

Rational Design of Human DNA Ligase Inhibitors that Target Cellular DNA Replication and Repair

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Abstract

Based on the crystal structure of human DNA ligase I complexed with nicked DNA, computer-aided drug design was used to identify compounds in a database of 1.5 million commercially available low molecular weight chemicals that were predicted to bind to a DNA-binding pocket within the DNA-binding domain of DNA ligase I, thereby inhibiting DNA joining. Ten of 192 candidates specifically inhibited purified human DNA ligase I. Notably, a subset of these compounds was also active against the other human DNA ligases. Three compounds that differed in their specificity for the three human DNA ligases were analyzed further. L82 inhibited DNA ligase I, L67 inhibited DNA ligases I and III, and L189 inhibited DNA ligases I, III, and IV in DNA joining assays with purified proteins and in cell extract assays of DNA replication, base excision repair, and nonhomologous end-joining. L67 and L189 are simple competitive inhibitors with respect to nicked DNA, whereas L82 is an uncompetitive inhibitor that stabilized complex formation between DNA ligase I and nicked DNA. In cell culture assays, L82 was cytostatic whereas L67 and L189 were cytotoxic. Concordant with their ability to inhibit DNA repair *in vitro*, subtoxic concentrations of L67 and L189 significantly increased the cytotoxicity of DNA-damaging agents. Interestingly, the ligase inhibitors specifically sensitized cancer cells to DNA damage. Thus, these novel human DNA ligase inhibitors will not only provide insights into the cellular function of these enzymes but also serve as lead compounds for the development of anticancer agents. [Cancer Res 2008;68(9):3169–77]

Introduction

Under normal circumstances, the genome is propagated and maintained by the combination of a highly accurate DNA replication machinery and a network of DNA repair pathways. The increased incidence of cancer associated with DNA repair-deficient human syndromes illustrates the role of these pathways in protecting against deleterious genetic changes that contribute to cancer formation. Conversely, many cancer therapeutic agents

exert their cytotoxic effects by damaging DNA. Unfortunately, these agents also kill normal cells, thereby limiting their utility. There is growing interest in the identification of DNA repair inhibitors that will enhance the cytotoxicity of DNA-damaging agents because combinations of DNA-damaging agents and DNA repair inhibitors have the potential to concomitantly increase the killing of cancer cells and reduce damage to normal tissues and cells if either the damaging agent or the inhibitor could be selectively delivered to the cancer cells (1).

Because DNA ligation is required during replication and is the last step of almost all DNA repair pathways, DNA ligase-deficient cell lines exhibit sensitivity to a wide range of DNA-damaging agents (2). Thus, DNA ligase inhibitors are predicted to have pleiotropic effects on cell proliferation and sensitivity to DNA damage. Human cells contain multiple species of ATP-dependent DNA ligase encoded by three genes, *LIG1*, *LIG3*, and *LIG4* (2). Although these enzymes have a conserved catalytic domain and use the same reaction mechanism, they are directed to participate in different DNA transactions by specific protein-protein interactions (2). To date, experimental screening of a synthetic chemical collection and a natural product library has led to the identification of several compounds that inhibit human DNA ligase I (hLigI) *in vitro*, although these compounds have not been fully characterized in terms of their specificity and mechanism of action (3, 4).

A problem with the screening of random chemical libraries for DNA ligase inhibitors is that many of the hits are likely to be nonspecific inhibitors that either bind to the DNA substrate or are nucleotide analogues that inhibit a large number of ATP-dependent enzymes. Recently, a crystal structure of hLigI complexed with nicked DNA substrate was determined (5). Notably, this structure revealed three domains of hLigI that encircle and contact the nicked DNA. In addition to the adenylation (AdD) and OB-fold (OBD) domains that constitute the catalytic core of DNA and RNA ligases as well as other nucleotidyl transferases, hLigI has a DNA-binding domain (DBD) located NH₂-terminal to the catalytic core that is a conserved feature of eukaryotic DNA ligases (5).

Using the atomic resolution structure of hLigI complexed with nicked DNA (5), a rational approach using computer-aided drug design (CADD) was taken to identify potential inhibitors of hLigI by virtual screening of a database of commercially available, low molecular weight chemicals. Subsequent experimental evaluation of the candidate inhibitors led to the identification and characterization of novel inhibitors with different specificities for human DNA ligases I, III, and IV.

Materials and Methods

CADD screening. A DNA-binding pocket between residues Gly⁴⁴⁸, Arg⁴⁵¹, and Ala⁴⁵⁵ of the hLigI DBD (5) was chosen as the target for CADD (6–10). Details of the *in silico* screening will be described elsewhere.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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A total of 233 compounds were selected for biochemical and biological assays.

Chemicals. Compounds identified by CADD screening were purchased from ChemBridge, ChemDiv, MayBridge, MDD, Nanosyn, Specs, Timtec, and Tripos. L189 was from Specs, and L82 and L67 were from Chemdiv. Ten-millimolar stocks were prepared in DMSO and stored at -20°C . The molecular mass and purity of L67, L82, and L189 were confirmed by mass spectrometry in the University of Maryland School of Pharmacy facility.

Proteins. Purification of human DNA ligases is described in the Supplementary Material. T4 DNA ligase was purchased from NEB.

DNA joining assays. Candidate ligase inhibitors identified by CADD were assayed for their ability to inhibit hLigI and T4 DNA ligase using a high-throughput, fluorescence energy transfer-based DNA joining assay (11). Duplicate reactions (30 μL) containing 10 pmol of nicked DNA substrate and either 0.25 pmol of hLigI or 10 units of T4 DNA ligase were incubated in the presence or absence of 100 $\mu\text{mol/L}$ of the putative inhibitor.

A radioactive gel-based DNA ligation assay was performed as previously described (11). A 25-mer (5'-CGC CAG GGT TTT CCC AGT CAC GAC C-3'), and a 5'-[^{32}P] end-labeled 18-mer (5'-GTA AAA CGA CGG CCA GTG-3') were annealed to a complementary 44-mer oligonucleotide, generating a linear duplex with a central nick. DNA joining reactions (30 μL) containing 0.5 pmol of labeled DNA substrate, and hLigI (0.02 pmol), hLigIII β (0.02 pmol), hLigIV/XRCC4 (0.1 pmol), or T4 DNA ligase (0.02 pmol) in ligation buffer were incubated in the absence or presence of ligase inhibitors at 25°C for 30 min.

Assays for steps 2 and 3 of the ligation reaction. To analyze step 2 of the ligation reaction, labeled ligase-AMP intermediates (ref. 11; 10 pmol) were incubated overnight at 25°C with an unlabeled nonligatable version (dideoxy residue at the 3'-terminus of the nick) of the DNA oligonucleotide substrate (10 pmol), either in the presence or absence of the ligase inhibitors (100 $\mu\text{mol/L}$).

