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Small-molecule inhibitors of DNA damage-repair pathways: an approach to overcome tumor resistance to alkylating anticancer drugs

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Abstract

A major challenge in the future development of cancer therapeutics is the identification of biological targets and pathways, and the subsequent design of molecules to combat the drug-resistant cells hiding in virtually all cancers. This therapeutic approach is justified based upon the limited advances in cancer cures over the past 30 years, despite the development of many novel chemotherapies and earlier detection, which often fail due to drug resistance. Among the various targets to overcome tumor resistance are the DNA repair systems that can reverse the cytotoxicity of many clinically used DNA-damaging agents. Some progress has already been made but much remains to be done. We explore some components of the DNA-repair process, which are involved in repair of alkylation damage of DNA, as targets for the development of novel and effective molecules designed to improve the efficacy of existing anticancer drugs.

Cancer resistance to drugs

The war on cancer has gone on around the world for more than 40 years, with the expenditure of billions of US dollars. As a result, the age-adjusted cancer death rate has been reduced by approximately 12% for both males and females between 1975 and 2008, with some major improvements in colon, breast and some hematological cancers [201,202]. A significant fraction of the overall reduction in death rates, specifically since the late 1980s, is linked to reduced lung cancer deaths due to reduced tobacco consumption. There are also improvements in 5-year survival rates for a number of important cancers, which reflect earlier detection and better treatments. Still, widespread cures remain elusive despite increased cancer screening, using improved methods for early diagnosis and an increase in the number and nature of the therapies to treat the different diseases that we call cancer. It has long been known that cancer is multiple diseases with multiple etiologies, involving genetic and epigenetic events, and that a single therapeutic agent would not be sufficient to lower cancer mortality [1]. However, the lack of significant progress in the development of approaches to eliminate cancer in patients rather than incrementally increase survival, would suggest a basic flaw in our therapeutic strategies, and a disconnect between the clinic and the laboratory. A classic example of the success and failure of cancer therapy is illustrated by trastuzumab, which targets the HER2 receptor that is over-expressed in a significant

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percentage of breast cancers and induces a cytostatic G1 arrest [2]. There is an initial excellent response to trastuzumab but, eventually, the disease reoccurs [3]. There are several mechanisms responsible for the development of **resistance**:

- Reduced trastuzumab binding due to mutations in the HER2 gene that lead to a truncated, but active, receptor [4];
- Upregulation of downstream signaling (e.g., loss of PTEN or increase in PI3K/Akt activity) that compensates for reduced receptor activation [5];
- Compensation due to alternative signaling pathways involving other members of the HER family of receptors and other receptors (e.g., IGF-1R [6]);
- Impaired immune-mediated activity [7].

Many other effective drugs eventually become ineffective, due to similar issues related to mutations in the binding domain that they target and epigenetic changes that activate alternative compensating signaling pathways.

For cytostatic compounds, which do not kill tumor cells and that continually need to be administered, cancer cell survival due to resistance appears to be inevitable owing to genetic diversity in the tumor mass and selection pressure for epigenetic changes. However, the same scenario occurs even for cytotoxic DNA-alkylating therapies [8,9]. Hence, the development of strategies to block resistance should be developed upfront rather than to adopting a passive 'wait and watch' approach [10–12]. We discuss below the role of **DNA repair** in tumor resistance to alkylating agents and the progress in developing agents that inhibit enzymes involved in the different pathways of repair.

DNA-damaging drugs

The history of anticancer therapies began with the development of World War I mustard gas agents [13] that at low doses had therapeutic activity due to the covalent modification of DNA. The first effective molecule clinically used in the treatment of cancer was the nitrogen mustard, *bis*(2-chloroethylamine) (Figure 1). The nitrogen mustards and related analogues, for example, *bis*(2-chloroethyl)-*N*-nitrosourea [14] and cyclophosphamide [15], react with DNA in all cells that they reach to produce complex arrays of monofunctional and bifunctional lesions that qualitatively and quantitatively differ with the different drugs [16]. The bifunctional lesions include intra- and inter-strand cross-links. The latter are extremely cytotoxic since they prevent the DNA strand separation that is required for replication by DNA **polymerases** and transcription by RNA polymerases. However, if not repaired, the monofunctional and intrastrand crosslink lesions generated by these compounds are also cytotoxic based upon their ability to block the processivity of DNA polymerases. There are a number of anticancer agents that only produce monofunctional lesions. Regardless, toxicity due to alkylation damage generally requires cell division, which is one reason why tumor cells (assuming that they are replicating) are selectively more sensitive to DNA-damaging agents than most noncancer cells. Over the years, numerous DNA-modifying drugs with superior specificity and bioavailability have been developed (Figure 2). Therefore, this class of compounds continues to constitute an important tool in the treatment of many cancers, despite efforts to produce mechanistically based noncytotoxic antineoplastic drugs.

The major limitation of DNA-damaging drugs is that they are generally cytotoxic in any cell type that is rapidly dividing; for example, tumor cells, epithelial cells in the GI tract and the hematopoietic cells in bone marrow. Accordingly, the difference between a therapeutic and toxic dose can be small and dosing must be carefully monitored. A second important drawback of DNA-alkylating agents is that they are mutagenic as well as cytotoxic; therefore, they increase the risk of secondary cancers derived from the initial round of

therapy [17–20]. Efforts to restrict DNA alkylation to the formation of lesions that are cytotoxic but marginally mutagenic have been reported but none of these compounds have yet made it into clinical trials [21–24].

Tumor resistance to alkylating agents

In addition to the undesirable side effects mentioned above, tumors can develop resistance to specific types of genotoxic insults via a number of mechanisms, including the upregulation of gene products that enhance DNA-repair capacity. This mode of resistance is in addition to the more generic multidrug resistance pathways involving increased expression of transporters (e.g., P-glycoprotein MRP1/ABCC1) [25–28] and higher levels of cellular nucleophiles that can scavenge the reactive intermediates generated from this class of antineoplastic drugs [29,30]. There is experimental evidence using genetic manipulations that reducing the expression of many, albeit not all (e.g., mismatch repair [MMR]) DNA-repair proteins makes cells and animals more sensitive to DNA-damaging agents [31–41]. Conversely, increasing repair protein expression can confer resistance [42,43]. Therefore, DNA-repair pathways afford valid targets to potentiate clinically useful chemotherapeutic agents and to block cellular resistance to the same drugs.

It is worth some discussion on whether the strategy to potentiate DNA-damaging agents to overcome resistance represents a rational approach to drug design. The genetic deletion of many DNA-repair proteins shows virtually no phenotype, so inhibitors of specific repair targets are not expected to be toxic in the absence of a DNA-damaging drug. There are notable exceptions to this; for example, APE-1 and polymerase β (Pol β) (see below). Clearly, the significant off-target toxicities associated with antineoplastic agents may also be affected by any agent that diminishes DNA repair. However, it has been proposed that the overexpression of repair enzymes in some tumors may indicate that these proteins are critical to tumor growth or survival, which may make these cells selectively sensitive to repair inhibitors, even in the absence of alkylating agents [44]. It is also important to determine the effect of repair inhibitors on tumor stem cells, which are critical to the survival and expansion of the tumor [45], and how repair inhibitors in combination with alkylating agents alter the sensitivity of these cells. The role of DNA-repair pathways in tumor stem cells has been explored with some reports suggesting that human-induced pluripotent cells have enhanced levels of DNA-repair proteins, including those involved in repair of double-strand breaks [46]. A similar effect of DNA repair has been suggested for hematopoietic stem cells [47]. Embryonic stem cells are thought to be sensitive to **DNA damage** and undergo apoptosis more rapidly because of genotoxic insults [48]. Similarly, murine embryonic stem cells show higher repair activity than the corresponding fibroblast cells [49]. While the question is still not resolved, the inhibition of specific repair pathways may selectively induce tumor cells to undergo apoptosis.

Below, we discuss the major pathways associated with the repair of DNA damage induced by anticancer agents, as well as some of the many proteins that can be targeted to increase the efficacy of existing antineoplastic drugs. We have focused specifically on the proteins that are enzymatically involved in the repair of lesions. As mentioned above, it is reassuring that the genetic ablation of many of these repair proteins has no obvious phenotype, except when animals or cells are challenged with DNA-damaging agents. It is important to note that the interaction between unrepaired DNA lesions and the biological pathways that they eventually trigger is not discussed in any detail (Figure 3). Clearly, tumor cells may respond very differently to lesions due to differences in, for example, cell cycle checkpoints and apoptotic pathways, and there are already small-molecule inhibitors that target some of these pathways. With the development of DNA-repair inhibitors and research to understand how

they can be used in combination with drugs that target these pathways, it may be possible to develop a systems biology approach to selectively kill tumor cells and eliminate resistance.

Potential drug targets to overcome

Resistance to DNA damage

The four major routes to repair DNA damage induced by alkylating anticancer agents are discussed below (Figure 4). The determination of the predominant pathway in response to DNA damage depends on the type and extent of damage but, in most cases, there is some overlap because of the diversity of DNA lesions that are generated by DNA-alkylating agents. This review focuses on proteins that are enzymatically involved in the removal of DNA damage created by anticancer drugs. There are more than 100 other proteins that are associated with DNA-repair pathways and each may constitute a viable target for inhibitors designed to sensitize cells to DNA-damaging drugs.

Base excision repair

Base excision repair (BER) removes specific types of damaged bases from DNA. The specificity is a function of the glycosylase protein that initially 'recognizes' the lesion and then excises it off the DNA backbone. There are 11 human DNA-repair glycosylases associated with BER but only a few are involved in recognition of lesions caused by anticancer drugs. After the initial stage of excision to afford an abasic site, the remaining enzymes and steps in the BER pathway are universal; that is, it does not apparently matter how the abasic site originated. Therefore, specificity for inhibition of the repair of drug-generated DNA lesions has to be achieved at the glycosylase stage of BER.

