Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases

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Abstract | Nucleoside analogues have been in clinical use for almost 50 years and have become cornerstones of treatment for patients with cancer or viral infections. The approval of several additional drugs over the past decade demonstrates that this family still possesses strong potential. Here, we review new nucleoside analogues and associated compounds that are currently in preclinical or clinical development for the treatment of cancer and viral infections, and that aim to provide increased response rates and reduced side effects. We also highlight the different approaches used in the development of these drugs and the potential of personalized therapy.

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Nucleosides and nucleotides are endogenous compounds that are involved in several cellular processes such as DNA and RNA synthesis, cell signalling, enzyme regulation and metabolism. Nucleoside and nucleotide analogues are synthetic, chemically modified compounds that have been developed to mimic their physiological counterparts (FIG. 1) in order to exploit cellular metabolism and subsequently be incorporated into DNA and RNA to inhibit cellular division and viral replication. This action has potential therapeutic benefits — for example, in the inhibition of cancer cell growth, the inhibition of viral replication as well as other indications (BOX 1). In addition to their incorporation into nucleic acids, nucleoside and nucleotide analogues can interact with and inhibit essential enzymes such as human and viral polymerases (that is, DNA-dependent DNA polymerases, RNA-dependent DNA polymerases or RNA-dependent RNA polymerases), kinases, ribonucleotide reductase, DNA methyltransferases, purine and pyrimidine nucleoside phosphorylase and thymidylate synthase.

The seminal work of Gertrude B. Elion and George H. Hitchings led to the development of agents such as the nucleobase 6-mercaptopurine and the antiviral nucleoside analogue acyclovir[1,2]. Further pioneering work by Erik De Clercq and Antonín Holý allowed the development of several of the nucleoside and nucleotide analogues that are currently in clinical use[3,4]. However, despite the availability of several nucleoside and nucleotide analogues in the clinic, the development of newer agents with improved properties is needed to overcome issues of resistance, poor oral bioavailability, long-term toxicity and inter-individual variability requiring dose adaptation.

In this Review, we first highlight approved agents, their mechanisms of action and mechanisms of resistance, and then focus on recent progress in the development of new nucleoside and nucleotide analogues for the treatment of cancer and viral disease, which are two of the main indications for this drug class.

Approved agents

Since the initial approval of cytarabine in 1969 by the US Food and Drug Administration (FDA) for the treatment of acute myeloid leukaemia, numerous nucleoside analogues have been synthesized and evaluated in patients for the treatment of cancers. There are currently six FDA- and European Medicines Agency (EMA)-approved cytotoxic nucleoside analogues, all of which are nucleosides, that are derivatives of deoxycytidine, deoxyadenosine or deoxyguanosine (Supplementary information S1 (table)). Two compounds, azacitidine (approved in 2004) and decitabine (approved in 2006), are used as demethylating agents but have also shown antiproliferative activity against cancer cells.

The first antiviral nucleoside analogue, edoxudine, which is not used in the clinic anymore, was also approved by the FDA in 1969; there are currently over 25 approved nucleoside and nucleotide analogues that are used as antiviral agents for several indications such as hepatitis, HIV and herpesvirus infections (Supplementary information S1 (table)). In addition, antiviral nucleoside