To analyze step 3 of the ligation reaction, an adenylated labeled version of the 18-mer was prepared as described (12). The DNA substrate containing a preadenylated nick (0.5 pmol) and hLigI (0.05 pmol), hLigIII β (0.05 pmol), hLigIV/XRCC4 (0.1 pmol), or T4 DNA ligase (0.05 pmol) were incubated in ligation buffer without ATP, either in the presence or absence of the ligase inhibitors (100 $\mu\text{mol/L}$). Reactions were stopped by the addition of an equal volume of gel loading dye (95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol). After heating at 95°C for 5 min, DNA was separated by denaturing polyacrylamide gel electrophoresis. Labeled oligonucleotides were detected and quantitated in the dried gel by phosphorImager analysis (Molecular Dynamics).

Kinetic analysis of ligase inhibitors. To measure the initial rates of ligation, hLigI (0.05 pmol) was incubated with 0.5 to 100 pmol of the fluorescent, nicked DNA substrate and various concentrations of the ligase inhibitors. K_i values were obtained from Lineweaver-Burk double reciprocal plots and curve-fitting using PRISM v3.03 (GraphPad).

Electrophoretic mobility shift assay. A labeled linear duplex with a nonligatable nick was incubated with hLigI in ligation buffer (30 μL total volume) with or without ligase inhibitors for 120 min at 25°C . After the addition of an equal volume of native gel buffer [160 mmol/L Tris-HCl (pH 6.8), 20% glycerol, 1.4 mol/L 2-mercaptoethanol, and 0.05% bromophenol blue], samples were separated by electrophoresis through a 12% native polyacrylamide gel and detected in the dried gel by phosphorImager analysis.

Cell extract assay of DNA replication and repair. Extracts were prepared from HeLa cells as described previously (13, 14). For base excision repair (BER) assays, the extraction buffer contained 100 mmol/L of KCl, whereas for nonhomologous end-joining (NHEJ) assays, extraction buffer contained 400 mmol/L of KCl. Where indicated, DNA ligases were immunodepleted from the extracts as described (15) using protein A or G Sepharose beads (GE Healthcare) and anti-LigI, anti-LigIII (GeneTex), or anti-LigIV (ABCAM) antibodies. Depletion was confirmed by immunoblotting.

A labeled 5'-flap substrate (0.1 pmol; ref. 14) was incubated with 20 μg of extract in the absence or presence of ligase inhibitors (100 $\mu\text{mol/L}$) at

25°C for 5 min in ligation buffer (final volume, 50 μL). For short patch BER, a linear duplex containing a single uracil residue was preincised by treatment with uracil DNA glycosylase and APE1 (both from NEB) to generate a strand break with 3' hydroxyl and 5' deoxyribose phosphate termini. Reactions (50 μL) containing 0.3 pmol of the incised DNA substrate, 10 μCi of [$\alpha^{32}\text{P}$]dTTTP, and 20 μg of extract either in the absence or presence of ligase inhibitors (100 $\mu\text{mol/L}$) were incubated at 25°C for 2 min in ligation buffer. After separation by denaturing polyacrylamide gel electrophoresis, labeled oligonucleotides were detected in the dried gel by phosphorImager analysis (Molecular Dynamics).

To assay NHEJ (13), a 1 kb end-labeled *Bam*HI fragment (0.1 pmol; ref. 16), and 20 μg of extract were incubated in ligation buffer (final volume, 20 μL), for 120 min at 25°C either in the presence or absence of ligase inhibitors (100 $\mu\text{mol/L}$). DNA fragments were resolved by separation through a 0.8% agarose gel. Labeled DNA fragments were detected in the dried gel by phosphorImager analysis (Molecular Dynamics).

Cell culture assays. The culture conditions for normal human breast epithelial MCF10A cells, human colon cancer HCT116 cells, human cervical cancer HeLa cells, and human breast cancer MCF7 cells are described in the Supplementary Material. Assays to measure cell proliferation and survival and the effect of the ligase inhibitors on cell cycle progression assays were carried out as described in the Supplementary Material.

Immunocytochemistry. The effect of ligase inhibitors on the subcellular distribution of tubulin was examined by fluorescence microscopy as described in the Supplementary Material.

Results

In silico screening for putative DNA ligase inhibitors.

Because the DBD is the predominant DNA-binding activity within hLigI (5), and both the AdD and OBD are likely to undergo significant conformational changes during the ligation reaction (2), we chose a DNA-binding pocket between residues Gly⁴⁴⁸, Arg⁴⁵¹, and Ala⁴⁵⁵ of the DBD (Fig. 1A) for the initial CADD screen. A database of 1.5 million commercially available, low molecular weight chemicals was subjected to an *in silico* screen for molecules that may bind within the DNA-binding pocket using the program DOCK (6–10). From this virtual screen, a total of 233 compounds were selected for biochemical and biological assays.

***In vitro* screening of putative hLigI inhibitors.** Of the 233 compounds selected from the *in silico* screen, 192 compounds were available from commercial sources. Using a fluorescence-based DNA joining assay (11), these compounds were assayed at 100 $\mu\text{mol/L}$ for their ability to inhibit purified hLigI (Fig. S1). To eliminate compounds that nonspecifically inhibit DNA joining, the same 192 compounds were also assayed for their ability to inhibit T4 DNA ligase, an enzyme that uses the same reaction mechanism as hLigI, has similar AdD and OBDs but lacks a DBD (2, 5). Under these conditions, 10 compounds inhibited hLigI activity by >50% but had no significant effect on T4 DNA ligase. Thus, the overall hit rate for specific inhibitors of hLigI from the *in silico* database screen was $\sim 5\%$.

Effect of hLigI inhibitors on DNA joining by human DNA ligases III and IV. Because the DNA-binding pocket within the DBD of hLigI that was used as the target for CADD is likely to be conserved in human DNA ligases III (hLigIII) and IV (hLigIV), we determined whether the inhibitors of hLigI also inhibit DNA joining by purified hLigIII β and hLigIV/XRCC4 (Fig. S1). Based on the inhibition profile of each compound for the three human DNA ligases, the 10 compounds originally identified as inhibitors of hLigI were divided into three groups, and one member of each group was chosen for further analysis. The chemical structures and predicted binding of the three chosen compounds, L67, L82, and

