Pharmacological and Therapeutics agents that Target DNA Replication

By

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In

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Introduction

DNA replication inhibitors are commonly used as anticancer and antiviral agents (see Appendix - Table VIII). This review focuses on their molecular pharmacology. Drugs inhibit DNA synthesis by two mechanisms that are generally associated: 1/ direct interference with molecules required for DNA polymerization or/and initiation of replication; and 2/ checkpoint response(s). The direct sites of drug action ("pharmacological targets": nucleotide precursor pools, chain elongation, DNA polymerases, the DNA template, and cyclin-dependent kinases) are outlined in Figure 1. Structures of the corresponding drugs and detailed molecular mechanisms of actions are summarized in Figures 2-7. Checkpoint response ("Intra S-phase checkpoint") was first identified by its deficiency in cells from patients with Ataxia Telangiectasia (AT). Checkpoints allow the repair of the drug-induced DNA lesions. Checkpoints can also activate programmed cell death (also referred to as apoptosis). Functionally, checkpoint response can be differentiated from direct replication block when replication inhibition can be alleviated by checkpoint inhibitors such as ATM/ATR or Chk1 or Chk2 inhibitors.

Nucleotide Triphosphate Inhibitors

Historically there have been several approaches in developing cancer therapeutics that bear chemical similarity to the various "building blocks" of nucleic acids and inhibit the formation of functional nucleotide triphosphates needed to synthesize either DNA or RNA. Many of these agents have been labeled "antimetabolites" because of their structural similarities to naturally occurring metabolites (Daher et al. 1994; Pizzorno et al. 2003). These include the antifolates (e.g., methotrexate), pyrimidines like 5-Fluorouracil (5-FU), and purines like 6-mercaptopurine and 6-thioguanine (Fig. 2). Other drugs like hydroxyurea are not "antimetabolites" from the perspective of mimicking nucleic acid "building blocks", but have unique inhibitory effects on important steps in the conversion of nucleotides (Fig. 2-J and K). Some of these agents like hydroxyurea are relatively pure S-phase inhibitors, while others like 5-FU have activities extending beyond S-phase itself. As a class, all of the agents inhibit DNA synthesis and affect the S-phase of the cell cycle. Thus rapidly growing cancers theoretically should be potentially the most responsive. They also share toxicities toward the most rapidly growing normal "host" cells (e.g., hematopoietic cells - white blood cells, red

blood cells, and platelets; gastrointestinal mucosal cells and hair) such that common side effects are produced (e.g. myelosuppression, anemia, thrombocytopenia, diarrhea, and hair loss).

Antifolates: Methotrexate and Related Drugs

Historically one of the first antimetabolites to be synthesized was methotrexate (MTX) (Fig 2A-C), an analog of the natural folate intermediate dihydrofolate. MTX is actually a better substrate for dihydrofate reductase (*DHFR*) than the natural folate, dihydrofolate (Fig 2B). As a result MTX effectively inhibits *DHFR*, the enzyme responsible for the conversion of dihydrofolate (FH_2) to tetrahydrofolate (FH_4), a critical precursor in the formation of 5,10-methylene tetrahydrofolate (CH_2-FH_4). CH_2-FH_4 is a 1-carbon donor utilized for the synthesis of both purines and the pyrimidine thymine. The 2 and 8 carbons in the purine ring derive from this source as does the methyl group at the 5 position of the pyrimidine ring converting uracil into a thymine. Not only are the monoglutamates of FH_2 and FH_4 involved in these folate interconversions, but also the polyglutamylated forms of FH_2 and FH_4. MTX is converted to methotrexate polyglutamates (MTX-PG) (Fig. 1A), which inhibit thymidylate synthetase (TS) (Fig. 2B) and glycinamide ribonucleotide transformylase (*GAR transformylase*) (Fig. 2C).

MTX is widely used in the treatment of human cancers (Table VIII) and as immunosuppressor for instance in rheumatoid arthritis. There have been several attempts to develop new antifolates with emphasis placed on compounds that may affect other (additional) steps in the folate pathway. While many antifolates have been developed with some even reaching clinical evaluation, the only FDA-approaved new drug of this subclass is pemetrexed (Alimta®). Pemetrexed is currently used for treatment of mesothelioma and lung cancer. Pemetrexed inhibits not only *DHFR*, but also *TS* (Fig. 2B) *GAR transformylase* (Fig. 2C), which are less effectively inhibited by MTX-PG.

Pyrimidine Antimetabolites: 5-Fluorouracil and Related Drugs

5-Fluorouracil (5-FU) also blocks thymidylate synthesis as MTX, but works specifically on *TS* (Fig. 2D-E). *TS* converts deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP). *TS* is inhibited non-competitively by the 5-FU-deoxyuridine

monophosphate (5-fluorodeoxyuridylate; FdUMP) (Fig. 2E). The resultant depletion of dTMP inhibits DNA synthesis and cell division. In addition, the accumulation of dUMP as well as the FdUMP pool formed from 5-FU can be incorporated into DNA. The repair enzymes (e.g., uracil glycosylase) can remove the incorporated uracil or 5-FU from the DNA resulting in DNA breaks, which further contribute to the S-phase directed cytotoxicity of 5-FU. Lastly, the ribonucleotide of 5-FU (FUMP) can be mis-incorporated into RNA. Because RNA dysfunction is not cell cycle specific, the resultant cytotoxicity effect is not confined only to the S-phase (Daher et al. 1994; Pizzorno et al. 2003).

There have been many attempts over the years to design a better drug than 5-FU. In particular has been the desire to develop a 5-FU that could be administered orally, motivated by studies demonstrating that prolonged infusion of 5-FU had therapeutic advantages. A number of oral fluoropyrimidines have been synthesized (Diasio 1999). Capecitabine is the only oral 5-FU drug approved for use in the US, although several other analogs or prodrugs are available elsewhere. This prodrug has an added biochemical benefit in that the final step of activation to 5-FU occurs within the tumor thereby lessening release of 5-FU into the general circulation, where it potentially can affect sensitive host tissues like bone marrow, gastrointestinal mucosa, or the integument (skin, hair, and mucosal membranes).