DNA glycosylases—As the name suggests, the BER pathway involves the initial recognition and removal of a single modified base by a DNA glycosylase (Figure 5). There are two distinct classes of DNA glycosylases; monofunctional, which cleave the modified base off the DNA leaving an abasic lesion, and bifunctional, which both remove the lesion and then excise the DNA at the abasic site [50]. While many of the DNA glycosylases repair lesions that predominately form from endogenous reactions of cellular metabolites with DNA, for example 8-oxoguanine and 5-hydroxycytosine, which are generated by the production of reactive oxygen species [51,52], there are proteins that efficiently remove some of the alkylation lesions produced by anticancer drugs. Specifically, *N*-methylpurine-DNA glycosylase (MPG) efficiently excises *N*3-alkyladenine and *N*7-alkylguanine adducts from DNA, although it also processes hypoxanthine, which is derived from the deamination of adenine [53,54], and 1,N^6-ethenoadenine, which is created from the reaction of adenine with lipid peroxidation byproducts [54]. The *N*3-alkylA adduct blocks DNA replication and is cytotoxic [55,56], while N7-methylG appears innocuous to polymerization [57]. The *N*3-alkylA lesion is generated in significant amounts by many DNA-alkylating drugs, so its efficient removal by MPG constitutes a mode of resistance. In fact, the repair of *N*3-alkylA lesions is normally so efficient and rapid in cells and animals that the lesion can only be isolated using high concentrations of alkylating agent and at short incubation times. However, in high-dose chemotherapy, or in cells with low MPG levels, the cytotoxicity of the lesion becomes apparent [57,58].

MPG inhibitors—The crystal structure of MPG in the presence of DNA and a hypoxanthine substrate is shown in Figure 6 [59]. There are relatively few reports on small-molecule inhibitors of the glycosylase [60]; the most active inhibitors in this study were Trp-P-1 and 2-thioxanthine (Figure 7) with the latter having an IC_{50} of 76 μM against human MPG. Both compounds clearly resemble potential substrates for MPG and presumably bind in the enzyme's active site. A number of other groups have conducted extensive screening,

without success, to identify potent and selective MPG inhibitors. The lack of more potent glycosylase inhibitors is probably because these proteins have electropositive surfaces that nonspecifically electrostatically interact with DNA through salt bridges to the phosphate backbone while they scan for damaged bases. The higher proportion of disordered regions in glycosylases, which may contribute to initial steps of recognition and binding [61,62], also pose a challenge to structure-based rational drug design, due to implied changes in global topology of the glycosylase that are not yet well understood. In addition, the active site, which is buried in the protein, is relatively small. The modified bases are excised off the deoxyribose ring upon extrusion from the base pair stack into the active site, which is a scenario common to all DNA glycosylases. A tyrosine residue stacks in the void in the DNA left by the lesion when it enters the active site. The modified bases make relatively few enthalpic interactions with the active site, which sterically blocks nonsubstrate bases from entering. This prevents the inadvertent excision of canonical bases and the formation of AP sites. In fact, overexpression of MPG is a weak mutator phenotype, presumably due to an increase in the background level removal of natural bases [63]. A potential alternative to reduce BER-mediated repair is to interfere with the displacement of the glycosylase from the DNA after the base excision reaction (Figure 5). However, exactly how the displacement of the glycosylase by the next enzyme in the BER pathway (i.e., APE-1; Figure 5) occurs is not well understood, but blocking this step would involve interfering with a protein–protein interaction. Because the molecular interactions between the different glycosylases and APE-1 have not been determined, and because of the challenges in targeting protein–protein binding sites with small-molecule inhibitors, there has been no reported effort to block the interaction between the different proteins in the BER pathway.

AP endonuclease—All of the DNA glycosylases, including MPG, produce an abasic site as a result of their initial excision of damaged bases (Figure 5). An abasic site, which has no coding information, can also be formed by ‘spontaneous’ hydrolysis of an unmodified base. The rates of ‘spontaneous’ depurination/depyrimidination at near neutral pH and physiological temperature are quite slow. However, the formation of a charged nucleic acid base due to alkylation on a ring nitrogen greatly accelerates the hydrolysis of the glycosidic C-N bond [64]. It is estimated that there are 10,000 abasic sites formed per day per cell without any treatment [65,66]. Apparently for this reason, neither animals nor cells can survive without the main enzyme (APE-1) that repairs abasic sites [67]. The lethality of APE-1 knockouts has been linked to loss of the repair activity and the mechanism of cell death appears to involve apoptosis [68–70]. There is also evidence that APE-1 expression can be induced by genotoxic agents, including cancer drugs [10]. The lethality of clinically used anticancer treatments can be enhanced by a temporal decrease in APE-1 using antisense technology [35,71,72]. Therefore, temporarily decreasing APE-1 activity could be an important adjuvant to DNA-damaging antineoplastic agents. In addition to its endonuclease activity, APE-1 also plays a role as a redox co-factor for a number of transcription factors [73–75]. The cellular effects of this redox activity are still not fully understood [76].

APE-1 inhibitors—The structure in the region around the active site of hAPE-1 with DNA containing a stable tetrahydrofuran abasic site is shown in Figure 8A [77]. As is the case with the binding of many of the repair enzymes, the DNA molecule is significantly distorted from linearity, the lesion is extrahelical and the protein introduces a side chain (Arg-177 for APE-1) into the void left in the base stack. Based upon the structures of the protein without and with DNA containing a lesion, rearrangement of this arginine residue into the DNA stack is a major structural change that takes place in the protein when APE-1 binds to DNA with a substrate. Therefore, the molecular modeling to design active site inhibitors of APE-1 requires some attention to the dynamic movement of the loop with the arginine side chain.

The enzymatic center of APE-1, where a bound Mg^{2+} activates a water molecule for the nucleophilic attack on the 5′-phosphate, lies in a pocket that is deep inside the protein and is lined with residues that can form H-bonds, π-cation and hydrophobic interactions with potential inhibitor ligands (Figure 8). Most of the nonlesion interactions between the protein and DNA are salt bridges with the phosphate backbone on both strands in the vicinity of the lesion.

A number of small-molecule inhibitors of APE-1 have been reported (Figure 9 shows some examples along with their IC_{50} values) [78–82]. Many of the compounds identified are based on dianionic molecules (e.g., 3-CCPPA and 2-BBIDA) that may mimic the diphosphate linkage that is embedded in the DNA substrate structure [78]. In a search for APE-1 inhibitors to enhance the cytotoxicity of alkylating compounds in melanoma and glioma cells, a number of structurally related compounds were identified, including 'compound **4**' (Figure 9) [79]. The molecule has low micromolar activity against APE-1 and enhances the cytotoxicity of both methyl methanesulfonate and temozolomide in glioma and melanoma cell lines. However, the level of abasic sites detected in the cells, which should be a biomarker of APE-1 inhibition, did not indicate any synergistic effect between 'compound **4**' and the alkylating agent, methyl methanesulfonate. Both the APE-1 inhibitor and methyl methanesulfonate alone increased the number of abasic sites by approximately 2.7-fold but together the increase was only fourfold. This raises the question regarding the mechanism of action for this compound. A benzo[*b*][1,8]naphthyridine (Figure 9; AR03) molecule, which was isolated after a large screening effort, *in vitro* showed a 2-μM IC_{50} against APE-1 activity and some potentiation when used with temozolomide and methyl methanesulfonate in cell culture [80]. Included in this class of APE-1 inhibitors is a series of arylstibinic acids (Figure 9; compounds 13755 and 13743), which although potent at nanomolar concentrations in biochemical experiments, lacked activity in cells [81]. Lucanthone, which inhibits APE-1/REF-1 activity and binds to the protein [82], also interacts with other cellular targets, including DNA via intercalation [83], so the mechanism of action remains uncertain. E3330 (Figure 9), which inhibits APE-1 endonuclease activity at low micromolar concentrations, was originally identified as a specific inhibitor of APE-1 redox activity [84]. However, NMR experiments have recently shown that the quinone binds to the active site of APE-1 (Figure 8B) and it also blocks the endonuclease activity [85]. We have confirmed that E3330 is an endonuclease inhibitor in two biochemical assays with an IC_{50} in the low micromolar range [Srinivasan A, Gold B, unpublished data]. Treatment of T98G glioma cells with E3330 also caused a modest increase in the number of abasic sites based on an aldehyde colorimetric assay [Srinivasan A, Gold B, unpublished data]. Because of these recent results, it has been suggested that E3330 bound in the catalytic site associated with endonuclease activity also blocks the binding of transcription factors to APE-1. If this overlap of activities is common, it may be difficult to discover the mechanism(s) of action of small-molecule APE-1 inhibitors. Regardless, there is much interest in developing APE-1 inhibitors for clinical use. Another approach to block APE-1 activity is to chemically modify the AP site using compounds such as methoxyamine. Methoxyamine forms an imine with the aldehyde group in the ring-open form of the AP site (Figure 10), which prevents the APE-1-mediated cleavage of the AP site [40,86]. Although methoxy-amine will react with any aldehyde (or ketone), it is being clinically evaluated in a Phase I trial as an adjunct therapy in combination with the DNA-methylating agent temozolomide (Figure 2). The fact that this simple reactive compound, which has no structural specificity for abasic sites, is being tested in humans illustrates the desperate need to block resistance to DNA-alkylating agents.

The interactions of APE-1 with other repair proteins, such as DNA glycosylases, Pol β and PARP, are also topics of interest for medicinal chemistry. APE is proposed to associate with MPG and in doing so influences base excision turnover [87,88]. The association of APE

with Pol β is expected, considering that the latter enzyme follows the phosphodiesterase activity of APE [89]. PARP has been known to compete with APE for binding to the same APE-cleaved BER intermediate [90]. The activities of Flap endonuclease and DNA ligase are also proposed to be coordinated by APE activity [91]. While all these interactions are of general academic interest to understand repair pathways and the interacting domains/surfaces, they also provide unique opportunities to design small molecules aimed at blocking these interactions so that the substrate 'hand-off ' does not proceed, thereby arresting the process of lesion repair.