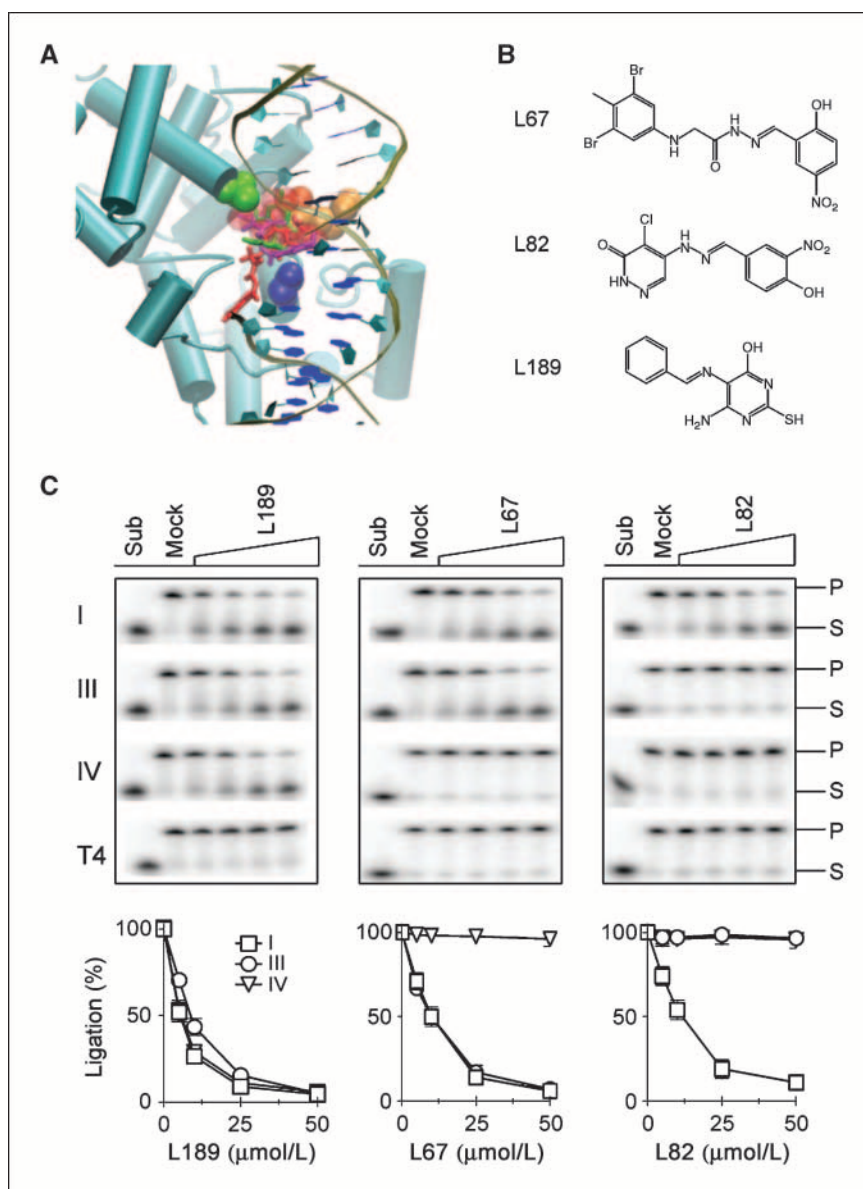


Figure 1. Small molecule inhibitors of human DNA ligases identified by CADD. **A**, key residues in the DNA-binding pocket, Gly⁴⁴⁸ (green), Arg⁴⁵¹ (orange), and Ala⁴⁵⁵ (blue), within the hLigI DBD (aqua ribbon format) in VDW representation with the nicked DNA in cartoon format. The sphere set used to direct the docking of small molecules (red transparent spheres). Docked orientations of the three characterized compounds: L67 (purple), L82 (red), and L189 (green). **B**, chemical structures of L67, L82, and L189. **C**, representative gels of DNA ligation assays. The results of three independent experiments are shown graphically. For clarity, the data for T4 DNA ligase, which was not significantly inhibited, was omitted (hLigI, □; hLigIIIβ, ○; hLigIV/XRCC4, ▽).

L189 to the DNA-binding pocket within the DBD of hLigI, are shown in Fig. 1. L189 inhibited hLigI, hLigIIIβ, and hLigIV/XRCC4; L67 inhibited hLigI and hLigIIIβ; and L82 only inhibited hLigI (Fig. 1C). The IC₅₀ values of L67, L82, and L189 for the three human DNA ligases and T4 DNA ligase are shown in Table S1.

Effect of the ligase inhibitors on the three steps of the ligation reaction. The ligase inhibitors had no detectable effect on formation of the covalent enzyme-AMP intermediate, which occurs independently of the DNA substrate and involves the active site lysine residue within the AdD of DNA ligases (17), by the three human DNA ligases (Fig. S2). To examine the second step of the ligation reaction, DNA ligases with a covalently linked labeled AMP moiety were incubated with an unlabeled linear DNA substrate containing a single nonligatable nick (Fig. 2A). Transfer of the labeled AMP moiety to the 5'-phosphate terminus of the nick results in the formation of a labeled DNA-adenylate intermediate. In accord with the results of the DNA joining assays (Fig. 1C), L189 inhibited step 2 of the reaction catalyzed by all three human DNA ligases by at least 90%, but had only a minor effect on T4 DNA

ligase. DNA-adenylate formation by hLigI was inhibited by L189 in a concentration-dependent manner with an estimated IC₅₀ of ~7 μmol/L (Fig. S3). Although less effective than L189, L67 and L82 also inhibited step 2 of the reaction and reiterated the specificity for the human DNA ligases observed in the DNA joining assays (Fig. 1C). L67, which inhibited DNA joining by hLigI and hLigIIIβ but not hLig IV/XRCC4 (Fig. 1C), was more effective at inhibiting DNA-adenylate formation by hLigI and hLigIIIβ compared with hLig IV/XRCC4 or T4 DNA ligase (Fig. 2A). Similarly, L82, which specifically inhibited DNA joining by hLigI, was more effective at inhibiting DNA-adenylate formation by this enzyme compared with hLigIII, hLigIV, and T4 DNA ligase (Fig. 2A).

In the third and final step of the ligation reaction, the nonadenylated enzyme catalyzes phosphodiester bond formation, releasing AMP. Similar to the second step, L67, L82, and L189 generated the same pattern of inhibition of the third step of the ligation reaction catalyzed by the three human DNA ligases and T4 DNA ligase (Fig. 2B) as was observed in the DNA joining assays (Fig. 1C). L67 and L82 were more effective inhibitors of step 3 than