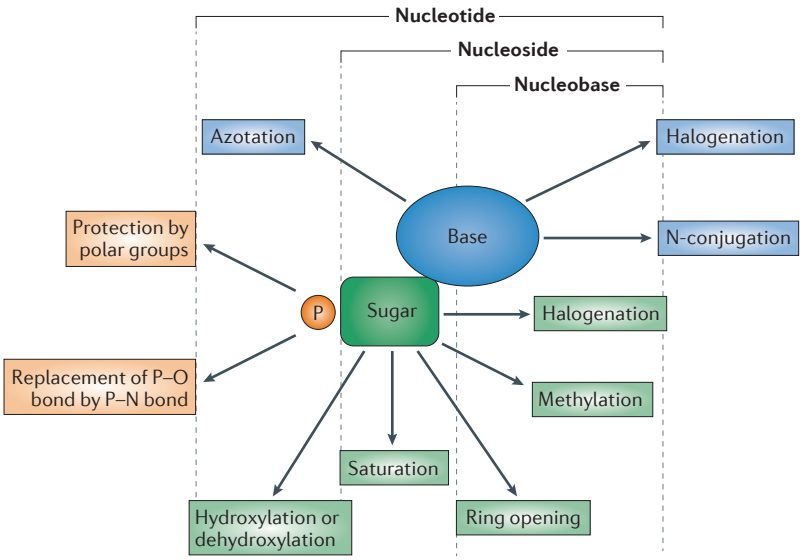

Figure 1 | **General structural and chemical modifications of nucleoside and nucleotide analogues.** Nucleoside and nucleotide analogues consist of a nucleobase (a purine or pyrimidine derivative) linked to a sugar moiety, and nucleotide analogues have a phosphate group (P) linked to the sugar moiety. The chemical diversity of these compounds is based on modifications such as halogenation, azotation, protection by polar groups and other modifications shown in this figure. N, nitrogen; O, oxygen.

and nucleotide analogues are structurally more diverse than anticancer nucleoside analogues, as they consist of nucleosides, nucleotides and acyclic nucleosides[5]. A major difference between anticancer nucleoside analogues and antiviral nucleoside and/or nucleotide analogues is that antiviral nucleoside and/or nucleotide analogues have low activity on mammalian enzymes, which results in a better tolerance profile than anticancer nucleoside analogues.

Mechanisms of action of nucleoside analogues

Currently used therapeutic nucleoside and nucleotide analogues exploit the same metabolic pathways as endogenous nucleosides or nucleotides, and they also act as antimetabolites (FIG. 2). Nucleoside and nucleotide analogues enter cells through specific nucleoside transporters[6,7] and there is growing evidence that organic anion or cation transporters as well as peptide transporters are involved in the cellular uptake of certain antiviral analogues. Inside the cells, the drugs are subsequently phosphorylated by a nucleoside kinase and a nucleoside monophosphate kinase, and then a nucleoside diphosphate kinase, creatine kinase or 3-phosphoglycerate kinase catalyses the last phosphorylation step. This leads to the accumulation of di- and triphosphorylated nucleoside analogues in cancer or virus-infected cells.

In cells infected by some DNA viruses (such as herpesvirus-infected cells) the first and second phosphorylation steps of thymidine are also performed by a virus-encoded thymidine kinase[8]. Herpesvirus thymidine kinases have broader substrate specificity than mammalian counterparts; this difference in substrate specificity forms the basis for the selectivity of nucleoside and nucleotide analogues as anti-herpes molecules[9,10]. Mono-, di- and triphosphorylated nucleosides are the active forms of these drugs and they act by inhibiting intracellular enzymes, such as viral or human polymerases or ribonucleotide reductase, as well as by being incorporated into newly synthesized DNA and RNA. The incorporation of nucleoside or nucleotide analogues into DNA may induce either the termination of chain elongation, the accumulation of mutations in viral progeny or the induction of apoptosis (BOX 2). Supplementary information S2 (table) details the consequences of inhibiting viral enzymes using nucleoside or nucleotide analogues of four different viruses that affect human health.

An in-depth knowledge of the mechanism of action of currently used compounds is of great value for the development of new compounds. These data have led to the development of compounds that act independently of membrane transporters or activating kinases and are less susceptible to degradation. A better understanding of the mechanism of action of these compounds will also contribute to the rational development of synergistic combinations of nucleoside or nucleotide analogues with drugs that have different and/or complementary mechanisms of action.

Mechanisms of resistance

Understanding the mechanisms that cause resistance to currently used nucleoside and nucleotide analogues is a prerequisite for the development of novel agents that could circumvent these mechanisms and therefore be prescribed to patients with relapsing or refractory disease. Resistance of cancer cells to the effects of nucleoside analogues is thought to be largely due to somatic changes in the tumour cells. Resistance to the inhibitory effects of nucleoside and nucleotide analogues on viral replication seems to be due to specific mutations in the viral genomes but may also be partly due to mutations and/or single nucleotide polymorphisms in the host genome; however, this needs to be further investigated. For example, the interferon λ3 (*IFNL3*; also known as *IL28B*) polymorphism strongly predicted the response to interferon and ribavirin in patients with hepatitis C virus (HCV) genotype 1 infection, via a mechanism that may involve the responsiveness of the infected host to interferon[11].

Metabolic resistance profile. Studying the pathways involved in the transport, activation or inactivation of nucleoside analogues has allowed the identification of mechanisms of resistance to these drugs, many of which have subsequently been clinically validated[12]. In cancer cells, a deficiency in nucleoside transporters such as equilibrative nucleoside transporter 1 (ENT1; also known as SLC29A1) or intracellular nucleoside kinases such as deoxycytidine kinase (DCK), as well as increased activity of ribonucleotide reductase and expression of 5′-nucleotidases, are all correlated with a reduced cytotoxicity of nucleoside analogues in cell models and in clinical samples[12–15].