Pol β—After APE-1 has removed the abasic lesion, the remaining break in the DNA backbone, with 3′-hydroxyl and 5′-deoxyribose-phosphate termini, is processed by Pol β. In short patch repair, the enzyme trims the 5′-deoxyribose terminus to a phosphate via its lyase activity and inserts the appropriate complementary base into the vacant position via its polymerase activity (Figure 5). In long patch repair, the enzyme performs a strand-displacing synthesis, leaving an extended nucleotide flap that is degraded by FEN1 endonuclease [92]. Mice deficient in Pol β die at birth and show neurogenic developmental deficiencies [93]. However, Pol β null cells are viable, although sensitive to DNA-alkylating agents [94]. Overexpression of Pol β has been observed in a number of cancers but it is not certain that it is required for tumor maintenance [95]. In addition, a variety of human tumors often express mutated forms of Pol β, including variants that have reduced BER activity or reduced fidelity [96]. Some of the mutants appear to be dominant negative in that they maintain their DNA-binding affinity but are defective in the repair functionality [97]. The crystal structures of Pol β with a variety of DNA substrates have been solved [98].

Pol β inhibitors—Because of the sensitivity of Pol β-defective cells to alkylating agents, there have been several reports of molecules that inhibit both the lyase and/or polymerase activities of Pol β (Figure 5). The natural products oleanolic acid, edgeworin, harbinatic acid and myristinin A (Figure 11) have low micromolar activity in biochemical assays, little toxicity on their own and weakly potentiate bleomycin cytotoxicity [99–101]. Using the crystal structure as a guide (Figure 12) [98], Jaiswal *et al.* more recently identified a small-molecule inhibitor (NSC666715) of Pol β that dramatically potentiates the cytotoxicity of temozolomide *in vitro* and *in vivo*, and even had an effect on MMR-deficient cells [102]. Cells that are defective in MMR are generally resistant to temozolomide (see below). NSC666715 interacts with Pol β in such a way that it blocks both short and long patch repair but does not affect other BER proteins (i.e., APE-1 and ligase). Another molecule identified by the same research group that is effective at inhibiting Pol β is the deoxynucleotide analogue NSC124854 (Figure 11) [103]. It causes a concentration-dependent decrease in the strand-displacement synthesis activity of Pol β with an IC_{50} value of 5.3 μM [103]. Both NSC666715 and NCS124854 are proposed to bind in the same region on Pol β based upon molecular modeling studies (Figure 12). This region of Pol β is where the tumor suppressor adenomatous polyposis coli (APC) protein binds [104]. APC expression is induced by DNA-alkylating agents [105]. Mutations in the APC gene occur early in the neoplastic transformation of the colon [106], and APC blocks Pol β-mediated BER pathways and increases the sensitivity of cells *in vitro* to alkylation-induced DNA damage [107]. As expected, APC gene mutant cells become more resistant to alkylating treatment [108].

NSC124854 was evaluated in a xenograft model of colorectal cancer employing MMR-deficient and -proficient cell lines in the absence and presence of the methylating agent temozolomide (20 mg/kg). This is well below the maximum tolerated dose of temozolomide. NSC124854 was given intraperitoneally at 10 mg/kg, which is tenfold lower than the *in vitro* IC_{50} dose [103]. NSC124854 and temozolomide were given for 5 consecutive days and the mice maintained for approximately 6 weeks. Both compounds independently reduced *in vivo* tumor growth, with NSC124854 unexpectedly being more

effective than temozolomide. Both compounds in combination lowered the IC_{50} of temozolomide in cell culture experiments. However, there was no synergistic effect when the two molecules were combined in the animal tumor model experiment. The observation that NSC124854 was effective by itself in the mouse xenograft model in the absence of alkylating agent implies that the molecule may have other targets or that the formation of abasic sites in the tumor line may require a high constitute level of BER. Clearly, the challenges of using two drugs simultaneously *in vivo* will require additional studies.

FEN-1—FEN-1, a protein required for normal development [109,110], plays an important role in the long patch BER of alkylation damage DNA in addition to the cleavage of Okazaki fragments that arise during replication.

FEN-1 inhibitors—In order to potentiate the therapeutic efficacy of DNA-damaging drugs, a series of nanomolar FEN-1 inhibitors, which have some specificity for this endonuclease, have been identified [111]. These inhibitors have a 3-hydroxydihydropyrimidine-2,4(*1H,3H*) -dione core and IC_{50} values in the nanomolar range. The compounds also increase the toxicity of methyl methanesulfonate in cell culture. However, the one compound assayed in cells did not appear to show a clear dose-dependent effect.

DNA ligase—The last enzymatic step in BER (Figure 5), as well as in other DNA-repair pathways, is the ligation of the 5′-phosphate and 3′-hydroxy groups at the nick (i.e., single strand break) by ligase-I or ligase-III associated with XRCC1. The XRCC1 appears to act as a scaffold that facilitates the repair process but does not perform an enzymatic function. The crystal structure of ligase-I in complex with a nicked DNA template shows that the enzyme distorts the DNA and exposes the nick that is the target of ligation [112]. The DNA-binding domain of the protein completely encircles the DNA and stabilizes the distorted structure that is required to join the break.

DNA-ligase inhibitors—Small molecules were initially screened *in silico* using the crystal structure (Figure 13) [112]. The target for binding was the surface on the DNA-binding domain of the protein. Molecules identified in the screen were then tested in a DNA-joining assay and in binding assays using a nonligatable template. Four low micromolar inhibitors are shown in Figure 14 [113]. Although all of the compounds inhibited ligase activity, their mechanisms of action varied [114]. Compound 197, which was the most active, is a dicarboxylic acid and is reminiscent of the APE-1 inhibitors that mimic the DNA backbone (Figure 9). Compounds 67 and 189 were competitive inhibitors and cytotoxic. Compound 82 was a noncompetitive inhibitor and cytostatic. The cytostatic activity was attributed to activation of a G_1-S cell cycle checkpoint. A notable observation was that compounds 67 and 189 markedly enhanced the toxicity of the DNA-methylating agent, methyl methanesulfonate, and ionizing radiation in tumor cells but not in ‘normal’ breast epithelial cells. The authors measured the levels of ligase-I activity in the cancer and normal cells and found them to be higher in the former, as previously observed in other cancers [115]. The question has been raised whether the tumor selectivity of these inhibitors can be attributed mainly to overexpression of ligase-I in tumor cells. Whether high ligase-I activity is required for these tumors is not clear, but the data indicate that the amount of DNA ligase-I enzyme in malignant tumors is higher than that in benign normal tissues and peripheral blood lymphocytes. The level of DNA ligase-I in human tumors grown in nude mice was also shown to be very high. Therefore, the selective activity of the molecules in tumor cells makes this a potentially exciting target for additional medicinal chemistry.

PARP-1—The final protein that we will discuss in connection with BER is PARP-1; although PARP actually does not play a direct enzymatic role in repair. The PARP proteins are nuclear enzymes that bind as homodimers to both single- and double-strand breaks [116–119]. This binding activates its catalytic activity that transfers ADP-ribose subunits derived from NAD^+ onto a number of protein substrates. Depending on the level of strand breaks, this process can deplete cells of NAD^+, which results in low ATP levels and rapid cell death by necrosis [120,121]. In PARP null mice or depleted cells, the death pathway switches from necrosis to apoptosis [122–124]. Regardless of its role in BER, the development of small-molecule inhibitors of PARP has received significant attention since they can produce a build up of toxic single-strand breaks by trapping PARP on the break site [125]. There is also a synthetic lethality generated by PARP inhibitors in tumors that express defective mutant BRCA tumor suppressor proteins [126,127]. The BRCA proteins are involved in one pathway for repair of DNA double-strand breaks by homologous recombination [128–130]. These toxic lesions persist in PARP-inhibited BRCA-mutant cells. The synthetic lethal effect induced by PARP inhibitors in BRCA-mutated cells is the focus of clinical trials, since PARP inhibitors have virtually no toxicity in BRCA wild-type cells.

PARP-1 inhibitors—There has been a significant effort in the development of PARP inhibitors [131–134], and there are 88 clinical trials listed that have been completed or are ongoing [203]. Some examples of the inhibitors are shown in Figure 15. Sanofi's iniparib (BSI-201) is presently in a Phase I clinical trial in combination with temozolomide for the treatment of newly diagnosed malignant glioma and in an ongoing Phase III trial in combination with gemcitabine and carboplatin treatment in patients with previously untreated stage IV squamous non-small-cell lung cancer. However, an iniparib Phase III trial in triple negative (negative for estrogen receptor, progesterone receptor and HER2) breast cancer did not reach its goal [204]. Another PARP inhibitor, olaparib (AZD2281), is being used in combination with carboplatin for refractory breast and ovarian cancer in a Phase I trial [205]. However, AstraZeneca does not plan on pursuing this PARP inhibitor in hereditary BRCA-1 and -2-positive breast cancers, and will focus their efforts on ovarian cancer. Pfizer's rucaparib (PF-01367338) molecule is in a Phase II trial with carboplatin for the treatment of advanced breast and ovarian tumors [206]. Regardless of the less-than-spectacular results to date, based on the number of PARP-1 inhibitors that are under development, it seems likely that even more effective and selective drugs will soon be in clinical trials. Only then will the clinical value of PARP inhibitors be determined.

Nucleotide excision repair

Global nucleotide excision repair (NER) is fundamentally different from BER in that it lacks the same level of structure-based specificity, and repair involves excision of a long stretch of DNA containing the lesion by the coordinated action of multiple enzymes; more than 15 have been assigned a role in NER (Figure 4). The key element in the initiation of NER is the disruption of the canonical Watson–Crick helix, due to lesions that distort DNA. Most alkylating agents do not yield adducts that are substrates for NER. However, there are notable exceptions, including the cisplatin drugs that generate intrastrand crosslinks, protein–DNA crosslinks and mono-functional DNA lesions [135]. For the cisplatin intrastrand lesions, NER excises a fragment that contains both modified bases, leaving a gap that is eventually filled in by polymerase. One mechanism associated with resistance to cisplatin drugs is overexpression of NER proteins, specifically ERCC1-XPF [136], which acts as a single-stranded endonuclease in excising the stretch of DNA containing the damage. This effect on cisplatin toxicity was confirmed using antisense technology to decrease the levels of ERCC1 [137]. NER is also involved in a transcription-coupled process that allows RNA synthesis to proceed at lesion-halting modifications.