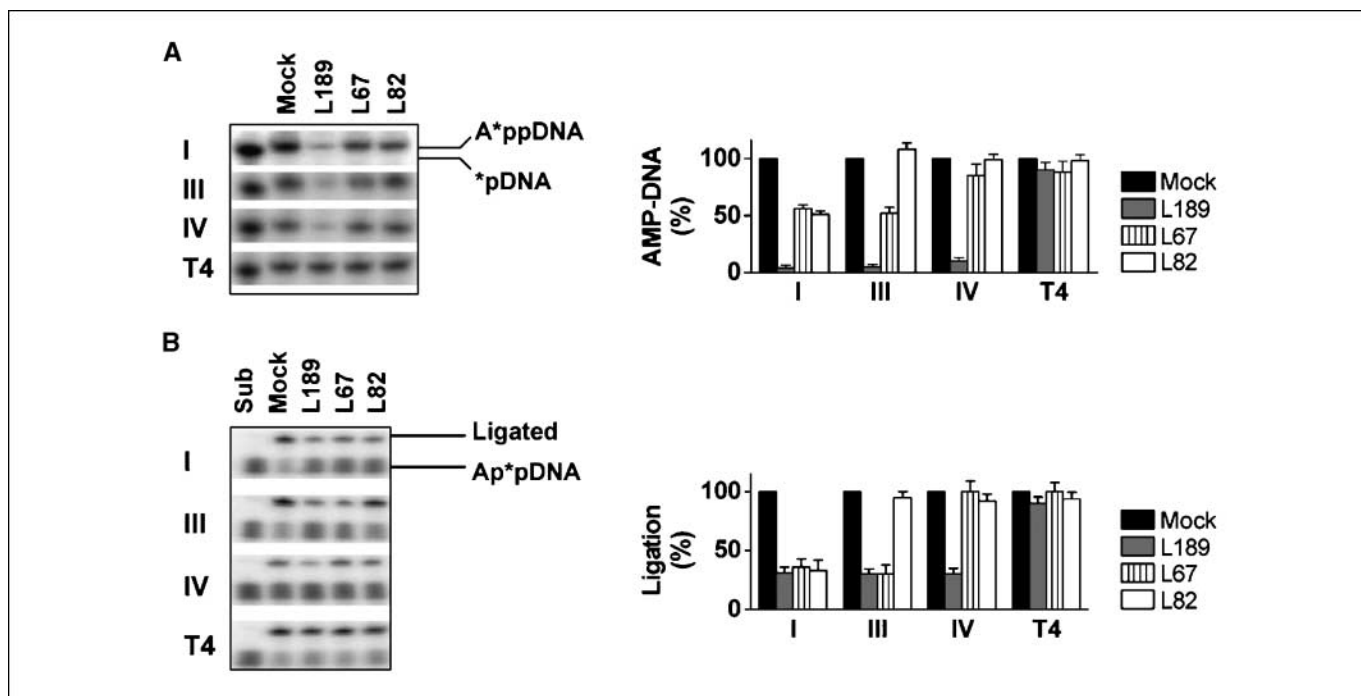


Figure 2. Effect of the ligase inhibitors on the second and third steps of the ligation reaction. *A*, formation of the DNA adenylate reaction intermediate. Labeled ligase-adenylate forms of hLigI (*I*), hLigIII β (*III*), hLigIV (*IV*), and T4 (*T4*) DNA ligase were incubated with a linear DNA substrate containing a single nonligatable nick in the absence or presence of L67, L82, and L189. *Left*, the positions of the labeled 19-nucleotide DNA-adenylate (A^*ppDNA) and a 5' end-labeled 18-mer oligonucleotide ($*pDNA$). *Right*, the results of two independent experiments. Formation of DNA-adenylate is expressed as a percentage of DNA-adenylate formed by the DNA ligase in the absence of inhibitor. *B*, phosphodiester bond formation. hLigI (*I*), hLigIII β (*III*), hLigIV (*IV*) and T4 (*T4*) DNA ligase were incubated with labeled linear DNA molecule containing a single ligatable nick with 3'-hydroxyl and 5'-adenylate termini in the absence (*Mock*) or presence of L67, L82, and L189. *Left*, the positions of the labeled 19-nucleotide DNA-adenylate (Ap^*pDNA) and labeled ligated product (43-mer). *Right*, the results of two independent experiments. Ligation is expressed as a percentage of DNA joining by the DNA ligase in the absence of inhibitor.

step 2, whereas the opposite was the case for L189. Thus, as expected, the inhibitors target the steps of the ligation reaction in which the enzyme interacts with nicked DNA.

Kinetic analysis of the inhibition of DNA joining: effect of the inhibitors on binding to nicked DNA. To further characterize the inhibition of hLigI, we measured the kinetics of DNA joining in the absence or the presence of L67, L82, and L189. For L67 (Fig. 3A, *middle*) and L189 (Fig. 3A, *left*), the resulting Lineweaver-Burk plots indicate that these compounds are simple competitive inhibitors with respect to the DNA substrate, with K_i values of 10 and 5 $\mu\text{mol/L}$, respectively. Because a DNA-binding pocket within the DBD of hLigI was used for the *in silico* modeling, it seems likely that L67 and L189 compete with DNA for binding to this site. To provide direct evidence for this, we performed electrophoretic mobility shift assays using a linear DNA molecule containing a nonligatable nick. As predicted, the addition of L189 (Fig. 3B) reduced the amount of hLigI-DNA complex. Similar results were obtained with the DBD of hLigI (Fig. S4). L67 was much less effective than L189 at inhibiting formation of the hLigI-DNA complex (data not shown). This may reflect the fact that L189, which is a better inhibitor of step 2 than step 3 of the ligation reaction (Fig. 2), acts at an earlier stage in the interaction with DNA than L67, which is a better inhibitor of step 3 than step 2. The inhibitory effect of L67 on step 3 of the ligation reaction was reduced by increasing the concentration of DNA-adenylate, confirming that L67 is a competitive inhibitor (Fig. S5).

The Lineweaver-Burk plots obtained with L82 (Fig. 3A, *right*) are strikingly different than those obtained with L67 (Fig. 3A, *middle*) and L189 (Fig. 3A, *left*), and indicate that this compound acts

uncompetitively. In accord with the prediction that L82 binds specifically to the enzyme-substrate complex, L82 increased the amount of hLigI-nicked DNA complex formed in a concentration-dependent manner (Fig. 3C). These results provide evidence that the inhibitors interact with the DBD and inhibit ligation either by blocking DNA-binding (L67 and L189) or stabilizing a reaction intermediate (L82).

Effect of ligase inhibitors on cell extract assays of DNA replication and repair. Cell extract assays for DNA replication and various DNA repair pathways have been developed and used to identify and purify the protein factors involved in these DNA transactions (13, 18–20). To determine whether L67, L82, and L189 retain their activity and specificity in cell extracts, we examined DNA replication and repair pathways for which there is an established cell extract assay (13, 21–23), and the DNA ligase involved has been identified by complementary genetic and biochemical evidence (13, 21, 24–27). During Okazaki fragment processing and the long patch subpathway of BER, short 5' single-strand flaps are removed and the resulting nicks ligated by the sequential action of FEN-1 and hLigI. In Fig. 4A, we show the processing and joining of a DNA flap substrate, which mimics a common pathway intermediate, by a HeLa cell extract. As expected, immunodepletion of hLigI but not hLigIII α reduced the amount of ligated product (Fig. 4A, compare *lanes 2, 6, and 7*). The addition of L67, L82, and L189, each of which inhibits purified hLigI (Fig. 1C), reduced the amount of ligated product (Fig. 4A, *lanes 3–5*). Notably, an intermediate generated by flap removal accumulated in these reactions, and the reaction with the extract immunodepleted hLigI, indicating that the ligase inhibitors do not inhibit FEN-1.

The DNA ligase III α /XRCC1 complex is the predominant DNA ligase activity responsible for completing the short patch subpathway of BER following insertion of a single nucleotide and removal of the 5' deoxyribose phosphate by DNA pol β (27). In Fig. 4B, we show the processing and joining of a pathway intermediate, a DNA duplex with an incised abasic site, by a HeLa extract. As expected, immunodepletion of hLigIII α , but not hLigI, reduced the amount of the ligated product (Fig. 4B, lanes 1, 5, and 6). L67 and L189, which are active against hLigIII, inhibited the formation of the repaired product (Fig. 4B, lanes 2 and 3), whereas, L82, the hLigI-specific inhibitor, had no effect (Fig. 4B, lane 4). The ligase inhibitors had no detectable effect on gap-filling synthesis by pol β which generates a labeled 31-mer (Fig. 4B). Similar results were obtained with DNA substrates containing larger gaps that were filled by DNA pol δ and/or pol ϵ (data not shown). Thus, the ligase inhibitors do not negatively affect gap-filling synthesis by the major DNA polymerases involved in DNA replication and excision repair.