5-FU has enjoyed extensive use in the treatment of a number of solid malignancies over the past 45 yrs (Table VIII). Today it is the major agent for advanced colorectal cancer and continues to be used in other gastrointestinal malignancies, such as stomach, esophageal and pancreatic cancer. 5-FU is also used in advanced breast and skin cancers. The deoxyribonucleoside of 5-FU, 5-fluorodeoxyuridine (FUDR or FdUrd) has been used for hepatic arterial infusion to treat liver metastases particularly from colorectal cancer. As noted above, Capecitabine has now been approved for advanced breast cancer and for both advanced and adjuvant treatment of colorectal cancer.

Purine Antimetabolites

6-Mercaptopurine (6-MP):

6-MP is a hypoxanthine derivative antimetabolite (see Fig. 2F) whose metabolites inhibit endogenous *de novo* purine synthesis at several steps (Fig. 2G) (Pizzorno et al. 2003). One of the most important metabolic activation is the formation of the nucleotide 6-MP ribose-5'-

phosphate also known as thioinosine monophosphate (TIMP) in the presence phosphoribosylpyrophosphate (PRPP) and the enzyme hypoxanthine –guanine phosphoribosyltransferase (*HGPRT*). Once formed TIMP can inhibit several steps in *de novo* purine synthesis. The most important of these inhibited steps is the formation of phosphoribosylamine from PRPP and glutamine (Fig. 2G). This step however is under feedback control being naturally inhibited by adenine or guanine nucleotides (AMP or GMP). TIMP causes a pseudo feedback inhibition mimicking the effect of AMP or GMP. TIMP is thought to inhibit *de novo* purine synthesis at two other steps (Fig. 2G): the conversion of IMP to GMP and the conversion of GMP to AMP. 6-MP is not taken up to any great extent into nucleic acid itself but rather affects the synthesis of purine nucleotides needed for both RNA and DNA synthesis. Thus, while S-phase may be primarily affected, 6-MP is not a pure S-phase inhibitor. 6-MP is used mainly in acute leukemias. As might be expected because of the affect mainly on rapidly proliferating cells, toxicities are seen in rapidly growing normal host cells in particular hematopoietic cells.

6-Thioguanine (6-TG):

Although structurally very similar to 6-MP (see Fig. 2H), 6-TG has a very different mechanism of action from 6-MP (Pizzorno et al. 2003). As is shown in Fig. 2I, 6-TG is metabolized to 6-TG-deoxyribonucleotide triphosphate (6-TdGTP), which can then be incorporated into DNA in place of dGTP. Futile mismatch repair and DNA fragmentation occur as the cell attempts to excise 6-TG from the DNA. As a result 6-TG affects S-phase cells primarily. 6-TG is mainly used in acute leukemias with toxicities being observed in rapidly growing host tissues like hematopoietic and mucosal cells.

Hydroxyurea (HU)

Although HU shares structural similarity to urea (except for the addition of a hydroxyl group in place of a hydrogen - See Fig. 2J), it is not a true antimetabolite, since antimetabolites are analogs of naturally occurring metabolites in nucleic acid synthesis and urea is actually an end product of metabolism. As shown in Fig. 2K, HU has a unique affect on ribonucleotide reductase a critical enzymatic step in the synthesis of DNA by which the two major pyrimidine (UDP, CDP) and two major purine (ADP, GDP) ribonucleotide diphosphates are

converted to their corresponding deoxyribonucleotide diphosphates (dUDP, dCDP, dADP, dGDP). This highly specific effect on a critical step in the DNA synthetic pathway results in hydroxyurea being essentially a pure S-phase inhibitor. HU is used in the management of chronic granulocytic leukemia in particular treatment during the blast phase of the disease (Table VIII). Toxicities include rapidly growing host tissues like hematopoietic and mucosal cells.

Chain Elongation Inhibitors

Some drugs in this group have traditionally been labeled "antimetabolites" (e.g, cytosine arabinoside and gemcitabine) because they are related to the naturally occurring "building blocks" in nucleic acid synthesis. Others (e.g., aphidicolin and Foscarnet) have unique chemical structures. Despite the differences, these drugs share a similar site of action, inhibiting chain elongation of the deoxyribonucleotide strand being synthesized. Because the major effect of these agents ison DNA synthesis and S-phase, such agents have found use in rapidly growing tumors (e.g., leukemias) and affect host tissues that also are rapidly dividing (bone marrow cells, mucosal membranes, hair and skin). Chain elongation inhibitors are useful not only as anticancer drugs, but also as antivirals.

Cytosine Arabinoside (AraC)

AraC is a pyrimidine antimetabolite in which a cytosine is linked to an arabinose sugar (Fig. 3A). Arabinose is an isomer of glucose. At the 2' position, the hydroxyl group is oriented such that the sugar has a conformation resembling a deoxyribose sugar. As a result cytosine arabinoside is "recognized" by DNA polymerase alpha as a deoxycytidine following conversion to the nucleotide triphosphate (AraCTP) (see Fig. 3C). While it becomes linked to the elongating DNA stand being synthesized, the orientation of the arabinoside sugar is unable to "stack" properly resulting in termination of DNA elongation (Chrencik et al. 2003).

Since the effect of AraC is specifically on DNA synthesis, it is essentially a "pure" S-phase inhibitor. Its anticancer activity is therefore expected in tumors where a large proportion of cells are in S-phase. This indeed is the case for acute leukemias (Table VIII). Its host cell effects are also typically on rapidly growing normal cells (bone marrow cells, mucosal membranes, hair and skin). In the past cytosine arabinoside was also used to treat

DNA viral infections, although its use in antiviral chemotherapy has been replaced by a number of newer more effective agents.