Inhibitors—A fluorinated epipodophylloid molecule, F-11782 (Figure 16), which inhibits topoisomerases I and II, is also an inhibitor of NER [138]. F-11782 inhibits the incision step in repair and the target may involve the ERCC1-XPF or XPG endonuclease activity associated with NER removal of the strand containing the damage. It was suggested that F-11782, in combination with DNA cross-linking agents would be a candidate for future clinical trials. Small-molecule inhibitors have also been reported for XPA (Figure 16), which is a factor associated with initial recognition of DNA damage in the NER pathway [139]. It is of interest that the molecules have a dianionic flavor similar to other repair inhibitors, including those that target APE-1 (Figure 9) and ligase (Figure 14). It may well be that these small dianions with different linker regions structurally mimic the DNA diphosphate backbone, electrostatically interact with DNA via salt bridges and physically block access of the specific repair protein to DNA. It would be interesting to determine if some of the molecules identified in a screen for XPA inhibitors would also inhibit APE-1.

Another NER target that has attracted interest is replication protein A, which is necessary for formation of the pre-incision complex that is required for NER removal of damaged DNA [140]. The repair of cisplatin intrastrand cross-links was shown to be inhibited by gemcitabine (Figure 17), which has a number of mechanisms of action, and the use of gemcitabine has been explored in a carboplatin Phase II trial in platinum-resistant ovarian cancer [141].

Interstrand crosslink repair

Interstrand crosslinks (ICL) formed from bifunctional alkylating agents (e.g., nitrogen mustards, cisplatin, nitrosoureas and mito-mycin C) physically prevent the complementary DNA strands from separating and are extremely toxic. However, even these lesions can be repaired by an excision reaction on both sides of one of the crosslinked bases to afford an unhooked intermediate, translesion synthesis past the unhooked intermediate by specialized polymerases, NER nonremoval of the unhooked monofunctional adduct and homologous recombination (Figure 18) [142–144]. As expected, the sensitivity to crosslinking drugs has been correlated with the efficiency of ICL repair [145].

Inhibitors—At this time, there have been no reports on small-molecule inhibitors that selectively affect ICL repair. Because many of the same proteins associated with NER are involved in ICL repair, some of the inhibitors developed for NER may also be effective against ICL repair. Finally, the unique translesion synthesis polymerases that can bypass damaged bases, albeit often with reduced fidelity, constitute another potential target to inhibit ICL repair.

Direct reversal repair

Direct reversal repair by alkylguanine DNA-alkyltransferase (AGT) is highly specific for the repair of O^6-alkylguanine lesions, which are commonly produced during exposure to alkylating agent chemotherapy by the nitrosoureas and triazenes (Figure 2). The toxicity of this type of monofunctional lesion requires functional MMR that performs a futile cycle of removing and replacing a natural base opposite the O^6-alkylG lesion. Eventually, this process leads to strand breaks and cell death by apoptosis [146]. It should be noted that some O^6-alkylG lesions, such as those formed from chloroethylating agents, also exert their toxicity via DNA crosslinking [147]. Tumors that are tolerant to drugs that produce O^6-alkylguaine lesions (e.g., temozolomide) are often defective in MMR and/or overexpress the AGT protein, which transfers the alkyl group from the imidate ester lesion onto the protein in a suicide reaction with a turnover number of unity. The question has been raised whether tumors are tolerant of temozolomide because they become defective in MMR or whether they are tolerant because they are MMR defective. This is a chicken-or-egg question.

However, it is likely that MMR-defective cells exist in any sizable tumor mass and they may be selected for by the chemotherapy treatment with temozolomide.

Inhibitors—There are many inhibitors of the AGT protein and they are all based on analogues that closely resemble the imidate ester substrate; for example, 2-amino-6-[(4-bromo-2-thienyl) methoxy]-9H-purine (lomeguatrib) (Figure 19), O^6-benzylguanine and related O^6-alkylguanine derivatives [148]. There are 32 clinical trials listed for O^6-benzylguanine [207]. In all of these studies, the inhibitors are being used as an adjuvant to a DNA alkylating drug; for example, temozolomide and carmustine [149]. An example of the combination of AGT inhibitor with an alkylating agent is the clinical trial with 2-amino-6-[(4-bromo-2-thienyl)methoxy]-9H-purine in combination with temozolomide in metastatic colon cancer patients [150]. In spite of the trial obtaining the goal of depleting AGT activity, no beneficial response was observed. The lack of response in this trial could be attributed to the decision to use temozolomide to treat colon cancer and/or the presence of MMR-deficient cells in the tumors that are resistant to the toxicity of 6-methylgua-nine lesions produced by temozolomide. Another reason for the lack of efficacy in this trial, and probably for many others, is the late stage of the cancer in the enrolled patients. It is conceivable that early-stage cancers with less genetic diversity may be more sensitive to specific therapies than advanced cancers that are genetically and epigenetically more heterogeneous.

In terms of adverse effects, the main difference between temozolomide without and with 2-amino-6-[(4-bromo-2-thienyl)methoxy]-9H-purine was a more pronounced myelosuppressive. This raises the issue of specificity; if there is no differentiation between cancer cells and other proliferating cells, the difference in the therapeutic index will remain constant. To combat this problem, clinical trials have explored the use of autologous infusion of AGT-transduced hematopoietic progenitors into patients with gliomas [207]. This would provide the sensitization of tumors to alkylating agents but protect the hematopoietic system.

At this time, there are no viable gene therapy strategies to restore MMR activity, which is required for tumor cells to be sensitive to O^6-alkylguanine adducts formed by many DNA-alkylating agents.

Future perspective: targeting DNA-repair pathways

We have presented an overview of many of the enzymes that can be targeted to directly interfere with normal DNA repair in order to potentiate existing DNA-damaging anticancer drugs and to overcome tumor resistance that results from the upregulation of tumor cell DNA-repair capacity. In addition, there are a large number of potential protein targets that do not directly participate in DNA repair but transmit the signals induced in cells as a consequence of DNA damage, which eventually leads to cell growth arrest and/or death. These are also attractive drug targets.

The rationale for combining DNA-damaging drugs with molecules that will specifically inhibit the repair of the DNA lesions that the drugs generate appears well founded. There are numerous *in vitro* and *in vivo* experiments in which proteins have been modulated genetically or by antisense or siRNA approaches, which demonstrate the potentiation of anticancer drugs by compromising DNA repair. Moreover, the deletion of many DNA-repair proteins does not cause toxicity in untreated cells or create a discernable phenotype in unchallenged null animals. Therefore, many of the molecular target that have been structurally characterized, which we discussed, have been validated and there are well-defined biochemical and cellular assays, animal models and biomarkers to evaluate new

inhibitor compounds. The ongoing clinical trials of several DNA-repair inhibitors in combination with alkylating drugs will provide additional insight into the potential of the approach, as well as the limitations. In this regard, the development of biomarkers to establish the appropriate patient population to target and the beneficial effect of the inhibitors is paramount. Unfortunately, the late-stage cancer patients enrolled in many of the clinical trials may confound an objective analysis of the clinical efficacy of the compounds.

The design of drugs to target DNA-repair proteins involves several significant technical challenges. Many of these enzymes electrostatically associate with DNA via basic amino acid sidechains that weakly and nonspecifically interact with the polyanionic phosphate backbone. While this allows the proteins that initially 'find' damaged bases to conduct an efficient 1D search, the requirement for numerous electrostatic contacts is not an attractive strategy in small-molecule drug design. In addition, the active sites for many repair proteins are relatively small and buried deep inside the protein and are lined with amino acid residues that present limited potential for selective stabilizing enthalpic interactions. Regardless, compounds with greater than micromolar IC_{50} values have been identified for many repair targets. Some of the compounds show the predicted synergistic cytotoxic effect when used with drugs that produce the relevant DNA lesions. In some cases, but not all, the appropriate biomarkers indicate that the mechanism of action is indeed related to DNA repair. Based upon many of the reported structures, converting these compounds into drugs will require a significant amount of medicinal chemistry engineering.

The ultimate challenge for the development of therapeutic molecules that target DNA repair is not a new one. It is the ability to selectively differentiate between tumor cells and normal dividing cells. There is already evidence in the clinical combination of DNA-methylating agents and AGT inhibitors that this problem will need to be addressed in order to achieve an increase in the therapeutic index of existing DNA-damaging drugs. Related to this issue of selectivity, research needs to be done to catalog the susceptibility of tumor stem cells to DNA-repair inhibitors since this is the critical cell population that must be eliminated for successful therapy.

Clearly, the upregulation of DNA-repair pathways through genetic and/or epigenetic changes is only one mechanism that allows tumor cells to avoid the toxicity of DNA-damaging agents. Regardless of the challenges and the potential pitfalls, the need to develop approaches to overcome drug resistance remains absolutely critical to improving cancer survival.

Key Terms

Resistance	Ability of selected cancer cells to survive drug treatment
DNA repair	Error free or error prone processing of DNA damage
Polymerase β	Key polymerase enzyme in base excision repair pathway
DNA damage	Lesions in DNA generated by anticancer drugs

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Executive summary

Background

- The development of drugs to prevent or limit tumor cell resistance is critical to improve long-term cancer survival.

Cancer resistance

- Many anticancer drugs initially work very effectively but eventually fail, because tumors reoccur due to drug resistance.
- Rather than the continuous cycle of developing new drugs, including those that specifically target cancers via nontoxic mechanism, and watching them fail due to resistance, we need to predict potential pathways for resistance and develop drugs to block them.
- Resistant cells may not be readily observable due to their number, but it should be assumed that they are present in tumors and this should be reflected in treatment therapies.

Tumor resistance to DNA damage

- The efficient repair of the DNA damage induced by genotoxic drugs (and ionizing radiation) by populations of cells within a tumor is a druggable target to address drug resistance.
- The targets to inhibit DNA repair should be chosen to reflect the nature of the lesions induced by the drugs used in chemotherapy and the potential resistance pathways.

DNA repair inhibitors

- The screening of existing chemical libraries has already provided many interesting candidate molecules that efficiently and selectively inhibit DNA repair, including some that act synergistically with DNA-damaging drugs. One example of this is the development of PARP inhibitors that create a synthetic lethal combination in breast tumor cells lacking functional BRCA-1 or -2. Unfortunately, nontarget tissue (e.g., hematopoietic system) is also sensitized to the drugs so the issue of specificity must be addressed.