Ribonucleotide reductase
A complex intracellular enzyme that converts ribonucleoside diphosphates into deoxyribonucleoside diphosphates, and is targeted by anticancer agents such as gemcitabine.

Nucleobase
A nitrogen-containing heterocyclic compound that can be grouped into purines (adenine and guanine) and pyrimidines (cytosine, thymine and uracil).

Dose adaptation
Determination of the dose that should be administered to a patient based on predicted or observed toxicity.

Demethylating agents
Compounds that modify the methylation status of regulatory sequences in DNA, thereby modifying the levels of expression of the corresponding gene.

Nucleoside transporters
Membrane pumps that allow the uptake and/or the efflux of nucleosides by cells.

Box 1 | **Other indications and actions of nucleoside and nucleotide analogues**

Besides their classical use in cancer and virology, some nucleoside and nucleotide analogues, and related compounds such as xanthine derivatives, have been used in various other indications.

Hyperuricaemia
Allopurinol, a structural isomer of hypoxanthine, is an inhibitor of xanthine oxidase and has been used for the treatment of chronic hyperuricaemia since 1966.

Immunosuppression
Azathioprine, a purine analogue that is a derivative of mercaptopurine, is used as an immunosuppressive drug in organ transplantation and autoimmune disease. Bone marrow suppression can be life-threatening in patients with low levels of thiopurine *S*-methyltransferase and so screening of this enzyme is recommended before azathioprine is prescribed. Cladribine also possesses specific activity on lymphocytes and has therefore been evaluated in patients with autoimmune diseases such as rheumatoid arthritis and multiple sclerosis.

Phosphodiesterase inhibitors
Theophylline, a methylxanthine analogue, acts as a nonselective inhibitor of phosphodiesterases, leads to an increase in intracellular cyclic AMP and is indicated in several situations including the treatment of chronic obstructive pulmonary disease and asthma.

Epigenetic modulators
Decitabine and azacitidine are DNA methyltransferase inhibitors and act as demethylating agents. Decitabine and azacitidine are currently approved for the treatment of myelodysplastic syndromes.

Neuroprotection and cardioprotection
The cellular uptake of adenosine as well as the activity of the intracellular enzyme adenosine kinase have been considered as potential targets for the protection of neurons and cardiac cells[159,160].

An increase in the activity of cytidine deaminase has also been correlated with decreased activity of cytidine derivatives (cytarabine and gemcitabine) *in vitro*[16]. Conversely, decreased cytidine deaminase activity in clinical blood samples was associated with increased exposure to the parental compound (gemcitabine), which subsequently resulted in the induction of toxicity[17]. Accordingly, an assessment of cytidine deaminase activity in the serum can be routinely performed before gemcitabine is administered to patients with cancer, and this could help clinicians to use a dose of gemcitabine that reduces the risk of severe toxicity[18].

Genomic resistance profile. The rate of spontaneous mutations within viral genomes is far higher than in mammalian genomes owing to absent or limited proofreading capabilities of viral polymerases. RNA viruses exist as complex quasi-species (that is, a wide population of related genomes that differ by less than 5%), which evolve over time depending on the selective pressure of the environment or exposure to therapy. This property is, at least in part, responsible for the high adaptability of viruses and represents a major hurdle to overcoming resistance because mutants that confer resistance to a given drug may appear or pre-exist in the population of genomes. Generally, resistance to a given nucleoside or nucleotide analogue is caused by a limited number of mutations (usually fewer than five) in a viral genome, which mainly affect the catalytic site of the polymerase to which the normal nucleotides or nucleotide analogues bind. Supplementary information S3 (table) lists the main mutations — within the genes encoding HCV, hepatitis B virus (HBV) and HIV polymerases — that confer resistance to the nucleoside or nucleotide analogues that are approved or in late-stage development.

We believe that the determination of resistance and toxicity factors before initiating treatment will become a major parameter that will, in the near future, enable the selection of the most appropriate nucleoside or nucleotide analogues for therapy. As selected mutations in viral genomes and clinically relevant polymorphisms in genes encoding proteins that are involved in nucleotide metabolism are increasingly being validated, these analyses — performed before initiating treatment — will contribute to better efficacy and tolerance.