Gemcitabine

Gemcitabine (2, 2-difluoroeoxycytidine; dFdC) is a relatively new pyrimidine antimetabolite drug analog of deoxycytidine (dC) (Fig. 3A). Because of its similarity to deoxycytidine, gemcitabine can be taken up by nucleoside transporters into cells and metabolized by many of the same anabolic and catabolic enzymes used by deoxycytidine (Fig. 3C). Gemcitabine does have however some important metabolic differences, in particular its affinity for the activating enzyme deoxycytidine kinase is 3-fold that of deoxycytidine while it is catabolized less effectively via cytidine deaminase than deoxycytidine. This results in elevated accumulation of gemcitabine nucleotide pools and contributes to the overall effectiveness of gemcitabine. The precise mechanism of gemcitabine's antitumor activity is not fully known. Unlike the AraC, which has an essentially pure S-phase effect, gemcitabine has cell cycle effect beyond the S-phase. Gemcitabine affects several critical steps including DNA polymerase, and ribonucleotide reductase. Gemcitabine triphosphate (dFdCTP) can be incorporated into DNA and lead to both chain termination (Fig. 3C). Misincorporation can also occur more efficiently than for AraC, as DNA polymerase can continue chain elongation past a single incorporated gemcitabine molecule. This misincorporation can trap topoisomerase I (Chrencik et al. 2003). This latter event is thought to be important because the gemcitabine remains hidden from repair enzymes which otherwise might excise it allowing DNA chain elongation to continue. Deamination of dFdCMP by dCMP deaminase is thought to require dCTP. Gemcitabine is thought to actually avoid what is otherwise a potential resistant mechanism as a result of depletion of dCTP secondary to inhibition of ribonucleotide reductase,

Gemcitabine was originally approved for use in the treatment of advanced pancreatic cancer (Table VIII). Gemcitabine is being evaluated for a number of other diverse solid tumors including lung cancer and breast cancer. Of interest preclinical and clinical studies have demonstrated that gemcitabine is a potent radiosensitizer. This effect has been exploited in planning treatment regimens of certain malignancies in particular pancreatic cancer.

Antiviral Drugs that Block Chain Elongation

There are a number of drugs used primarily in the treatment of viral infections including AIDS, which share a common history with anticancer drugs. As noted above, AraC was actually used for both indications. AZT (Azidothymidine) was initially developed as an anticancer agent.

Acyclovir

Acyclovir (Ac) is an antimetabolite analog of 2'-deoxyguanosine (Balfour 1999) (Fig. 3F). While the base is a guanine, the usual sugar locus of the nucleoside consists of an acyclic structure that still allows phosphorylation in a position similar to the location of a typical 5' carbon with a deoxyribose sugar. The initial activating step that converts acyclovir to acyclovir monophosphate (AcyMP) is catalyzed by herpes thymidine kinases (*TK*) or cytomegalovirus phosphotransferase (Fig. 2G). Key to its mechanism of action, acyclovir is not a good substrate for human *TK*, hence allowing for relative selectivity of the infected cells. Once formed AcyMP is further anabolized to AcyTP, which can then compete with dGTP as a substrate for viral DNA polymerase. It is after acyclovir is inserted (in place 2'-deoxyguanosine) into the elongating DNA strand that DNA synthesis stops, thus accounting for the drug's mechanism of action. Acyclovir incorporation into viral DNA is irreversible since the polymerase-associated 3', 5'-exonuclease is unable to excise AcyMP from the DNA. This further inactivates viral DNA polymerase and further contributes to the antiviral effect. Substrate selectivity is seen not only at the level of *TK*, but also at the level of DNA polymerase where AcyTP has been shown to be a 30-50 times more potent inhibitor of viral DNA polymerase than the human enzyme. As a consequence of this biochemical selectivity for the viral enzymes there is relative pharmacologic selectivity with most of the drug being excreted unchanged with minimal host cell toxicity. Acyclovir is used in the treatment of varicella-herpes zoster and herpes simplex infections.

Ganciclovir

Ganciclovir is a purine nucleoside-like antimetabolite that differs from acyclovir by inclusion of a hydroxymethyl group at the 3' position of the acyclic branch (Fig. 3F). Ganciclovir is

converted a nucleotide (GanMP) by viral phosphotransferase in cells infected by cytomegalovirus. Compared to acyclovir, ganciclovir is a better substrate for the viral phosphotransferase. Moreover, the cellular half-life of GanTP is 12 hrs compared to 1-2 hrs for acyclovir triphosphate. This biochemical selectivity "vis a vis" acyclovir is the basis for ganciclovir's use in cytomegalovirus infections.

Foscarnet

Foscarnet (see next section) is also used in cytomegalovirus infections. Its clinical use necessitates intravenous administration as a suitable oral formulation is not available. Foscarnet is not metabolized and thus is excreted essentially unchanged. Its antiviral activity is equivalent to ganciclovir. It is also used as a second-line agents for herpes simplex infections resistant to acyclovir.

DNA Polymerase Inhibitors

Direct inhibitors of DNA polymerases differ from AraC or acyclovir, which also act at the DNA polymerase step but in an indirect manner. As noted above AraC or acyclovir derive there inhibitory effect from the termination of chain elongation that occurs after the nucleotide of the drug is incorporated into the elongating DNA strand causing structural changes that prevent further DNA elongation. Two examples of direct DNA polymerase inhibitors are aphidicolin and foscarnet.

Aphidicolin (APH)

APH is a tetracyclic diterpene [(3-alpha, 4-alpha, 5alpha, 17 alpha)-3,17-dihydroxy-4methyl-9,15-cyclo-C,18-dinor-14,15-secoandrostane-44,17-dimethanol] derived from the fungus *Cephalosporium amphidicola* (see Fig. 3B). APH is a specific inhibitor of eukaryotic and viral encoded replicative DNA polymerases (α, δ and ε). APH derivatives have been evaluated for both anticancer and anti-AIDS activity. As anticancer agents they have not displayed significant activity as monotherapy in clinical studies, which have also shown these agents to have minimal toxicity. Preclinical studies have suggested a potential use for APH in modulating the resistance that develops to anticancer agents like platinum drugs and AraC. It has been suggested that it is possible to inhibit repair of DNA platinum adducts

using APH. Unfortunately the clinical effectiveness of APH as a modulator of drug resistance to cancer chemotherapy has not been comprehensively evaluated. From a pure laboratory perspective, APH is useful (at single digit micromolar concentrations) in tissue culture to synchronize cells in early S-phase.