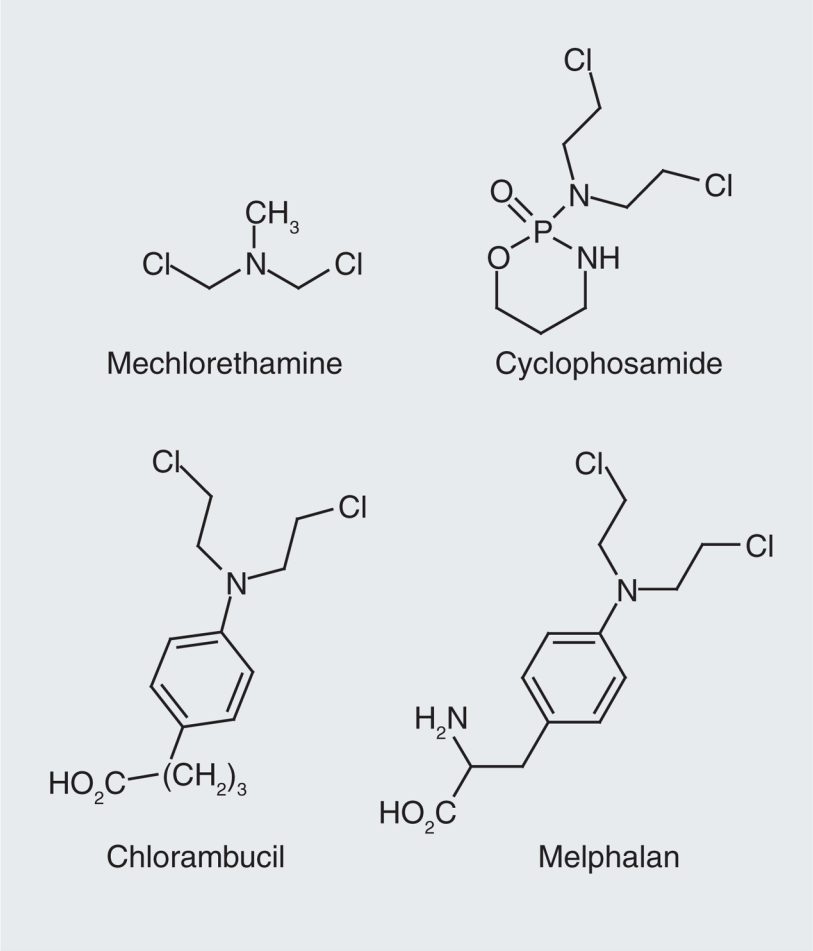

Figure 1.
Initial nitrogen mustard-based anticancer drug (mechlorethamine) and some clinically used analogues.

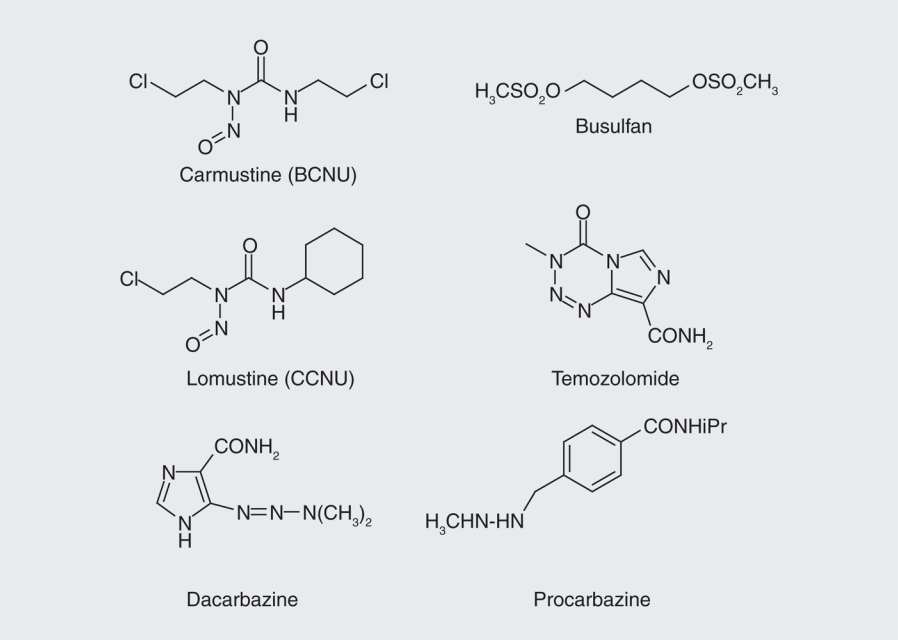

Figure 2.
Classes of clinically used alkylating anticancer drugs: nitrosourea, triazene and sulfonate ester.

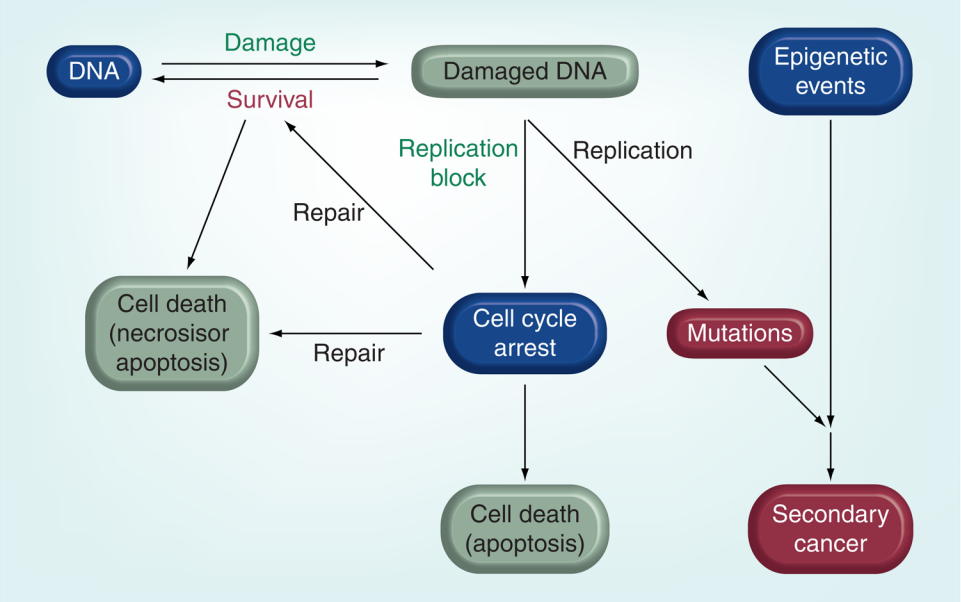

Figure 3. Biological effects of DNA damage and DNA repair

DNA damage blocks processivity of DNA polymerases, which induces cell cycle arrest due to the formation of stalled replication forks. DNA repair can occur during cell cycle arrest, which is a survival pathway, but persistent stalled replication forks eventually give rise to single-strand and double-strand breaks that lead to cell death by apoptosis. Persistent lesions can result in mutations and possibly secondary cancers due to error prone repair or translesion synthesis by low-fidelity polymerases. DNA repair can also lead to cell death due to overactivation of PARP, which results in depletion of cellular levels of ATP. The processes in green are desired effects for successful chemotherapy and red are undesirable effects.

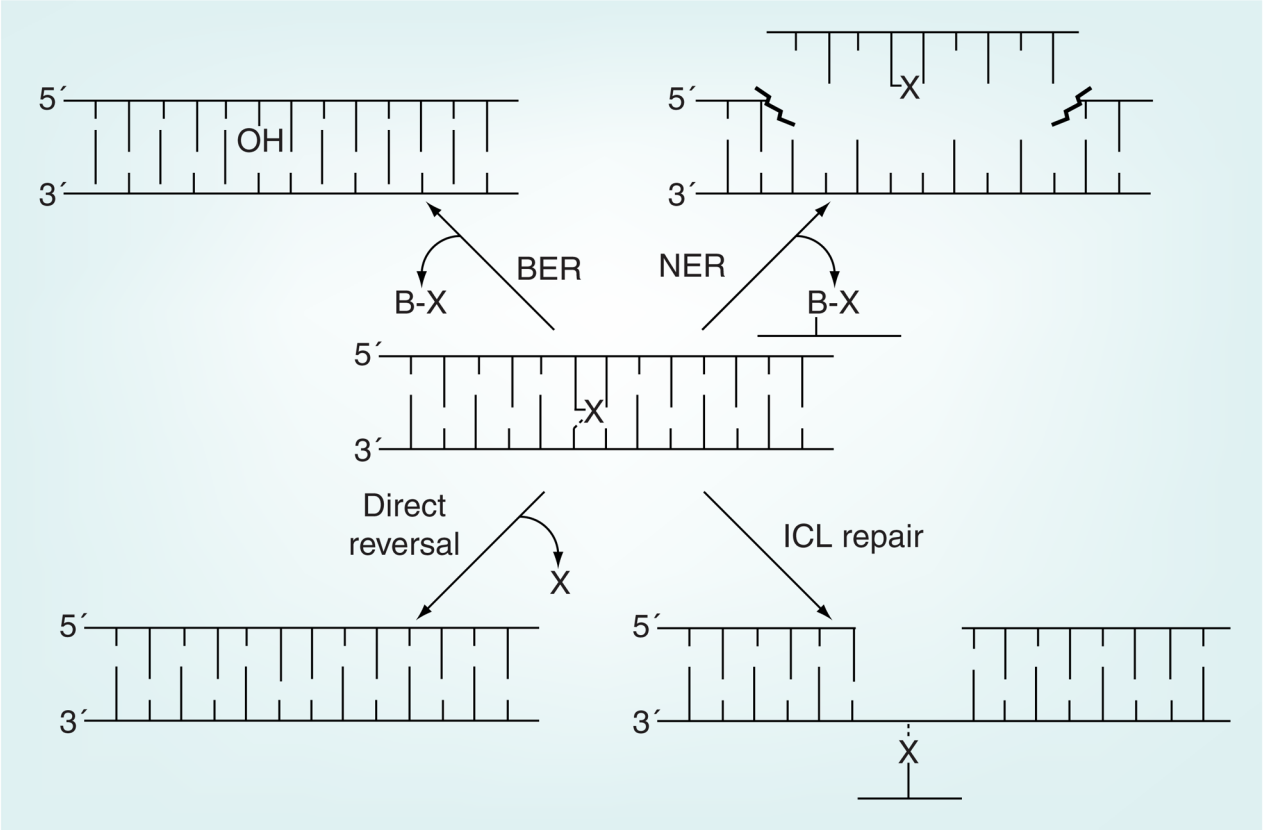

Figure 4. The four major pathways for the repair of DNA damaged by anticancer drugs

BER: in the initial step the adducted base is excised off the DNA backbone, leaving an abasic site that is sequentially processed by a number of enzymes (see Figure 5). NER: the lesion, which generally has a helix distorting effect, is initially recognized by proteins that then recruit endonucleases that cut a segment out of DNA including the lesion. The gap is filled in by DNA polymerase and the ends ligated. Direct reversal: the alkyl lesion is directly transferred from the DNA nucleobases to the repair protein in a one-step suicide reaction. ICL repair: in the initial step the crosslink is recognized by enzymes associated with NER and then one end of the crosslink is unhooked by endonucleases. The remaining lesion (shown in the figure) is then bypassed by a translesion polymerase. The remaining monofunctional lesion is then processed as in NER. If the repair occurs during DNA replication, potential errors made during the error-prone bypass step can be corrected by homologous recombination in which a strand from the sister chromatid serves as an undamaged template.