NHEJ is the major pathway for the repair of DNA double-strand breaks in human cells. In a cell extract assay that measures joining of cohesive-ended linear DNA fragments and is dependent on the key NHEJ factors, Ku, DNA-PKcs, and hLigIV/XRCC4 (13), L189, the only inhibitor with activity against hLigIV (Fig. 1C), was the only compound to inhibit end-joining by the HeLa cell extract (Fig. 4C, compare lane 4 with lanes 5 and 6). As expected, immunodepletion of hLigIV abolished end-joining (Fig. 4C, lane 9) whereas immunodepletion of either hLigI or hLigIII α had no significant effect (Fig. 4C, lanes 7 and 8), confirming that the majority of DNA joining events were catalyzed by hLigIV/XRCC4. Together, these results show that the ligase inhibitors retain their specificity for the different species of DNA ligases in cell extracts and thus can be used to determine the contributions of

individual human DNA ligase(s) to DNA repair pathways in cell extracts or partially purified fractions.

Effects of ligase inhibitors on cultured human cells. If L67, L82, and L189 enter human cells, it is likely that they will inhibit DNA proliferation and may be cytotoxic because they all inhibit hLigI, the major replicative DNA ligase. As shown in Fig. 5A, each of the compounds reduced the proliferation and/or viability of four human cell lines, including a normal breast epithelial cell line MCF10A and the cancer cell lines MCF7, HeLa, and HCT116 established from breast, cervical, and colon cancers, respectively, in a concentration-dependent manner. In colony-forming assays, L67 and L189 were cytotoxic (Figs. 5B and 6A), whereas L82 was cytostatic, reducing the size but not the number of colonies formed by MCF7 (Fig. 5B) and the other cell lines (data not shown).

To determine the effects of L82 on cell cycle progression, asynchronous MCF7 cells were enriched for the G₀-G₁ phase by serum starvation and then released into serum-containing medium either with or without 50 μ M of L82. In the presence of L82, there was a transient accumulation of cells at G₂-M after 12 hours followed by an accumulation at G₀-G₁ that peaked after 24 hours and then remained relatively stable (Fig. 5C). Because the increase in the G₀-G₁ fraction occurred concomitantly with a decrease in the S phase cell fraction (Fig. 5C), it seems that the cytostatic activity of L82 is primarily due to activation of the G₁-S checkpoint. In accord with this conclusion, >80% of the cells treated with 50 μ M of L82 had enlarged nuclei and a single centrosome, morphologic characteristics of G₀-G₁ cells (Fig. 5D, compare top and bottom). Moreover, deformed nuclei and abnormal spindle separation occurred in cells treated with 10 μ M of L82 (Fig. 5D, middle) even though this lower concentration had much less of an effect on cell cycle distribution (data not shown).

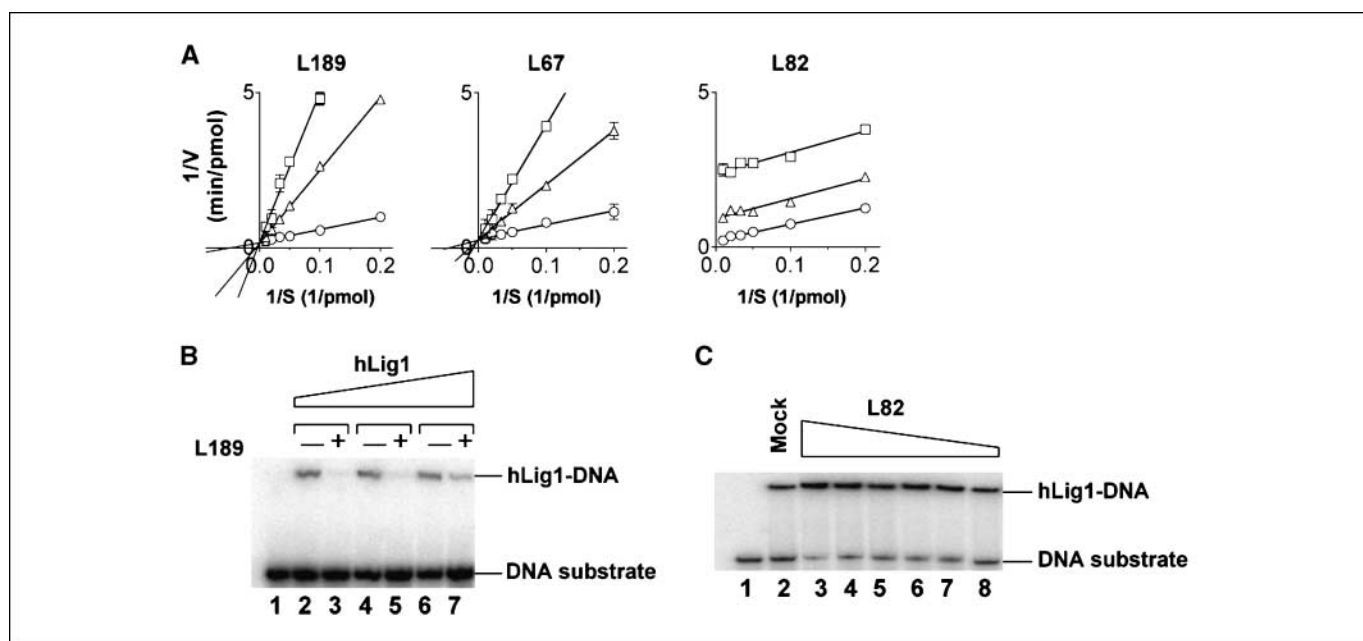


Figure 3. Michaelis-Menten analysis of ligase inhibitors. Effect of ligase inhibitors on DNA-protein complex formation by hLigI. A, hLigI (0.05 pmol) was incubated in the absence (○) and presence of L189 (left), L67 (middle), and L82 (right) at 25 μ M (Δ) and 50 μ M (\square) with increasing amounts of a linear nicked DNA substrate. Lineweaver-Burk double reciprocal plots of initial reaction velocity ($1/V$) versus substrate concentration ($1/S$). B, a labeled linear substrate with a single nonligatable nick (1 pmol) was incubated with no addition (lane 1), 0.25 pmol of hLigI (lanes 2 and 3), 0.5 pmol of hLigI (lanes 4 and 5), and 1 pmol of hLigI (lanes 6 and 7) in the absence (-) or presence (+) of 100 μ M of L189. C, a labeled linear substrate with a single nonligatable nick (1 pmol) and hLigI (3 pmol) were incubated with either no addition (lane 2) or 100 μ M of L189 (lane 3), 60 μ M of L189 (lane 4), 50 μ M of L189 (lane 5), 30 μ M of L189 (lane 6), 20 μ M of L189 (lane 7), or 10 μ M of L189 (lane 8) of L82. Lane 1, 1 pmol of DNA substrate alone. The positions of the labeled DNA substrate and DNA-protein complexes are indicated.