Predicting response

The ability to choose a treatment that has the highest probability of invoking a positive response would be beneficial to patients, their environment (in the case of contagious diseases) and the health-care system overall as it would reduce costs. As illustrated below, our current ability to predict patient response is essentially based on our knowledge of drug metabolism and targeting as well as mechanisms of resistance.

Genetic polymorphisms and somatic phenotypes. The response to currently used regimens based on nucleoside or nucleotide analogues can be partially predicted by the genetic make-up of patients. Many studies that aimed to identify biological markers of treatment outcome have collectively highlighted the link between genetic polymorphisms and patient response to a nucleoside or nucleotide analogue; the main results of these studies are presented in Supplementary information S4 (table).

Target cell phenotype. Studies performed directly on tumour cells to identify markers that predict the activity of nucleoside and nucleotide analogue-based treatments have shown that clinically relevant markers include

Chain elongation
The increase in length of DNA or RNA strands during replication or transcription.

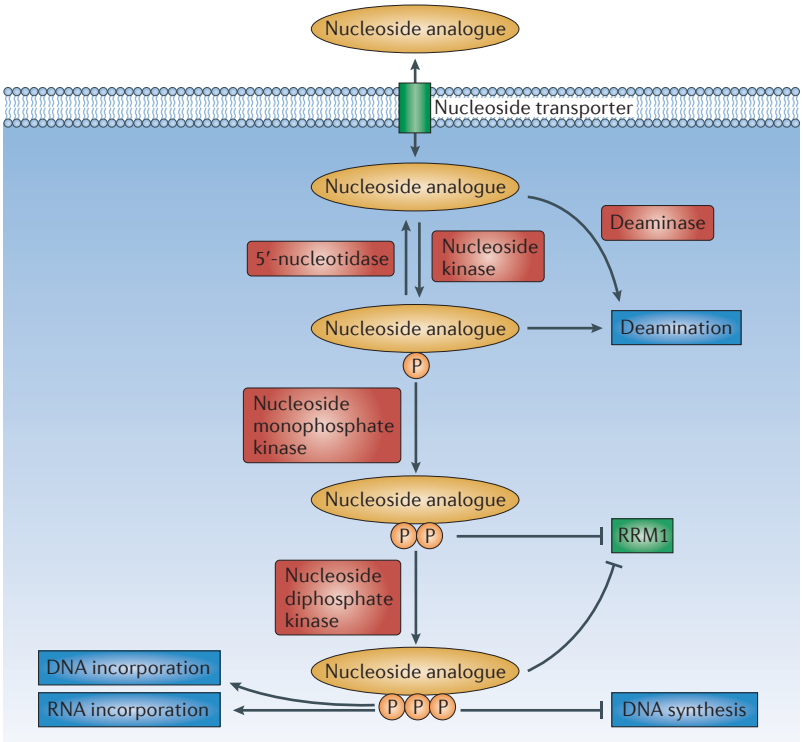

Figure 2 | **Mechanism of action of nucleoside analogues.** Cellular uptake of nucleoside analogues is an active process involving concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs). There are three CNTs and four ENTs described in humans. Once inside the cell, the nucleoside analogue undergoes an initial rate-limiting phosphorylation step by a nucleoside kinase, which leads to the production of a monophosphate metabolite. A second phosphorylation step is then performed by nucleoside monophosphate kinase, and the third phosphorylation step is performed by nucleoside diphosphate kinase. Triphosphates can be incorporated in nucleic acids, in competition with their normal counterparts, or they can inhibit nucleic acid synthesis by inhibiting essential enzymes such as polymerases. Ribonucleotide reductase M1 (RRM1), a key enzyme involved in nucleotide metabolism, can be inhibited both by diphosphorylated and triphosphorylated analogues. Catabolic enzymes may reduce the amount of active metabolites, including deaminases and 5′-nucleotidases. The cellular effects induced by nucleoside and nucleotide analogues are described in BOX 2.

ribonucleotide reductase subunit M1 (RRM1)[13,19], the membrane transporter ENT1[15,20], the activating kinase DCK[21,22], the cytosolic 5′ nucleotidase II[23,24] as well as uridine phosphorylase and dihydropyrimidine dehydrogenase. A routinely applicable functional assay of membrane transport or enzymatic activity would therefore certainly be of great interest for predicting the activity of these drugs. A clinical trial evaluating ENT1 transport activity with a fluorescent nucleoside probe is currently being carried out in patients receiving gemcitabine for pancreatic cancer (ClinicalTrials.gov identifier: NCT00414570)[25].