BER: Base excision repair; ICL: Interstrand crosslink; NER: Nucleotide excision repair; X: Monofunctional or crosslinked lesion.

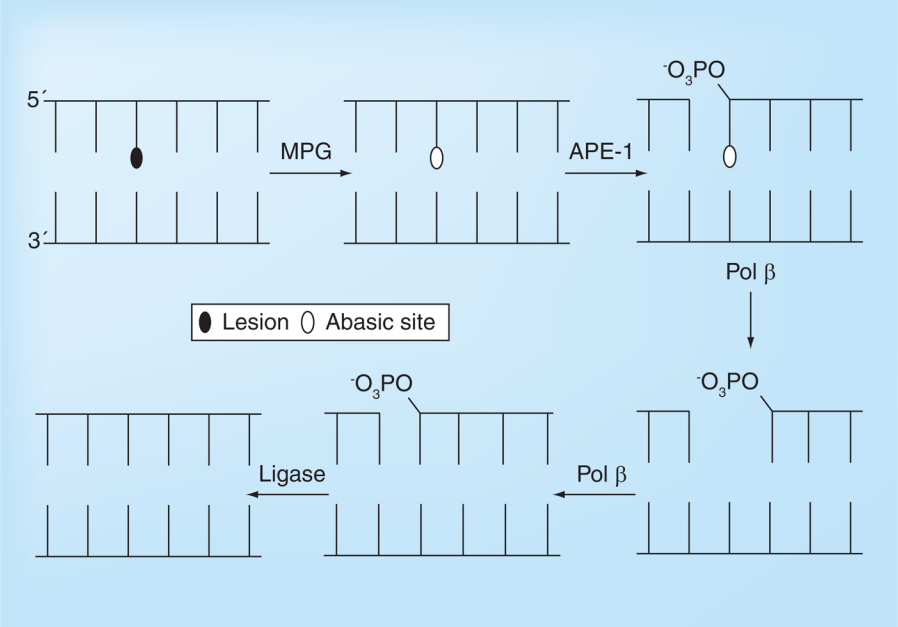

Figure 5. Short patch base excision repair involving sequential processing of lesion by *N*-methylpurine-DNA glycosylase, APE-1, Pol β and DNA ligase

In long patch repair (not shown), the polymerase fills in the gap and displaces a number of bases (2–10) leaving a dangling flap, which is digested by FEN-1.

MPG: *N*-methylpurine-DNA glycosylase.

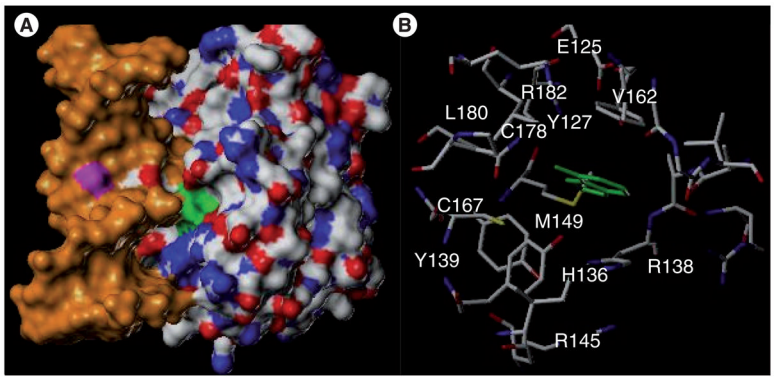

Figure 6. *N*-methylpurine-DNA glycosylase

(A) Structure of human *N*-methylpurine-DNA glycosylase in the presence of DNA (orange) and the extrahelical hypoxanthine modification (green) buried in the enzyme's active site.
(B) View of hypoxanthine (green) substrate in active site of *N*-methylpurine-DNA glycosylase.
Adapted from PDB entry: 1EWN [58].

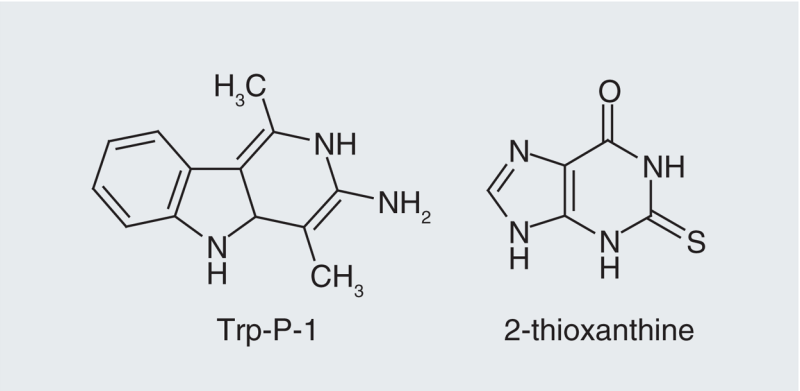

Figure 7.
Small-molecule inhibitors of *N*-methylpurine-DNA glycosylase.

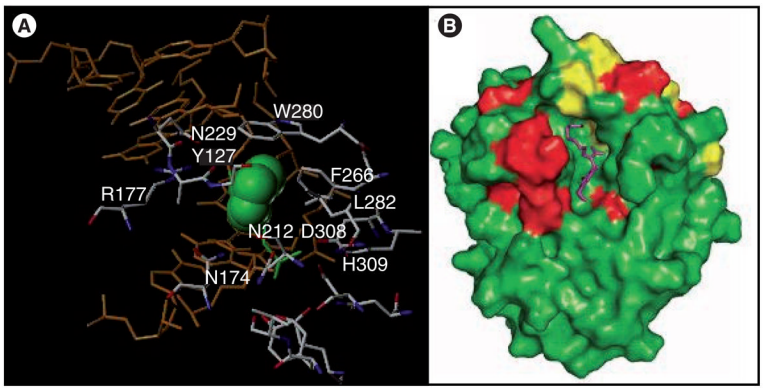

Figure 8. Human APE-1 protein

(A) The amino acids that flank the enzymatic site of APE-1 are shown along with the DNA (orange) and the abasic site (green) adapted from PDB entry 1DEW [76]. **(B)** The proposed binding of E3330 (purple; see Figure 9 for structure) to APE-1 based on NMR studies (red and yellow represent amino acid residues whose chemical shifts change by >0.01 and >0.02 ppm, respectively, when E3330 is present) [84].

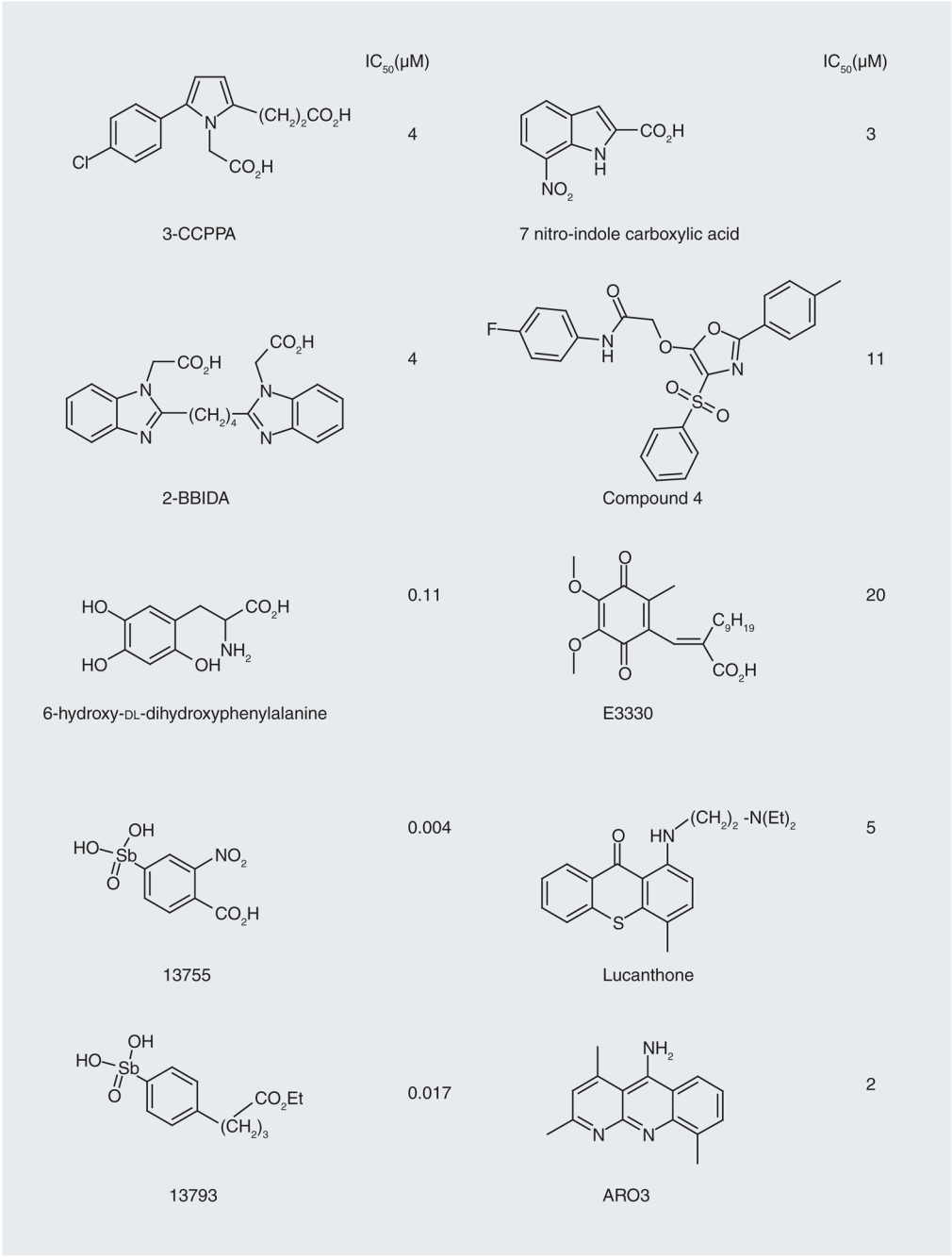

Figure 9.
APE-1 inhibitors and their reported *in vitro* IC_{50} values.