APH binds reversibly to the polymerase-DNA complex and preferentially blocks dCTP incorporation opposite to a guanine on the DNA template. APH blocks the replicative polymerases (α, β and ε) but not the repair polymerase (β). Because APH cannot be viewed as a dCTP analog and blocks selectively the elongation phase of Pol α, it has been proposed that APH forms a ternary complex with the polymerase bound to its DNA substrate and product (Huberman 1981; Sheaff et al. 1991). We recently proposed that APH acts as an "interfacial inhibitor" (Pommier and Cherfils 2005; Pommier and Marchand 2005). However, there is yet no atomic structure for such complexes to demonstrate this mechanism. High concentrations of APH (above 10 μM) can induce DNA double-strand breaks that can be detected as γ-H2AX foci (Furuta et al. 2003). APH is commonly used to reveal "Fragile Sites (FRA)" that can be visualized in mitotic cells following APH treatment. Twenty fragile sites including the fragile X site represent over 80% of the lesions observed in lymphocytes treated with APH. The molecular mechanisms generating these breaks ("replication fork collapses" followed by recombinations and sister chromatid exchanges) at fragile sites remain to be elucidated. The ATR and Chk1 kinases and BRCA1 have been implicated in preventing the occurrence of breaks at fragile sites by stabilizing replication forks in these late replicating regions with high DNA flexibility (Arlt et al. 2003). Fragile sites and their stabilization is implicated in oncogenic mutations and gene amplification (Hellman et al. 2002). Fragile sites are preferred sites for plasmid integration and may be favored targets for papillomavirus integration (Casper et al. 2002).

Foscarnet

Foscarnet (FOS) is an organic analogue of the natural inorganic pyrophosphate (Fig. 3E). Foscarnet interferes with the polymerization step by complexing with the pyrophosphate binding site of DNA polymerase, preventing cleavage of pyrophosphate from nucleoside triphosphates, thereby blocking elongations (Fig. 3C). Foscarnet has a specific effect of viral

DNA polymerases at concentrations that do not inhibit cellular DNA polymerases. This is thought to contribute to its antiviral activity particularly for cytomegalovirus infections.

DNA Template damaging drugs

Three main DNA template lesions induced by anticancer agents can block replication fork progression: DNA adducts, DNA strand breaks and DNA-protein crosslinks.

DNA alkylating agents

Methylmethanesulfonate (commonly referred to as MMS) is commonly used in biological experiments because it is effective on a broad range of cells including yeast. MMS is used to study post-replication repair (Pfander et al. 2005). MMS reacts with guanine N7 and forms methyl-guanine adducts (Fig. 4).

Nitrogen mustards were the first non-hormonal anticancer drugs scientifically reported in the 1940's (Kohn 1996; Tew et al. 1996). Platinum derivatives were the first drugs to cure metastatic testicular cancer (Einhorn and Donohue 1977). DNA alkylating agents have since remained a major component of the anticancer armementarium. Selected compounds are shown in Figure 5. All these compounds contain an electrophilic center that reacts with cellular nucleophiles, primarily nucleic acids (oxygens of phosphates, oxygens of bases [adenine O6], amino groups of purines [guanine N7], and pyrimidine (cytosine N3]), but also with amino groups of proteins, sulfur atoms of methionine and thiol groups of cysteinyl residues of glutathione. Hence, increased cellular levels of glutathione increase resistance to alkylating agents and the activity of alkylating agents is enhanced by glutathione depletion. The activity of alkylating agents is correlated with inhibition of DNA synthesis, which is due to alteration of the nucleic acid template rather than to inactivation of DNA polymerase or other enzymes responsible for DNA synthesis.

Cisplatin, carboplatin and oxaliplatin:

Cisplatin in combination with bleomycin is curative against most metastatic testicular tumors (Einhorn and Donohue 1977; Einhorn 1997). The most critical platinum lesions for anticancer activity are DNA-DNA crosslinks between adjacent guanines N7 (Fig. 5A, right). Each adduct bends the DNA toward the drug-bound in the DNA major groove. Platinum

derivative also form interstrand DNA-DNA crosslinks between adjacent guanines on opposite DNA strands, as well as DNA-protein crosslinks, in particular with HMG proteins (Bruhn et al. 1992). Carboplatin has been developed to limit cisplatin's renal and neurological toxicities. Oxaliplatin has a different spectrum of activity from cisplatin (Rixe et al. 1996) and has recently become a major drug for the treatment of colorectal cancers. The platinum DNA crosslinks interfere both with transcription and replication. Cells with deficient transcription-coupled nucleotide excision repair (TC-NER) are hypersensitive to cisplatin (Furuta et al. 2002). Conversely, resistance to cisplatin is commonly associated with mutations in the TC-NER pathways. This can be explained by the removal of platinum-DNA adducts by TC-NER. DNA replication also contributes to the antiproliferative activity of platinum derivatives in at least two ways. First, post-replication mismatch repair (MMR) signals the presence of platinum-DNA adducts to the apoptotic pathways and thereby induces cell death. Second, deficiencies in bypath polymerases sensitizes cells to cisplatin (Wu et al. 2004). Hence, one could envisage the following scenario to include the various pathways listed above. Replication of DNA containing platinum-DNA adducts needs bypass polymerases (see review by Kunkel et al.) in non-transcribed regions, as they are not removed by TC-NER.

Nitrogen mustards (Melphalan, Chlorambucil and Cyclophosphamide) and nitrosoureas (CCNU, BCNU):

Like platinum derivatives, nitrogen mustards form DNA adducts preferentially at the guanine N7 position. However, the cytotoxic crosslinks are *inter*-strand crosslinks (Fig. 5B). Alkylation of DNA phosphates also occurs and can lead to DNA breaks by chemical hydrolysis of the resulting phosphotriester. However, this reaction is much slower than guanine alkylation and seems unlikely to be a major determinant of drug action.

Cyclophosphamide and ifosfamide must be metabolized to produce alkylating compounds (Fig. 5B, lower left) and cannot be used in tissue culture because liver activation is required. Cyclophosphamide is a widely anticancer agent (Table VIII).

Nitrosoureas (Fig. 5C) are lipophilic compounds that cross the blood brain barrier and show activity in brain tumors (Tew et al. 1996). They are also used in lymphomas, myelomas and various carcinomas. Their cholorethyl group generates adducts with guanine N7 but also

cytosine N3. A second alkylation from the other arm of the molecule (R in Fig. 5-C) can produce guanine-cytosine DNA-DNA crosslinks. A key site of DNA attack for the nitrosoureas as well as nonclassical methylating agents such as procarbazine and dacarbazine is the O6 methyl group of guanine. Enhanced repair of this site is associated with drug resistance, and O6 methylguanine transferase inhibitors enhance the antitumor activity of nitrosoureas.