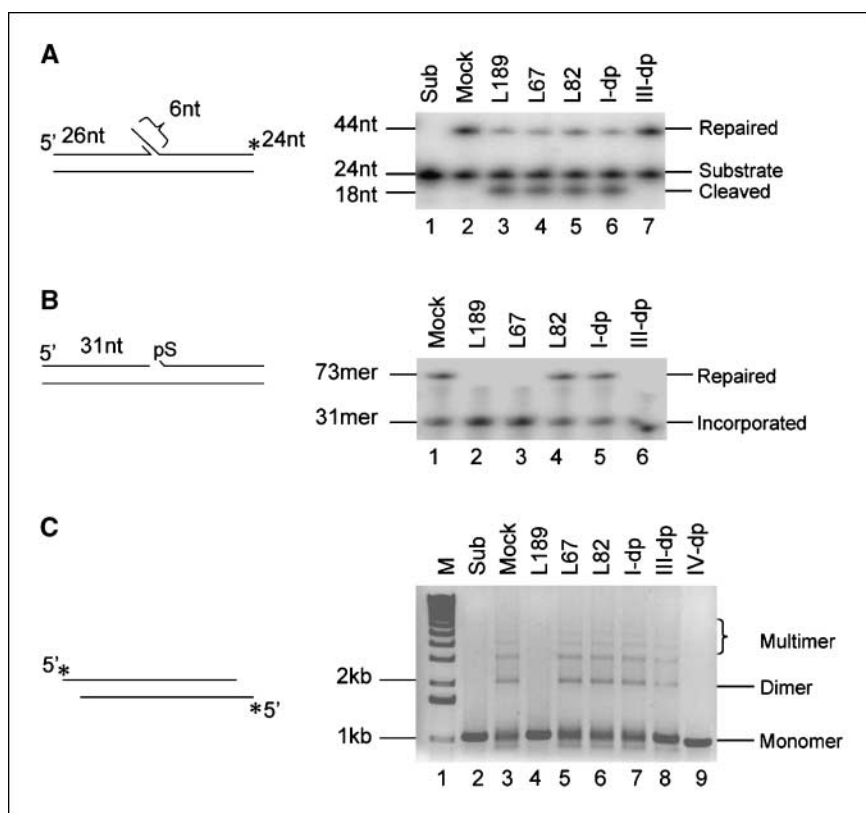


Figure 4. Effect of ligase inhibitors on replication and repair reactions catalyzed by human cell extracts. **A**, the flap substrate (0.1 pmol) was incubated with cell extract (20 μ g) in the absence (lane 2, *Mock*) or presence of 100 μ mol/L of L189 (lane 3), L67 (lane 4), or L82 (lane 5). hLigI (lane 6, *I-dp*) and hLigIII α (lane 7, *III-dp*) were immunodepleted from the cell extracts prior to incubation with the DNA substrate. Lane 1, DNA substrate alone (*Sub*). The positions of the DNA substrate (24-mer), cleaved product (18-mer), and fully repaired product (43-mer) are indicated. **B**, the linear DNA substrate with an incised AP site (0.3 pmol) was incubated with a cell extract (20 μ g) and [α^{32} P]dTTP in the absence (lane 1, *Mock*) or presence of 100 μ mol/L of L189 (lane 2), L67 (lane 3), or L82 (lane 4). hLigI (lane 5, *I-dp*) and hLigIII α (lane 6, *III-dp*) were immunodepleted from the cell extracts prior to incubation with the DNA substrate. The positions of the single nucleotide insertion reaction intermediate (31-mer, *Incorporated*) and the ligated product (73-mer, *Repaired*) are indicated. **C**, a 1 kb fragment with cohesive ends (0.1 pmol) was incubated with cell extract (20 μ g) in the absence (lane 3, *Mock*) or presence of 100 μ mol/L of L189 (lane 4), L67 (lane 5), or L82 (lane 6). hLigI (lane 7, *I-dp*), hLigIII α (lane 8, *III-dp*), and hLigIV (lane 9, *IV-dp*) were immunodepleted from the cell extracts prior to incubation with the DNA substrate. Lane 1, molecular mass standard (*M*). Lane 2, DNA substrate alone (*Sub*). The positions of the DNA substrate and dimers and multimers of the substrate are indicated.

In contrast with the cytostatic activity of L82, treatment of normal and cancer cells with either L67 or L189 (Fig. 6A) resulted in a concentration-dependent reduction in cell survival. Cells with a DNA content of less than 2n, indicative of apoptosis, were detected by fluorescence-activated cell sorting after treatment with either L67 or L189. This was confirmed by the detection of the chromosome fragmentation pattern characteristic of apoptotic cell death (data not shown).

Because DNA ligase-deficient mammalian cells exhibit increased sensitivity to DNA damage (2), we determined whether exposure to subtoxic concentrations of ligase inhibitors potentiate the cytotoxic effects of DNA-damaging agents. L67 markedly increased the killing of MCF7 breast cancer cells by the DNA alkylating agent, methyl methane sulfonate (Fig. 6B, left). Similar results were obtained with the other cancer cell lines, HCT116 and HeLa (data not shown). Notably, the presence of L67 had no obvious effect on the sensitivity of normal breast epithelial MCF10A cells to DNA alkylation (Fig. 6B, left). L189 also enhanced the killing of cancer cell lines but not the normal cell line by MMS (data not shown). Furthermore, L189 markedly increased the killing of HCT116 colon cancer cells (Fig. 6B, right) and the other cancer cell lines (data not shown) by ionizing radiation. Once again, the ligase inhibitor had very little effect on the sensitivity of normal breast epithelial MCF10A cells to DNA damage induced by ionizing radiation (Fig. 6B, right). Similarly, L67 enhanced the killing of the cancer cell lines but not the normal cell line by ionizing radiation (data not shown).

The ability of ligase inhibitors to specifically sensitize cancer cells to DNA damage prompted us to compare the levels of DNA ligases in the normal and cancer cell lines. As expected (28), the level of hLigI was elevated in the cancer cell lines compared with the normal cell line (Fig. 6C). Interestingly, there were changes in

the levels of hLigIII α and hLigIV in the cancer cell lines compared with the normal cell line (Fig. 6C). Specifically, the levels of hLigIII α were elevated, whereas the levels of hLigIV were markedly reduced in the three cancer cell lines, suggesting that these reciprocal changes in the levels of hLigIII α and hLigIV may be a characteristic feature of cancer cells.

Discussion

There is emerging interest in the development and use of therapeutics that target DNA repair pathways in the treatment of cancer (1). Because almost all DNA repair pathways are completed by a ligation event, the DNA ligases encoded by the three human *LIG* genes are attractive therapeutic targets. In this study, we have identified novel small molecule inhibitors of the human DNA ligases using CADD. Specifically, 1.5 million commercially available low molecular weight compounds were screened *in silico* for their ability to potentially bind to a DNA-binding pocket within the DBD domain of hLigI (5). Notably, 5% of the compounds identified by the CADD screen inhibited DNA joining by hLigI but not T4 DNA ligase, confirming the utility of this structure-based approach.