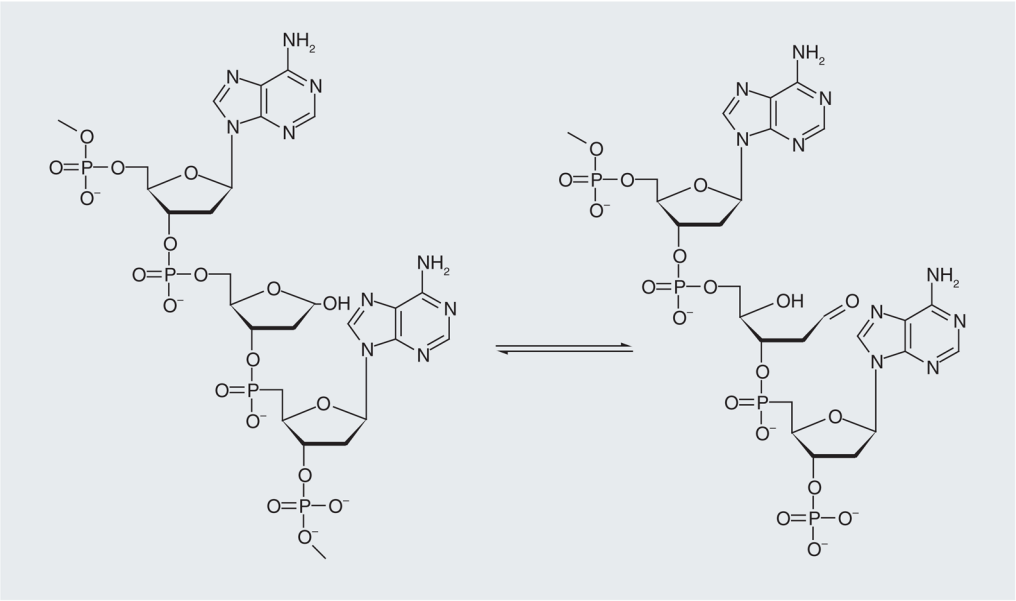

Figure 10.
Abasic lesion; cyclic and ring-opened form.

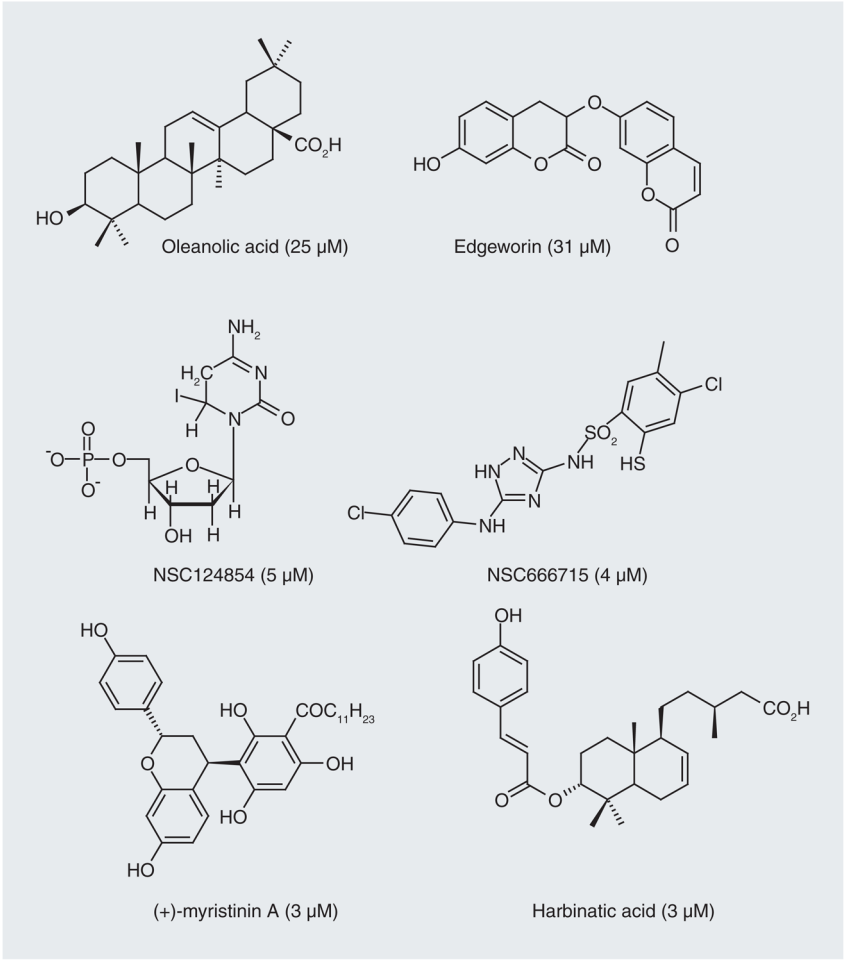

Figure 11. Inhibitors of DNA polymerase b and their reported IC_{50} values in parentheses
Different assays were used to generate the IC_{50} values.

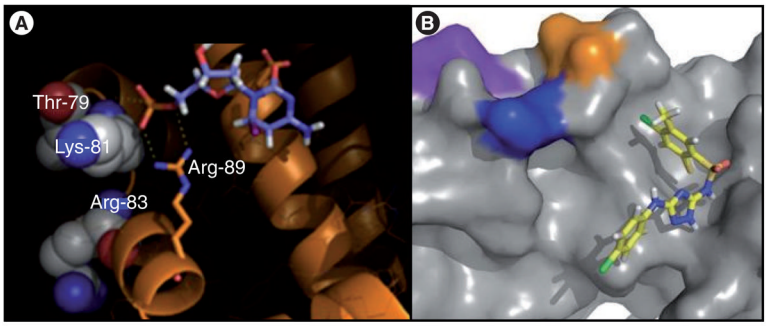

Figure 12. Models of inhibitors docked with Pol β

(A) NSC124854 (Figure 11) docked to Pol β via salt bridge between phosphate and Arg residue and hydrophobic contacts with heterocycle [102]; **(B)** NSC666715 docked in the same region of the protein (Lys-81: blue; Thr-79: orange; Arg-83: purple) [101].

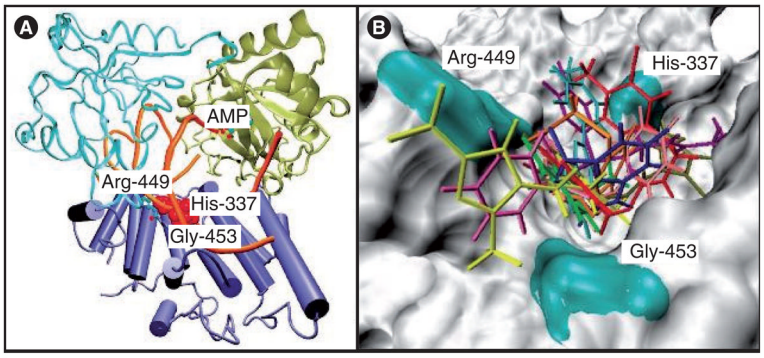

Figure 13. DNA Ligase-1

(A) Structure of the DNA-binding domain of DNA ligase-I with the predicted binding site in red and residues His-337, Arg-449 and Gly-453 that constitute the potential binding pocket. **(B)** Predicted docking of some of the ligase inhibitors (Figure 14) with the DNA-binding domain [112].

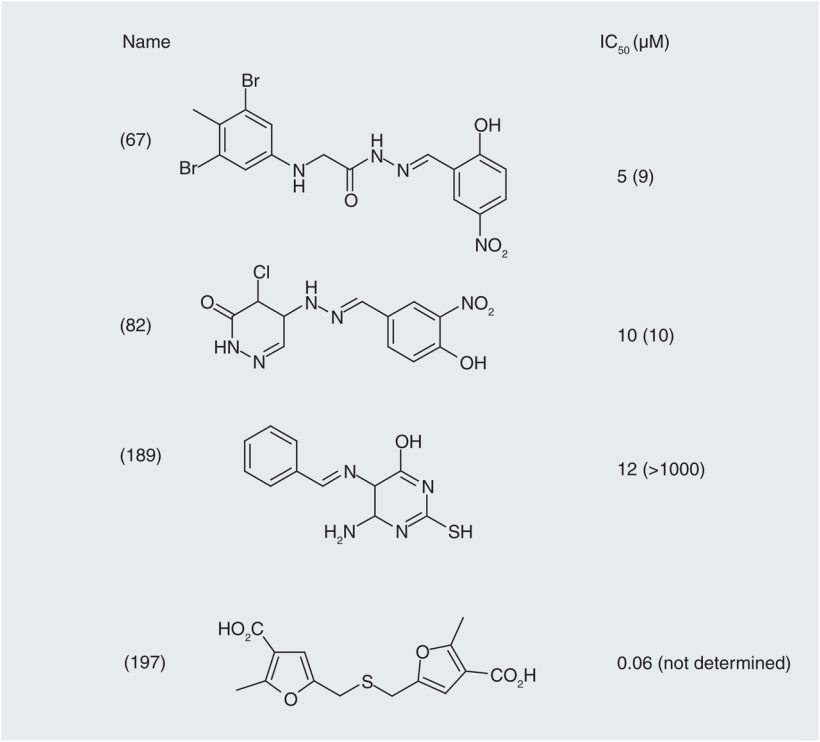

Figure 14.
DNA human ligase inhibitors against ligase-I and -III with *in vitro* IC_{50} values (μM) in parasentheses.

