agents Chemother.* 52, 2035–2042.
19. Gu, Z., Arts, E. J., Parniak, M. A., and Wainberg, M. A. (1995) Mutated K65R recombinant reverse transcriptase of human immunodeficiency virus type 1 shows diminished chain termination in the presence of 2',3'-dideoxycytidine 5'-triphosphate and other drugs. *Proc. Natl. Acad. Sci. U.S.A.* 92, 2760–2764.
  20. Reardon, J. E., and Spector, T. (1989) Herpes simplex virus type 1 DNA polymerase. Mechanism of inhibition by acyclovir triphosphate. *J. Biol. Chem.* 264, 7405–7411.
  21. Hewlett, G., Hallenberger, S., and Rubsamen-Waigmann, H. (2004) Antiviral against DNA viruses (hepatitis B and the herpes viruses). *Curr. Opin. Pharm.* 4, 453–464.
  22. Gao, W. Y., Shirasaka, T., Johns, D. G., Broder, S., and Mitsuya, H. (1993) Differential phosphorylation of azidothymidine, dideoxycytidine, and dideoxyinosine in resting and activated peripheral blood mononuclear cells. *J. Clin. Invest.* 91, 2326–2333.
  23. Gao, H. Q., Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2000) The role of steric hindrance in 3TC resistance of human immunodeficiency virus type-1 reverse transcriptase. *J. Mol. Biol.* 300, 403–418.
  24. Scatena, R., Bottoni, P., Botta, G., Martorana, G. E., and Giardina, B. (2007) The role of mitochondria in pharmacotoxicology: A reevaluation of an old, newly emerging topic. *Am. J. Physiol.* 293, C12–C21.
  25. Lim, S. E., and Copeland, W. C. (2001) Differential incorporation and removal of antiviral deoxynucleotides by human DNA polymerase  $\gamma$ . *J. Biol. Chem.* 276, 23616–23623.
  26. Lee, H., Hanes, J., and Johnson, K. A. (2003) Toxicity of nucleoside analogues used to treat AIDS and the selectivity of the mitochondrial DNA polymerase. *Biochemistry* 42, 14711–14719.
  27. Naeger, L. K., Margot, N. A., and Miller, M. D. (2001) Increased drug susceptibility of HIV-1 reverse transcriptase mutants containing M184V and zidovudine-associated mutations: Analysis of enzyme processivity, chain-terminator removal and viral replication. *Antiviral Ther.* 6, 115–126.
  28. Tuske, S., Sarafianos, S. G., Clark, A. D., Jr., Ding, J., Naeger, L. K., White, K. L., Miller, M. D., Gibbs, C. S., Boyer, P. L., Clark, P., Wang, G., Gaffney, B. L., Jones, R. A., Jerina, D. M., Hughes, S. H., and Arnold, E. (2004) Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. *Nat. Struct. Mol. Biol.* 11, 469–474.
  29. Meyer, P. R., Matsuura, S. E., So, A. G., and Scott, W. A. (1998) Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 95, 13471–13476.
  30. Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2001) Selective excision of AZTMP by drug-resistant human immunodeficiency virus type-1 reverse transcriptase. *J. Virol.* 75, 4832–4842.
  31. Crumpacker, C. S. (1992) Mechanism of action of foscarnet against viral polymerases. *Am. J. Med.* 92, 3S–7S.
  32. Yang, S. S., Fliakas-Boltz, V., Bader, J. P., and Buckheit, R. W., Jr. (1995) Characteristics of a group of nonnucleoside reverse transcriptase inhibitors with structural diversity and potent anti-human immunodeficiency virus activity. *Leukemia* 9, S75–S85.
  33. Maga, G., Ubiali, D., Salvetti, R., Pregnotato, M., and Spadari, S. (2000) Selective interaction of the human immunodeficiency virus type 1 reverse transcriptase nonnucleoside inhibitor efavirenz and its thio-substituted analog with different enzyme-substrate complexes. *Antimicrob. Agents Chemother.* 44, 1186–1194.
  34. Spence, R. A., Kati, W. M., Anderson, K. S., and Johnson, K. A. (1995) Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Science* 267, 988–993.
  35. Ren, J., Bird, L. E., Chamberlain, P. P., Stewart-Jones, G. B., Stuart, D. I., and Stammers, D. K. (2002) Structure of HIV-2 reverse transcriptase at 2.35-Å resolution and the mechanism of resistance to non-nucleoside inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14410–14415.