In accord with the selection of the DBD of hLigI as the target for CADD, the three compounds that have been most extensively characterized inhibit the second and third steps of the ligation reaction in which the enzyme interacts with nicked DNA. Interestingly, L189 preferentially inhibits step 2 whereas the other two compounds, L67 and L82, preferentially inhibit step 3. This may reflect differences in the conformation of the DBD and/or the position of the DNA substrate during steps 2 and 3 of the ligation reaction. Moreover, these results raise the possibility of generating step 2-specific and step 3-specific inhibitors that could be used to

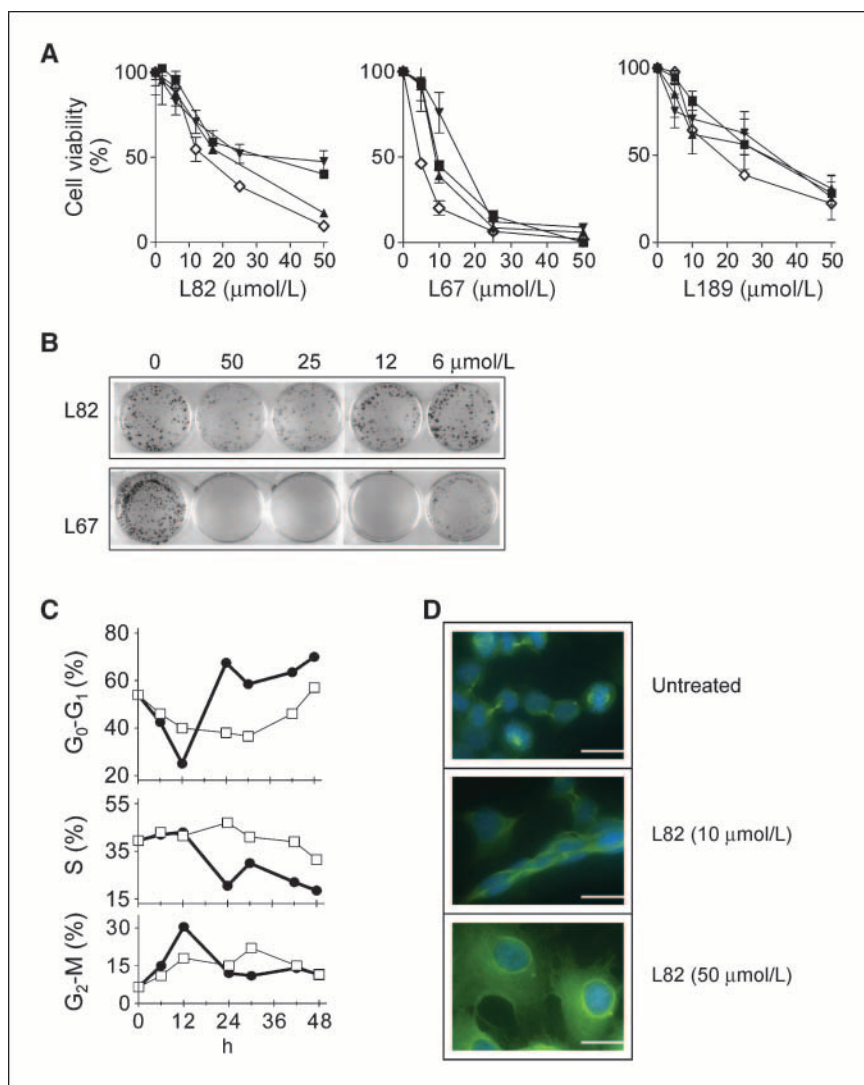
gain more detailed insights into the ligation reaction. As expected, two of the compounds, L67 and L189, were competitive inhibitors with respect to the nicked DNA substrate, indicating that their binding to the DBD of hLigI prevents interaction with nicked DNA. In contrast, L82 is an uncompetitive inhibitor, analogous to the prototypic topoisomerase I inhibitor camptothecin (29, 30). Camptothecin and other topoisomerase I inhibitors function as uncompetitive inhibitors by stabilizing the covalent linkage of topoisomerase I with the cleaved DNA, simultaneously binding to topoisomerase I and stacking within DNA base pairs adjacent to the cleavage site (31, 32). Because both human topoisomerase I and hLigI encircle and interact with nicked DNA (33), it is possible that L82 simultaneously contacts the DNA and DBD within the context of the ring structure formed by hLigI on nicked DNA.

Because the DBD is conserved among human DNA ligases, we examined whether the hLigI inhibitors were also active against hLigIII and hLigIV. Examples of compounds that were either specific for hLigI (L82), specific for hLigI and hLigIII (L67), or inhibited all the human DNA ligases (L189) were identified. These results raise the possibility that the CADD approach may identify compounds that are specific for hLigIII and hLigIV. The availability of inhibitors that target each of the human DNA ligases will

facilitate studies to identify the DNA ligase(s) in cell extracts participating in different DNA transactions. This has been problematic because of the larger repertoire of DNA ligases in mammals compared with lower eukaryotes, in particular, the multiple isoforms encoded by the *LIG3* gene (2) and the absence of viable *lig3* mutant cell lines (34). Indeed, recent studies have implicated DNA ligase III α in nucleotide excision repair (35) and an alternative NHEJ pathway (36), in addition to its previously known roles in the repair of DNA single-strand breaks and the short patch subpathway of BER (37).

Inhibitors that block ligation *in vivo* will be valuable reagents for elucidating the cellular functions of human DNA ligases. L67, L82, and L189, each of which inhibit hLigI, the replicative DNA ligase, all reduced cell proliferation and viability, indicating that they cross the cell membrane. Surprisingly, although L67 and L189 were cytotoxic, L82 was cytostatic. Because L82 is specific for hLigI, it is possible that hLigIII α , which is also inhibited by L67 and L189, is required for cell viability either in the presence of normal levels of hLigI or specifically when hLigI activity is reduced. Alternatively, the differing effects of the inhibitors *in vivo* may reflect the uncompetitive mode of inhibition by L82 compared with L67 and L189, which are competitive inhibitors.

Figure 5. Characterization of the cytostatic effect of L82. **A**, MCF10A (■), MCF7 (◇), HCT116 (▲), and HeLa (▼) cells were plated in the absence or presence of L82 (left), L67 (middle), and L189 (right). After 6 days, cell viability was measured and is expressed as a percentage of the value obtained with untreated cells. **B**, MCF7 cells were plated out in the absence or presence of L82 (top) and L67 (bottom) at the indicated concentrations. After 2 weeks, colonies were stained with crystal violet. **C**, after serum starvation for 4 days, MCF 7 cells were returned to serum-containing media either without (□) or with 50 μ mol/L of L82 (●). The cell cycle distribution at various time intervals was determined by fluorescence-activated cell sorting. **D**, asynchronous populations of MCF cells were either untreated (top) or treated with L82 at 10 μ mol/L (middle) and 50 μ mol/L (bottom). After 3 days, tubulin and DNA were visualized by fluorescence microscopy (bars, 0.5 mm).