Search for known mutations or resistant profile in circulating or intracellular viral strains. In viral diseases, mutations that are associated with resistance to nucleoside analogues are systematically identified during the preclinical development of a drug using sequencing-based technologies or *in vitro* phenotypic assays[26–28]. Indeed, the identification of drug-resistant viral strains is a prerequisite for obtaining investigational new drug (IND) status from the FDA or the EMA[29–31]. In addition, genotypic and phenotypic assays, such as sequencing-based or hybridization-based technologies, or assays based on the replication of isolated circulating viruses or molecular clones, are important for the management of patient treatment, particularly for patients with HIV. Moreover, as a result of the establishment of comprehensive databases on drug resistance, such as the Stanford University HIV Drug Resistance Database, virtual phenotyping can also be used by clinicians to make decisions on the treatment to be prescribed[26].

In current medical practice, physicians routinely take into account specific mutations associated with resistance to certain antiviral nucleoside and nucleotide analogues. However, the patient's genotype seldom influences the choice of anticancer nucleoside analogues prescribed in the clinic. A sobering example is that of thiopurine methyltransferase genotyping in patients receiving the nucleobase drug 6-mercaptopurine[32]. Although specific genotypes are associated with severe drug toxicity, genotyping (which has been routinely available for over a decade) has yet to gain widespread acceptance[33]. Several factors, such as the absence of a clear-cut impact on response rates or the toxicity observed in most cases, may explain why there is a relative lack of interest in genotyping patients with cancer[34].

Novel agents

The development of new nucleoside and nucleotide analogues is based on the need to identify new agents that have different mechanisms of action compared to existing agents, the need to provide drugs with improved bioavailability and solubility as well as the need to overcome resistance mechanisms and to improve the balance between efficacy and long-term toxicity for drugs that are administered over a long period of time (for example, patients with HIV or HBV require lifelong treatment). In this article, we have chosen to divide these novel drug candidates into the following categories: new nucleosides that have important modifications in their base and sugar moieties; pronucleotides; conjugates composed of nucleoside or nucleotide analogues and other chemical entities; liposomal formulations; and orally administered formulations of approved drugs. As there is a vast amount of information available in the scientific literature regarding new nucleoside and nucleotide analogues, we have mainly focused our discussion on those compounds that are in clinical development but we have also included compounds that are in preclinical stages of research. Chemical structures of some of these compounds are shown in FIG. 3.

New anticancer nucleosides

When objectively considering the contribution that nucleoside analogues have had in curing patients with cancer, examples are few (TABLE 1). Cytarabine, which has been in clinical use for many years, contributes to the cure of acute myeloid leukaemia in less than 30% of all adult patients, and 6-mercaptopurine is commonly used

Phenotypic assays
Assays in which biological function or cell response is measured as an index of drug action.

Pronucleotides
Phosphorylated nucleosides in which the phosphate is linked to a protective group to increase diffusion of the nucleoside across the cell membrane.

Box 2 | **Cellular effects induced by antiviral and cytotoxic nucleoside and nucleotide analogues**

Once the active phosphorylated metabolites of nucleoside analogues have been produced in cells (FIG. 2), they will induce the termination of chain elongation, the accumulation of mutations as well as apoptosis.

Termination of chain elongation

Nucleotide analogues are in competition with physiological nucleotides during the initiation and extension of DNA or RNA chains by cellular or viral polymerases. The incorporation of the analogues is therefore dependent on the relative affinity of polymerases for nucleotides. The absence of a 3′ hydroxyl group on the carbohydrate moiety of nucleoside and nucleotide analogues prevents the formation of 3′-5′ phosphodiester bonds between the analogue and incoming 5′ nucleoside triphosphates, resulting in an early termination of the growing viral DNA or RNA chain. Chain termination is also observed with anticancer compounds that possess the 3′ hydroxyl group, and the recognition of the nucleotide as non-human seems to be responsible for this effect. For viruses that have a reverse transcription step, chain termination can occur during RNA-dependent or DNA-dependent DNA synthesis[161]. Moreover, viral polymerases often have weaker specificity for nucleotides and are therefore more prone to incorporate analogues.