  36. Casado, J. L., Hertogs, K., Ruiz, L., Dronda, F., Van Cauwenberge, A., Arnó, A., Garcia-Arata, I., Bloor, S., Bonjoch, A., Blazquez, J., Clotet, B., and Larder, B. (2000) Non-nucleoside reverse transcriptase inhibitor resistance among patients failing a nevirapine plus protease inhibitor-containing regimen. *AIDS* 14, F1–F7.
  37. Basavapathruni, A., Vingerhoets, J., de Bethune, M. P., Chung, R., Bailey, C. M., Kim, J., and Anderson, K. S. (2006) Modulation of human immunodeficiency virus type 1 synergistic inhibition by reverse transcriptase mutations. *Biochemistry* 45, 7334–7340.
  38. Udier-Blagović, M., Tirado-Rives, J., and Jorgensen, W. L. (2003) Validation of a model for the complex of HIV-1 reverse transcriptase with nonnucleoside inhibitor TMC125. *J. Am. Chem. Soc.* 125, 6016–6017.
  39. Basavapathruni, A., Bailey, C. M., and Anderson, K. S. (2004) Defining a molecular mechanism of synergy between nucleoside and nonnucleoside AIDS drugs. *J. Biol. Chem.* 279, 6221–6224.
  40. Clementi, M. (2004) Can modulation of viral fitness represent a target for anti-HIV-1 strategies? *New Microbiol.* 27, 207–214.
  41. Loeb, L. A., Essigmann, J. M., Kazazi, F., Zhang, J., Rose, K. D., and Mullins, J. I. (1999) Lethal mutagenesis of HIV with mutagenic nucleoside analogs. *Proc. Natl. Acad. Sci. U.S.A.* 96, 1492–1497.
  42. Crotty, S., Maag, D., Arnold, J. J., Zhong, W., Lau, J. Y., Hong, Z., Andino, R., and Cameron, C. E. (2000) The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. *Nat. Med.* 6, 1375–1379.
  43. Huang, P., Chubb, S., and Plunkett, W. (1990) Termination of DNA synthesis by 9- $\beta$ -D-arabinofuranosyl-2-fluoroadenine. A mechanism for cytotoxicity. *J. Biol. Chem.* 265, 16617–16625.
  44. Plunkett, W., Huang, P., and Gandhi, V. (1990) Metabolism and action of fludarabine phosphate. *Semin. Oncol.* 17, 3–17.
  45. Datta, R., Kharbanda, S., and Kufe, D. W. (1992) Transcriptional and posttranscriptional regulation of H1 histone gene expression by 1- $\beta$ -D-arabinofuranosylcytosine. *Mol. Pharmacol.* 41, 64–68.
  46. Brueckner, B., Kuck, D., and Lyko, F. (2007) DNA methyltransferase inhibitors for cancer therapy. *Cancer J.* 13, 17–22.
  47. Issa, J. P. (2004) CpG island methylator phenotypes in cancer. *Nat. Rev. Drug Discovery* 4, 988–993.
  48. Veigl, M. L., Kasturi, L., Olechnowicz, J., Ma, A. H., Lutterbaugh, J. D., Periyasamy, S., Li, G. M., Drummond, J., Modrich, P. L., Sedwick, W. D., and Markowitz, S. D. (1998) Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. *Proc. Natl. Acad. Sci. U.S.A.* 95, 8698–8702.
  49. Oki, Y., Aoki, E., and Issa, J. P. (2007) Decitabine: Bedside to bench. *Crit. Rev. Oncol. Hematol.* 61, 140–152.
  50. Steitz, T. A. (1999) DNA polymerases: Structural diversity and common mechanisms. *J. Biol. Chem.* 274, 17395–17398.
  51. Gentil, A., Cabral-Neto, J. B., Mariage-Samson, R., Margot, A., Imbach, J. L., Rayner, B., and Sarasin, A. (1992) Mutagenicity of a unique apurinic/apyrimidinic site in mammalian cells. *J. Mol. Biol.* 227, 981–984.
  52. Strauss, B. S. (2002) The “A” rule revisited: Polymerases as determinants of mutational specificity. *DNA Repair* 1, 125–135.
  53. Zhang, X., Lee, I., and Berdis, A. J. (2005) A potential chemotherapeutic strategy for the selective inhibition of promutagenic DNA synthesis by nonnatural nucleotides. *Biochemistry* 44, 13111–13121.
  54. Devadoss, B., and Berdis, A. J. (2007) Non-natural nucleotide analogs as probes of DNA polymerase activity. *Curr. Chem. Biol.* 1, 241–264.
  55. Sheriff, A., Motea, E., Lee, I., and Berdis, A. J. (2008) The mechanism and dynamics of translesion DNA synthesis catalyzed by the *Escherichia coli* Klenow fragment. *Biochemistry*. (in press).

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