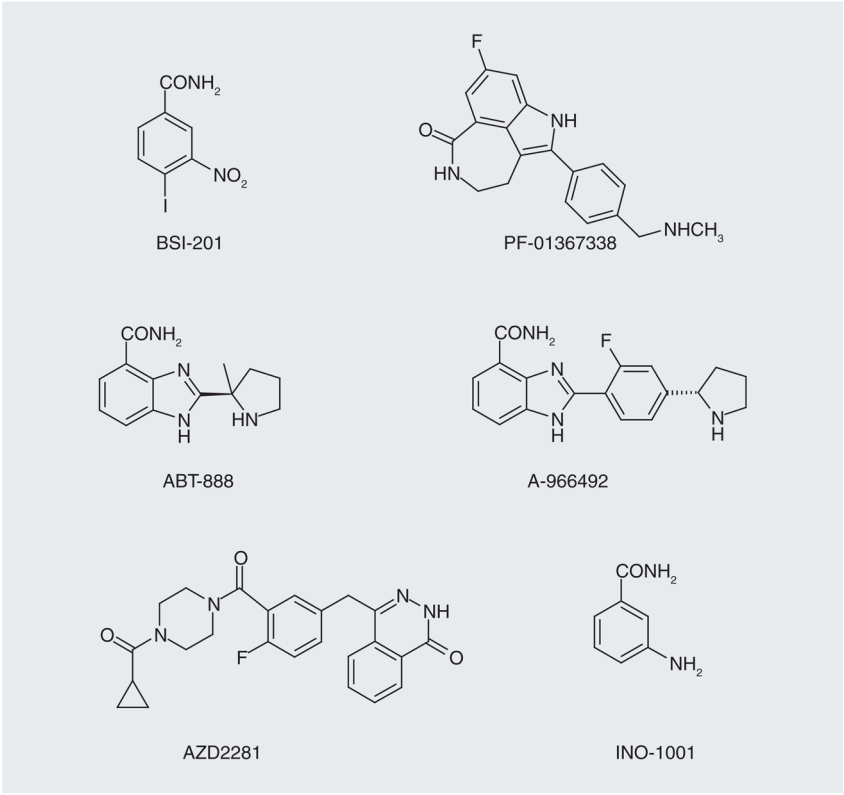

Figure 15.
Nanomolar inhibitors of PARP-1 activity.

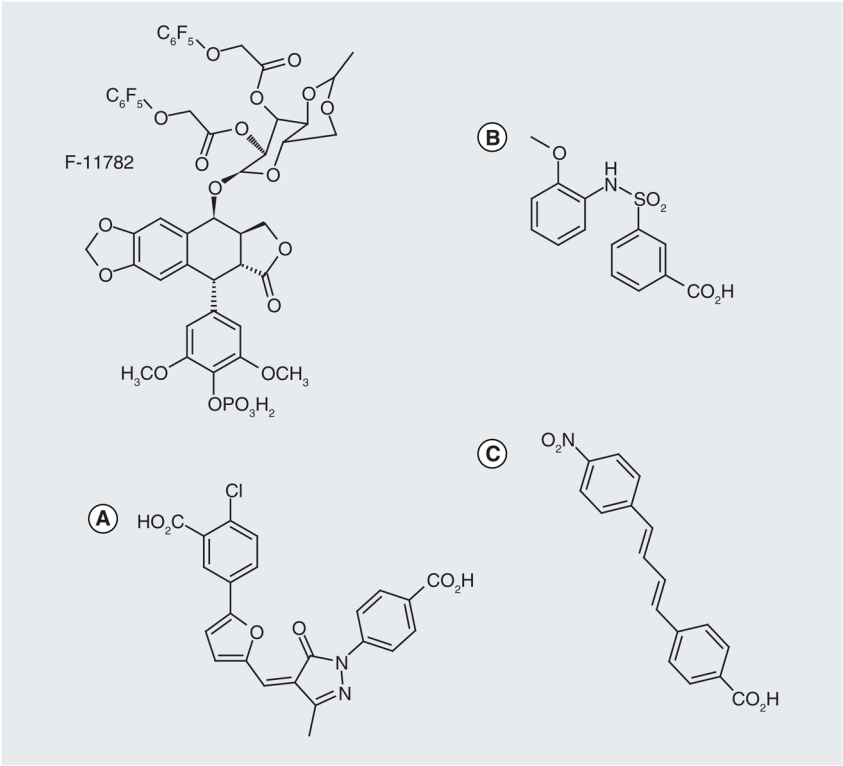

Figure 16. Small-molecule inhibitors of nucleotide excision repair

(A) Structure of nucleotide excision repair inhibitor F-11782 (formulated as the *N*-methyl-$_D$-glucamine salt) and XPA inhibitors **(B & C)**.

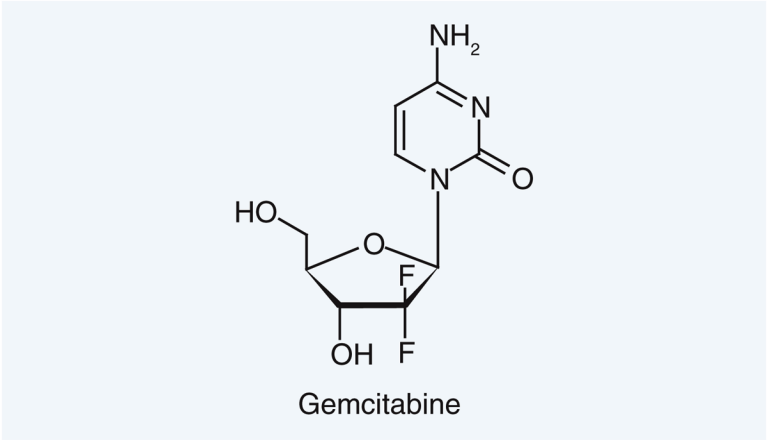

Figure 17.
Gemcitabine, which inhibits repair of cisplatin intrastrand crosslinks.

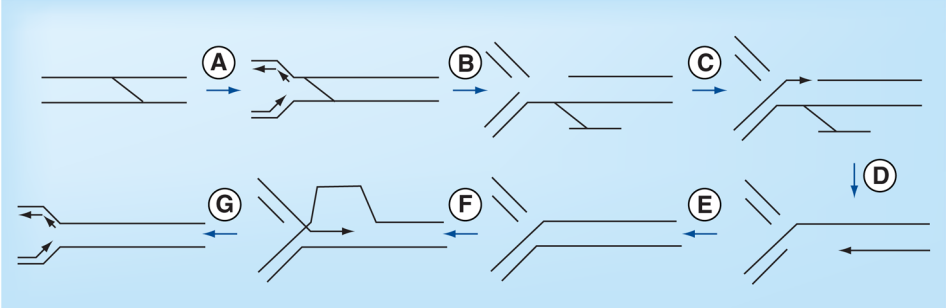

Figure 18. Pathway for interstrand crosslink repair

(A) The interstrand crosslink represents a block to polymerization; **(B)** one end of the crosslink is excised to yield a double strand break terminus, a gap and a lesion connected to the excised DNA; **(C)** the remaining unhooked monofunctional lesion is bypassed by translesion synthesis polymerase; **(D)** the remaining unhooked lesion is excised by nucleotide excision repair proteins; **(E)** polymerase fills in the gap in the DNA **(F)** homologous recombination is used to provide a template for DNA synthesis; **(G)** after resolution of the Holiday junction the DNA is fully repaired.

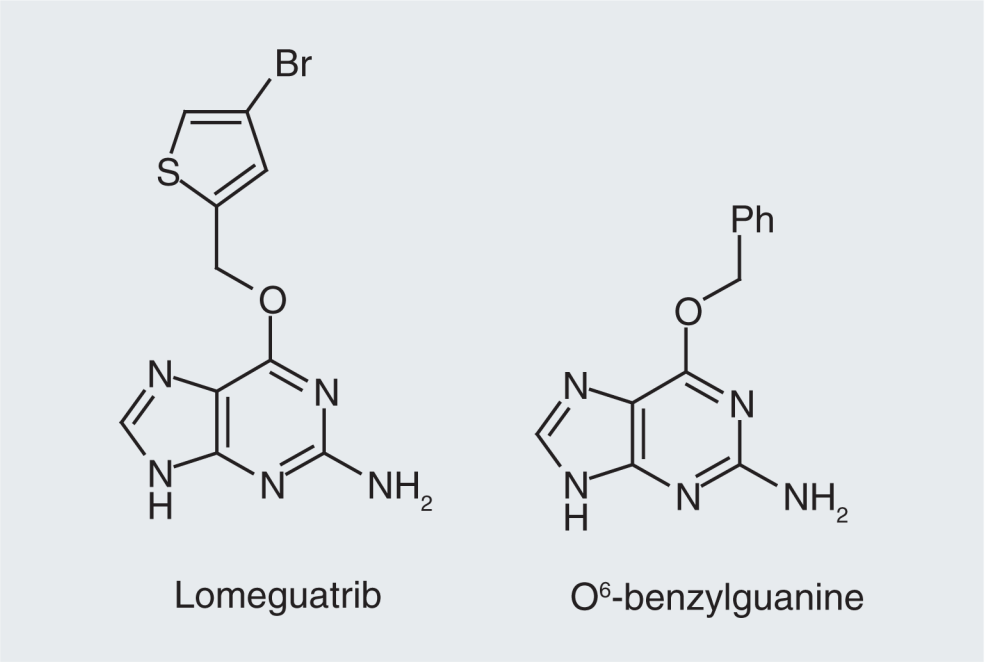

Figure 19.
Inhibitors of O^6- alkylguanine alkyltransferase.