Accumulation of mutations in viral progeny

Ribavirin has been reported to induce lethal mutations in the viral genome[162,163]. Other so-called DNA or RNA stealth nucleobases or nucleosides (such as 5-fluorouracil, 5-azacitidine or deoxyguanosine derivatives) have a 3′ hydroxyl group on their carbohydrate group, which enables sustained chain elongation after their incorporation into DNA or RNA. Subsequent mismatching may lead to mutagenesis, resulting in reduced infectivity; this concept has been validated for many riboviruses with the notable exception of hepatitis C virus[164,165].

Induction of apoptosis

The exact mechanism by which nucleoside analogues induce apoptosis in cancer cells is still not clearly understood[166]. Nucleoside analogues generally cause a block in the S phase of the cell cycle and may involve DNA mismatch sensors. Nucleoside analogues are commonly combined with agents that are toxic to DNA, such as alkylating or platinum compounds, as they both inhibit the repair of DNA lesions and may also cause DNA lesions per se. Fludarabine combined with cyclophosphamide, and gemcitabine combined with cisplatin, are commonly used for the treatment of chronic lymphocytic leukaemia and lung cancer, respectively. In addition, compounds such as gemcitabine have been reported to be potent radiosensitizers[167].

for the treatment of acute lymphoblastic leukaemia and contributes to the cure of this disease. The other cytotoxic nucleoside analogues provide temporary responses or prolonged survival but do not yield definitive cures. There is thus a strong need to improve the potency of currently available cytotoxic nucleoside analogues.

Some of the novel nucleoside analogues have original mechanisms of cytotoxicity compared to conventional nucleoside analogues. CNDAC ((1-(2-C-cyano-2-deoxy-beta-D-arabino-pentofuranosyl) cytosine) was initially synthesized as a cytarabine analogue capable of inducing DNA strand breaks after its incorporation into DNA because of the nucleophilic attack of the cyano groups. Both CNDAC and sapacitabine, an orally administered derivative of CNDAC, have shown good activity *in vivo* on human cancer xenografts. Several Phase I and II clinical trials of sapacitabine alone or in combination with other anticancer drugs are underway in patients with chronic lymphocytic leukaemia, acute myeloid leukaemia (AML)[35], non-small-cell lung cancer and other advanced solid tumours, and a pivotal Phase III study of sapacitabine has been initiated in elderly patients with newly diagnosed AML (ClinicalTrials.gov identifier: NCT01303796).

The adenosine analogues 8-chloro-adenosine and 8-amino-adenosine decrease RNA synthesis and induce cell death by decreasing the intracellular concentration of ATP[36,37]. 8-amino-adenosine induces a decrease in both RNA and DNA synthesis, whereas 8-chloro-adenosine only decreases RNA synthesis, with a preference for mRNA[38]. Although this inhibition of mRNA synthesis is associated with the reduced expression of a large number of proteins, the cytotoxicity induced by 8-chloro-adenosine is related to decreased MET expression in multiple myeloma cells and reduced cyclin E expression in breast cancer cells[39,40]. The triphosphorylated metabolite of 8-chloro-adenosine also inhibits the polymerization of actin and the activity of topoisomerase II[41,42]. 8-chloro-adenosine is currently being evaluated in a Phase I trial in patients with chronic lymphocytic leukaemia (ClinicalTrials.gov identifier: NCT00714103).

A recurrent question in the field of cytotoxic nucleoside analogues is the breadth of the potential oncology indications. The first cytotoxic nucleoside analogues to be introduced into the clinic, such as cytarabine and fludarabine, are active in some haematological malignancies but not in solid tumours. Gemcitabine, a bifluorinated pyrimidine nucleoside derivative, has activity in haematological malignancies and against several solid tumours. Several of the novel nucleoside analogues offer promise in multiple indications.

Based on the observations that both purine and pyrimidine cyclopentenyl derivatives have biological activity, Choi *et al.*[43] synthesized and evaluated fluorocyclopentenyl-cytosine (RX-3117). This compound has a broad cytotoxic activity *in vitro* and antitumour activity *in vivo*. It is a substrate of nucleoside transporters and is phosphorylated by uridine cytidine kinase before being incorporated into DNA and RNA, and it also inhibits DNA methyltransferase 1 (REF. 44). 4′-thioaracytidine (T-araC; also known as OSI-7836 and thiarabine)[45] is also a sugar-modified nucleoside analogue that is currently

Nucleophilic attack
A chemical reaction in which a negatively charged entity forms a bond with a positively charged atom.

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