Radiomimetic DNA cleaving agents: bleomycin and neocarcinostatin

Bleomycin (BLM) is a natural peptide antibiotic isolated from *Streptomyces* [for review see (Lazo and Chabner 1996)]. BLM is curative against metastatic testicular cancer in association with cisplatin and vinblastine (Einhorn 1997) (Table VIII). It is also used in combination chemotherapy for Hodgkin's disease, lymphomas, and various carcinomas. The human toxicity profile of BLM is different from other chemotherapies. Bleomycin virtually lacks hematopoietic toxicity but has cumulative lung toxicity in small number of patients. The chemical structure of BLM is complex (M.W. around 1500). The drug is activated by forming a 1:1 coordination complex with Fe(II) and oxygen. The binding of O_2 to the $Fe(II)^{\cdot}$BLM proceeds most rapidly in the presence of DNA. The binding of BLM to DNA involves electrostatic interactions, minor groove binding and partial sequence-specific intercalation between a G:C and T:A base pairs. DNA cleavage takes place 3' from a guanine. BLM produces both single-strand breaks (SSB) and double-strand breaks (DSB) with a ratio of approximately 10:1. The DSB:SSB ratio is approximately twice that of ionizing radiation, which produces approximately 20 SSB for each DSB. The high frequency of DSB has been attributed to the binding of a second BLM molecule, which is facilitated by the prior binding the first BLM. Cells exposed to BLM seem to be most sensitive at the end of S-phase in G2 and mitosis.

Neocarcinostatin (NCS) (Zinostatin®) is an antitumor antibiotic commonly used in experimental systems to generate DNA breaks. NCS consists of 2 components: a protein of 10.7 kDa bound to an otherwise labile non-protein chromophore that cleaves DNA. Bleomycin- and neocarcinostatin produce high frequency of DSB because they generate the reactive oxygen species (ROS) in the DNA. NCS like BLM is commonly used as radiomimetic agents and in studies exploring the ATM-Chk2, γ-H2AX pathways.

DNA Topoisomerase Inhibitors

DNA topoisomerase inhibitors are commonly used as anticancer drugs (Table VIII). The two main classes of clinical topoisomerase inhibitors target topoisomerases I and II.

Seven topoisomerase genes are encoded in the human nuclear genome. The enzymes (abbreviated Top) have been numbered in the order of their discovery except for the most recent enzyme, mitochondrial topoisomerase I (Top1mt). Vertebrate cells contain two Top1 (Top1 for the nuclear genome and Top1mt for the mitochondrial genome), two Top2 (Top2α and β) and two Top3 (Top3α and β). The seventh topoisomerase is Spo11, whose expression is restricted to germ cells. Top3α forms heterodimers with BLM (the gene product deficient in Bloom syndrome) and is mechanistically related to the resolution of post-replicative hemicatenanes and recombination intermediates. Top1's belong to the family of the tyrosine recombinases (which includes λ-integrase, Flp and Cre recombinases), and Top2 is related to bacterial gyrase and Topo IV, which are the target of quinolone antibiotics. Topoisomerases and tyrosine recombinases nick and religate DNA by forming a covalent enzyme-DNA intermediate between a specific enzyme catalytic tyrosine residue for each enzyme and the DNA via a DNA phosphodiester bond (Fig. 6A). Topoisomerases have also been classified in two groups depending whether they cleave and religate one strand (type I) or both strands (type II) of the DNA duplex. Type I enzymes include Top1 and Top3 and type II, Top2 and Spo11.

Top1 inhibitors: camptothecins;Trapping of Top1 cleavage complexes by endogenous DNA lesions and carcinogenic adducts

Top1 relaxes DNA supercoiling processively by nicking the DNA and allowing rotation of the broken strand around the Top1-bound DNA strand (Fig. 6D – curved arrow). Once the DNA is relaxed, Top1 religates the breaks by reversing its covalent binding (Fig. 6D->C). Under normal condition, the cleavage intermediates (Fig. 6D) are transient. Religation is much faster than cleavage. Camptothecins and non-camptothecin Top1 inhibitors trap Top1 cleavage complexes by binding at the enzyme-DNA interface. Hence Top1 inhibitors represent a paradigm for "interfacial inhibitors". Interfacial inhibition has recently accounts for the molecular mechanism of inhibition of many natural products that block specific

conformational states of macromolecular complexes. Aphidicolin and Top2 inhibitors have been proposed to follow the interfacial inhibition paradigm (Pommier and Cherfils 2005; Pommier and Marchand 2005). Interfacial inhibition is a special case of uncompetitive inhibition.

Two camptothecin derivatives are used in cancer therapy: hycamtin (Topotecan®) and CPT-11 (Irinotecan; Camptosar®). CPT-11 is an inactive prodrug. It needs to be converted to its active metabolite SN-38. Hence, it is preferable to use Topotecan and the parent drug, camptothecin for pharmacological studies. Top1 cleavage complexes can also be trapped by endogenous and frequent DNA lesions including abasic sites, mismatches, oxidized bases, and carcinogenic DNA adducts (Pourquier and Pommier 2001).

Top1 cleavage complexes on the leading strand are converted into replication double-strand breaks (Rep-DSB) by replication "run-off" (Fig. 6F). These Rep-DSB activate the ATM-Chk2 pathway with hyperphosphorylation of histone H2AX (γ-H2AX formation) (Furuta et al. 2003) and phosphorylation of BLM on threonine 99 (Rao et al. 2005). They also induce the rapid and sustained phosphorylation of RPA2 at least in part by DNA-PK (Shao et al. 1999). Top1 cleavage complexes also activate the ATR-Chk1 pathway by mechanisms that still remain to be elucidated. Camptothecins are now commonly used to induce Rep-DSB in vertebrate and yeast cells (Pommier et al. 2003). They complement hydroxyurea and aphidicolin as pharmacological tools to "collapse" replication forks. It can be argued that the nature of the Rep-DSB are better defined in the case of camptothecins than for hydroxyurea or aphidicolin. The repair of the Top1-DNA adducts needs to take place for replication restart and camptothecins can be used to study the genetic and molecular pathways implicated in this repair (for review see (Pommier et al. 2003)).

Top2 inhibitors: etoposide and anthracyclines;and Trapping of Top2 cleavage complexes by endogenous DNA lesions and carcinogenic adducts

Top2 enzymes functions as homodimers and generate DSB. Thesebreaks are staggered by 4 base pairs (Fig. 6I) and are generated by the covalent linkage of each monomer to the 5'-end of the broken DNA. By contrast to Top1, which allows controlled rotation (swiveling) of the broken DNA around the intact strand, Top2 catalyzes the passage of an intact duplex through the broken DNA held within the Top2 complex (Fig. 6I; dashed curved arrow). This strand

passage reaction allows reactions that are specific for Top2 such as decatenation of duplex circles (Fig. 6K) (and its reverse reaction, catenation) and knotting/unknotting (not shown in Fig. 6). Decatenation is essential at the end of replication to allow the segregation of newly replicated chromatin domains.