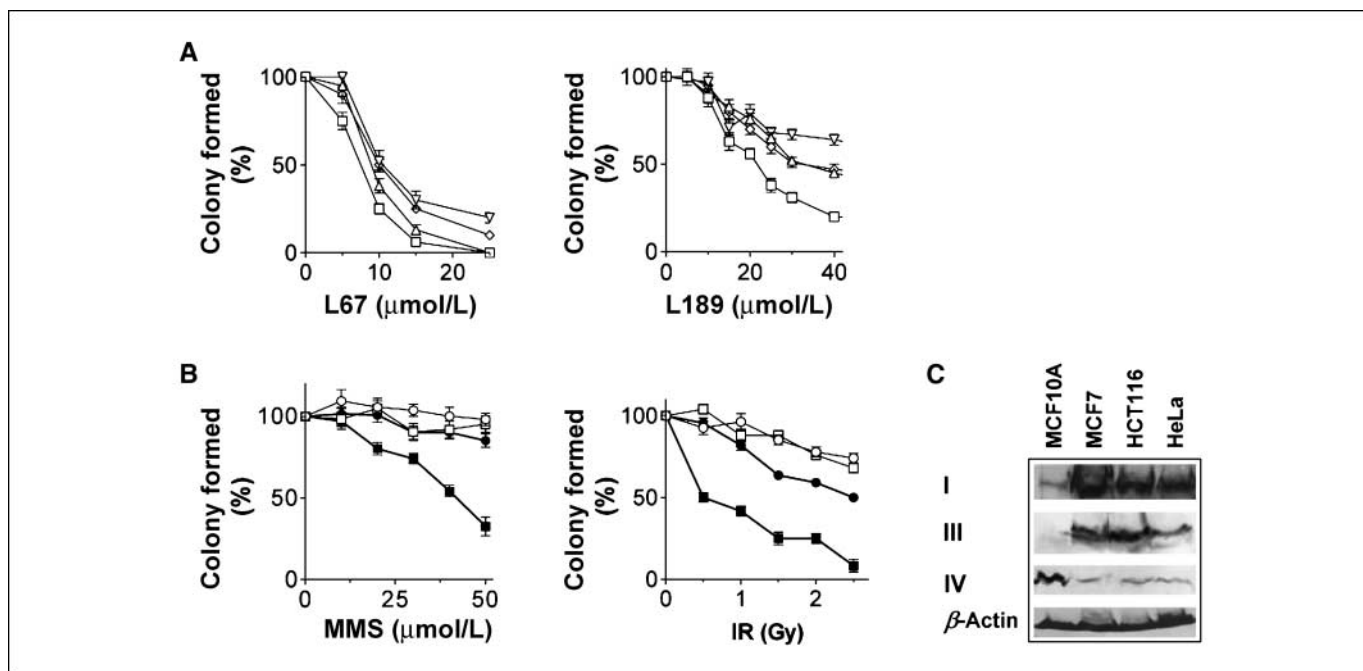


Figure 6. L67 and L189 are cytotoxic and potentiate the cytotoxic effects of DNA-damaging agents: altered levels of DNA ligase in cancer cells. **A**, effect of L67 (left) and L189 (right) on the survival of MCF7 (Δ), HCT116 (\square), HeLa (\diamond), and MCF10A (∇) cells. **B**, normal breast epithelial MCF10A cells (open symbols) and breast cancer MCF7 cells (filled symbols) in the absence (circles) or presence of 3 $\mu\text{mol/L}$ of L67 (squares) were exposed to increasing concentrations of MMS (left). Normal breast epithelial MCF10A cells (open symbols) and colon cancer HCT116 cells (filled symbols) in the absence (circles) or presence of 20 $\mu\text{mol/L}$ of L189 (squares) were exposed to increasing doses of ionizing radiation (right). **C**, hLigI (I), hLigIII α (III), and hLigIV (IV) were detected in extracts (400 μg) of the indicated cell lines by immunoblotting. To control for extract loading, β -actin was also detected by immunoblotting.

The hypersensitivity of DNA ligase-deficient cell lines to DNA damage (2) and the ability of the ligase inhibitors to inhibit BER and NHEJ *in vitro* suggested that subtoxic levels of DNA ligase inhibitors may significantly increase cell killing by DNA-damaging agents. In support of this idea, L67 and L189 increased DNA damage-induced cytotoxicity. Strikingly, the increased cytotoxicity occurred in cancer cell lines but not in a cell line established from normal breast epithelium. Differences in the level of DNA ligases may underlie the selective effect of the ligase inhibitors on the cancer cell lines. Because unregulated proliferation is a characteristic of cancer, it was not surprising that the level of the replicative DNA ligase, hLigI, was significantly higher in all three cancer cell lines compared with the normal breast epithelial cell line. Notably, the cancer cell lines also had elevated levels of hLigIII α but reduced levels of hLigIV. It is not known which, if any, of these changes is responsible for the specific sensitization of the cancer cells to DNA damage in the presence of a ligase inhibitor.

The reciprocal change in the levels of hLigIII α and IV was also observed in cell lines with the bcr-abl translocation established from acute myelogenous leukemias but not in comparable normal cells,⁵ suggesting that this may be a characteristic feature of malignant cells. Because NHEJ is a major pathway for repairing DNA double-strand breaks, the reduced levels of hLigIV may explain, at least in part, the increased sensitivity of cancer cells to ionizing radiation and suggest that cancer cell lines may be more susceptible to radiosensitization by a hLigIV inhibitor. In addition,

the reduced levels of DNA ligase IV may result in more DNA double-strand breaks being repaired by an error-prone DNA ligase III-dependent back-up NHEJ pathway, contributing to the increased genome instability that is a hallmark of cancer cells (38).

Differences in the network of pathways that maintain genome stability between cancer and normal cells constitute an opportunity to develop therapies that specifically target cancer cells. For example, loss of the function of either the *BRCA1* or *BRCA2* tumor suppressor genes results in defective homologous recombination (39–41). As a consequence of this abnormality, *brca* cancer cell lines are hypersensitive to killing by inhibitors of poly(ADP-ribose) polymerase (42, 43). Thus, the availability of a repertoire of inhibitors targeting different DNA repair pathways is likely to lead to the development of novel combinations of DNA-damaging agents and DNA repair inhibitors that exploit differences in the DNA repair properties of normal and cancer cells. Because of the multiplicity of human DNA ligases and the almost ubiquitous requirement for DNA joining to complete DNA repair, DNA ligase inhibitors have the potential to target one or different combinations of DNA repair pathways.

In conclusion, we have used an *in silico* screening approach based on the structure of hLigI complexed with nicked DNA to identify low molecular weight inhibitors of human DNA ligases that specifically block functional interactions between these enzymes and nicked DNA. This is not only the first example of this type of inhibitor but also the first characterization of a set of inhibitors with different specificities for the three human DNA ligases that can be used to identify the DNA ligase(s) acting in extract-based assays of replication and repair. In addition to their *in vitro* activities, the selected ligase inhibitors inhibit cell proliferation and, at subtoxic concentrations, they specifically potentiate the

⁵ F. Rassool, A. Sallmyr, and A.E. Tomkinson, unpublished results.

killing of cancer cells by DNA-damaging agents. Thus, these inhibitors are promising lead compounds for the development of novel therapeutic agents to treat human cancer.

Acknowledgments

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