Etoposide, doxorubicin, anthracyclines and other Top2 inhibitors trap the cleavage intermediate (Fig. 6J). This type of inhibition is probably mechanistically similar to Top1 trapping by camptothecin. The Top2 inhibitors are therefore candidate interfacial inhibitors (Pommier and Cherfils 2005; Pommier and Marchand 2005) by trapping Top2 cleavage complexes by forming ternary complexes with Top2 and the cleaved duplex (Fig. 6J).

As in the case of Top1, base alterations including oxidative lesions and carcinogenic adducts, as well as DNA single-strand break can interfere the DNA religation step, and therefore trap Top2 cleavage complexes under physiological condition (i.e. during normal cell cycle in the absence of drug/pharmacological inhibitors) (Kingma and Osheroff 1998).

Top2 cleavage complexes produce large protein-DNA adducts. Each enzyme subunit covalently linked to DNA has a molecular mass of 170 kDa (Top2α) or 180 kDa (Top2β). There is no evidence that heterodimers α/β form under physiological conditions. The trapped Top2 homodimer complexes can alter DNA replication by forming steric blocks on the DNA template. They also can interfere with other DNA metabolic processes including transcription and probably DNA repair, and chromatin remodeling.

Cdk Inhibitors and Checkpoint Inhibitors

Cyclin-dependent kinases (Cdk's) are required to activate DNA replication only once per cell cycle. By contrast, phosphatidyl inositol-like kinases (PIK's: ATM, ATR, TOR, DNA-PK) and the checkpoint kinases Cdk1 and Cdk2 negatively regulate replication under normal (ATR, TOR, Chk1) and stress (ATM, Chk2) conditions. Pharmacological inhibitors of these kinases have been identified and synthesized. A number of them are in clinical development.

Cdk Inhibitors

The cyclin-dependent kinase (Cdk) inhibitors, flavopiridol and roscovitine are in clinical trials as anticancer agents along with indisulam and BMS-387032 (Fig. 7) (Dancey and Sausville 2003; Fischer and Gianella-Borradori 2005). These drugs are competitive inhibitors

for ATP binding in the Cdk's. Many additional chemicals and potential clinical agents have been developed recently (Dancey and Sausville 2003; Pommier and Kohn 2003; Fischer and Gianella-Borradori 2005). Cdk inhibitors have also potential applications in therapeutic areas other than cancer where aberrant cell proliferation plays a key pathogenic role (restenosis, infectious diseases including HIV, degenerative neuropathies, and glomerulopathies) (Fischer and Gianella-Borradori 2005).

Cdk's control DNA replication by at least 3 mechanisms: cell cycle activation through the restriction point (G1/S transition), activation of replication origins, and inactivation of replication origins once they have fired (thereby ensuing that replication occurs only once per cell cycle). In the first mechanism, Cdk4/6 initiate replication by lifting the inhibitory effect of Rb on E2F1-DP1 complexes and thereby by inducing the transcription of structural proteins and enzymes required for G1/S transition ("restriction point"). Cdk4/6 and 2 also stimulate the degradation of the Cdk inhibitor $p27^{kip1}$, thereby enabling the G1/S transition. The second mechanism leading to DNA replication implicate Cdk2-cyclin E or A complexes, which are required for the firing of replication origins by activating cdc45. Finally, in a third control mechanism, Cdk1-cyclin B complexes prevent re-initiation by inactivating Orc's, Mcm's and Cdc6 (for details see Chapter by Aladjem) (Aladjem et al. 2004) (http://discover.nci.nih.gov/mim).

It is important to keep in mind that a subgroup of Cdk/cyclin complexes are required for transcription, and that a wide spectrum Cdk inhibitors such as flavopiridol and roscovitine also inhibit RNA polymerase II (Pol II) and therefore transcription. This property confers anti-HIV activity to flavopiridol because it block TAT-mediated transcription of viral genes. Cdk7 and Cdk9 phosphorylate Pol II at its C-terminal domain, which for human Pol II contains 52 homologous heptapeptide repeats (YSPTSPS). Phosphorylation at the underlined serines (2 and 5) is required for the Pol II to switch from pre-initiation to elongation. Cdk9/cyclin T complexes are part of transcription elongation p-TEFb and Cdk7/cyclin H are part of the TFIIH helicase and nucleotide excision repair complex.

Checkpoint Inhibitors

Checkpoint regulatory pathways are a major focus of attention (see Chapter XXX) because they are defective in cancer predisposing human diseases. They could also provide

new therapeutic approaches and be used as biomarkers for tumor response to DNA targeted therapies. Defects in the intra-S-phase checkpoint primarily lead to "radioresistant DNA synthesis" (RDS), a hallmark of cancer-prone diseases first exemplified in Ataxia Telangiectasia (AT) (Painter and Young 1980) and now expanding to a list of cancer predisposing genetic diseases such as ATLD (Mre11 deficiency), Nijmegen Breakage syndrome (NBS), and familiar breast and ovarian cancer (BRCA1 deficiency). Two main pathways have been elucidated recently: The ATM-Chk2 and the ATR-Chk1 pathways. The ATM-Chk2 pathway is activated in response to DNA double-strand breaks both in replicating and non-replicating DNA. It is regulated by the MRN (Mre11-Rad50-Nbs1) complex. Activation of the ATM-Chk2 pathway can activate both cell cycle checkpoints (by phosphorylating and inactivating Cdc25, by phosphorylating BRCA1 and p53-Mdm2) and pro-apoptosis molecular nodes (p53, E2F1, PML) (http://discover.nci.nih.gov/mim). The ATR-Chk1 pathway is activated by replication stress in the absence of double-strand breaks. The ATR-Chk1 pathway is regulated by claspin, ATRIP and the 9-1-1 (Rad9-Rad1-Hus1) complexes. It is critical for replicon stability and cell cycle checkpoint activation that allow DNA repair (see Chapter XXX). Hence, ATM, Chk1 and Chk2 inhibitors are actively pursued for medical development (Zhou and Bartek 2004; Pommier et al. 2005). The rationale for developing Chk1 inhibitors is to sensitize p53-deficient tumors to DNA-targeted agents. Chk2 inhibitors may have the same application but also be used as anti-apoptotic agents (Pommier et al. 2005). In which case, their medical use might expand to neurological, immunological and cardiovascular diseases where apoptosis contributes to the pathogenic process.

7-hydroxystaurosporine (UCN-01) (Fig. 7) is a remarkably effective abrogator of G2 and S cell cycle checkpoint, particularly in p53-deficient cells (Wang et al. 1996; Shao et al. 1997). UCN-01 synergizes the antiproliferative activity of S-phase specific DNA targeted agents including araC, 5-FU and camptothecins, while showing no additive effect with microtubule and Top2 inhibtors (Shao et al. 1997; Monks et al. 2000; Shao et al. 2004). UCN-01 inhibits Chk1 (Sarkaria et al. 1999; Graves et al. 2000) but also Chk2 (Yu et al. 2002) and PDK1, a kinase that activates Akt/PKB (Wu et al. 2001). Hence, UCN-01 cannot be considered a specific Chk1 inhibitor. Clinical trials with UCN-01 in association with DNA replication inhibitors and cisplatin are ongoing. Specific inhibitors of Chk2 and Chk1 are

being developed by multiple pharmaceutical companies and academic laboratories. Their clinical usefulness will be tested in the near future.

Caffeine was the first cell cycle checkpoint abrogator described (Lau and Pardee 1982), which led to the concept of cell cycle checkpoints. Caffeine can directly inhibit ATM and ATR kinases (Sarkaria et al. 1999) in addition to its known effect on adenyl cyclase. 2-aminopurine is an isomer of adenine (Fig. 7), which can also be used as cell cycle checkpoint abrogator. Both drugs are relatively non-specific and need to be used at millimolar concentrations.

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Figure Legends

Figure 1: Site of action of commonly used DNA replication inhibitors. 6-Mercaptopurine is abbreviated 6-MercaptoP., and 7-hydroxystaurosporine: UCN-01.

Figure 2: Structures and Mechanisms of Action of Purine and Pyrimidine Biosynthesis Inhibitors.

A. Structures of Methotrexate (MTX) and Methotrexate Polyglutamates (MTX-PG). Up to 6 glutamyl residue ($n \leq 6$) are added by folypolyglutamyl synthetase (FPGS).

B. Effects of MTX on Thymidylate Synthesis. The formation of thymidylate (dTMP) from deoxyuridylate (dUMP) is dependent on donation of a methyl group from the folate intermediate 5,10 methylene tetrahydrofolate or its polyglutamylated forms (CH_2-FH_4-Glu_n). Following donation of the methyl group, dihyfdrofolate (FH_2) or its polyglutamylated forms (FH_2-Glu_n) must be reconverted to a tetrahydrofolate (FH_4Glu_n). This is mediated by dihydrofolate reductase (*DHFR*). MTX can inhibit this step. Polyglutamylated forms of MTX (MTX-PG) can also inhibit *DHFR* thymidylate synthetase (*TS*).

C. Effects of MTX on *de novo* Purine Synthesis. Glycineamide ribonucleotide (GAR) plus Formyltetrahydrofolate polyglutamate (CHO-FH_4-Glu_n) in the presence of GAR transformylase yields aminoimidazole carboxamide ribonucleotide (AICAR) plus FH_4-Glu_n. AICAR plus N-10 Formyl FH_4Glu_n in the presence of AICAR transformylase yields inosine monophosphate (IMP), a precursor for adenine an guanine nucleotides (AMP and GMP) used in nucleic acid synthesis, and FH_4-Glu_n. Both GAR and AICAR transformylase are inhibited by MTX-PG.

D. Structure of 5-Fluorouracil (5-FU).

E. Effects of 5-FU on DNA and RNA Synthesis. Anabolism of 5-FU to 5-FU nucleotides is similar to that of uracil. 5-FU nucleotides result in anticancer activity primarily from inhibiting *TS*, but also from incorporation into RNA and DNA.

F. Structure of 6-Mercaptopurine (6-MP).

G. Inhibition of Purine Biosynthesis by 6-MP. MP can combine with phosphoribosylpyrophosphate (PRPP) in the presences of Hypoxanthine guanine phosphoribosyltyransferase (HGPRT) to yield 6-MP ribose-5'-PO_4 also known as thioinosine

monophosphate (TIMP). As can be seen, TIMP can inhibit 3 important purine interconversions important in *de novo* synthesis of purines needed for the formation of RNA and DNA.

H. Structure of 6-Thioguanine (6-TG).

I. Misincorporation of 6-TG into DNA. 6-TG is anabolized via the same enzymatic pathway as guanine, eventually being taken up into DNA. Following attempts to repair the DNA and remove the incorporated 6-TG, DNA fragmentation occurs resulting in the DNA specific effects.

J. Structure of Hydroxyurea (HU).

K. Inhibition of Deoxynucleotide synthesis by HU. Hydroxyurea has a specific inhibitory effect on ribonucleotide reductase, blocking the conversion of pyrimidine and purine nucleotide diphosphates to their corresponding deoxyribonucleotide diphosphates.

Figure 3: Structure and Mechanisms of Action of DNA Polymerase Inhibitors:

A. Structures of Cytosine Arabinoside (AraC) and Gemcitabine (dFdC).

B. Structure of Aphidicolin (APH).

C. Mechanisms of DNA Polymerase inhibition by AraC, dFdC and Foscarnet (FOS). AraC following anabolism via the same anabolic pathways used by deoxycytidine is eventually converted to araCTP. It can substitute for deoxycytidine triphosphate (dCTP) at DNA polymerase being incorporated into the elongating strand of newly synthesized DNA (open polygon), but terminating further elongation due to steric interference preventing addition of further nucleotides.

Gemcitabine (dFdC) has effects on multiple steps in the deoxycytidine (dC) metabolic pathway. Its effect is maximized by preferential anabolism to nucleotides (particularly the initial conversion to dCMP by the nucleotide kinase that is 300 x faster than with dC) with less deamination by dCMP deaminase than with dCMP. Similarly the incorporation of dFdCTP into DNA (black polygon) is preferential to that of dCTP resulting in some inhibition of DNA elongation, but also some potential effect after incorporation into DNA as well. Accumulation of dFdCDP also inhibits ribonucleotide reductase (*RR*) blocking the conversion of pyrimidine and purine nucleotide diphosphates to their corresponding deoxyribonucleotide diphosphates (see Fig. 2K).

FOS, an inhibitor of DNA elongation, interferes with the pyrophosphate binding site on DNA polymerase by complexing with it, preventing cleavage of pyrophosphate (pPO_4) from nucleoside triphosphates, thereby blocking further primer template extensions. As noted in the text, it can also be considered a DNA replication inhibitor.

Aphidicolin is a specific inhibitor of both eukaryotic and viral encoded replicative DNA polymerases (α, δ, ε).

E. Structure of Foscarnet (FOS).

F. Structures of Acyclovir (Acy) and Gancyclovir (Gan).

G. Mechanism of Inhibition of Viral Replication by Acy and Gan. Acy is converted efficiently by virally encoded thymidine kinase (*viral TK*) to the nucleotide monophosphate (AcyMP) and eventually to the nucleotide triphosphate (AcyTP) that can be incorporated by viral DNA polymerase into an elongating DNA strand where it results in termination of further elongation of viral DNA. In contrast there is minimal conversion of Acy to AcyMP by mammalian *TK* resulting in a very selective antiviral effect. The metabolism of Gan is very similar to Acy with the critical initial step being conversion by virally encoded phosphotransferasese to the nucleotide monophosphate (GanMP) with further anabolism to the nucleotide triphosphate (GanTP) that can be incorporated into viral DNA and block chain elongation.

Figure 4: DNA Alkylation by Methylmethanesulfonate (MMS). Nucleophilic attack from the guanine N7 (left) generates a methyl adduct at position 7 of guanine.

Figure 5: DNA Alkylating Drugs

A. Structure of Cisplatin (CDDP), Carboplatin and Oxaliplatin (Oxali) (*Left*), and Schematic Representation of the Intrastrand DNA-DNA Crosslinking Adducts (*Right*). DNA adducts are formed at the guanine N7 position and generate intra-strand crosslinks.

B. Nitrogen mustards. Drug Structures (*Left*), and Schematic Representation of the Interstrand DNA-DNA Crosslink Adduct (*Right*). (*1*) In the initial step, one of the chlorines is lost and the β-carbon reacts with the nucleophilic nitrogen atom to form the cyclic positively charged, and very reactive aziridinium. (*2*) Reaction of the aziridinium with a nucleophile (electron-rich atom) (shown is a guanine [G]) yields the first alkylated product.

(*3*) Formation of a second aziridinium by the remaining chloroethyl group allows for a second alkylation, which produces a crosslink between the two alkylated guanines.

C. Nitrosourea. Drug Structures (*Left*), and Summary of Biological Lesions (*Right*).

Figure 6: Topoisomerase Inhibitors:

A. Topoisomerase Reactions. Topoisomerases cleave DNA by forming reversible transesterification intermediates between a catalytic tyrosine and a DNA phosphodiester. The cleaved intermediates are referred to as cleavage (cleavable) complexes. Topoisomerase I (Top1) is the only enzyme forming a covalent bond with the 3'-end of the broken DNA, leaving a 5'-hydroxyl end. Topoisomerases II and III (Top2α and β and Top3α and β) have opposite polarity, forming a covalent bond with the 5'-end of the broken DNA and leaving 3'-hydroxyl ends. The curved arrows indicate the direction of the electron transfer. Top2's require ATP for DNA breakage-religation. Top1's and Top3's do not.

B. Structure of Camptothecin (CPT). CPT has a chiral center at position 20 and the natural alkaloid is 20-S (with the 20-hydroxyl above the plan). Synthetic 20-R camptothecin is inactive against Top1.

C-F. Replication Double-Strand Breaks (Rep-DSB) Induced by Top1 Cleavage Complexes. Under normal conditions, the religation reaction is favored over cleavage and more than 90% of the Top1-DNA complexes are non-covalent (**C**). The cleavage complexes (**D**) provide a break around which the DNA swivels (controlled rotation: curved arrow) until supercoiling has been dissipated. CPT binds at the Top1-DNA interface, forming a ternary complex (**E**).). The CPT molecule is represented as filled rectangle. Top1 cleavage complexes on the leading strand for DNA synthesis are converted into Rep-DSB (**F**). Aphidicolin (APH) prevents the conversion of the Top1 cleavage complexes into Rep-DSB.

G. Structures of Top2 Inhibitors: Etoposide (VP-16, Vepesid), Doxorubicin (Adriamycin) and Daunorubicin.

H-J. Trapping of Top2 Cleavage Complexes by Top2 Inhibitors. Under normal conditions, the religation reaction is favored over cleavage and more than 90% of the Top2-DNA complexes are non-covalent (**H**). Top2 cleavage complexes (**I**) allow the passage of another intact duplex through the Top2-DNA complex (dashed curved arrow). Strand passage allows decatenation of double-strand circular DNA molecules (**K**) and segregation of

replicated supercoiled DNA circles. Top2 cleavage complexes are trapped by the drugs probably as they bind in at least one of the DNA cleavage sites by forming ternary complexes (**J**). The drug molecule is represented as filled rectangle.

K. Top2-mediated decatenation. Interlocked duplex DNA circles can be separated by cleavage of one circle and transfer of the other circle trough the broken circle (see detail for strand transfer in panel **I**). The reaction is reversible (catenation; not shown).

Figure 7: Commonly Inhibitors of Cyclin-Dependent Kinases and Checkpoint Inhibitors. Flavopiridol is a broad spectrum Cdk inhibitor inhibiting the cdk's controling both the cell cycle and transcription. Roscovitine has a slightly narrower Cdk inhibitory profile with selectivity for Cdk1, Cdk2, Cdk7 and Cdk9. Roscovitine also inhibits both cell cycle and transcription Cdk's. Indisulam and BMS-387032 are less commonly used experimentally because they are primarily limited for clinical trials. They arrest cell cycle in G1/S. 7-Hydroxystaurosporine (UCN-01) has been in clinical trials for several years and is widely used and studied in experimental studies for its potent inhibition of cell cycle arrest in S and G2 in response to DNA damaging agents. Caffeine and 2-aminopurine inhibit ATM an ATR kinases. Both require millimolar concentrations to abrogate cell cycle checkpoints and inhibit ATR/ATM.