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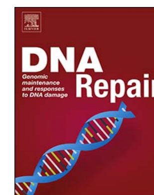

Structure-activity relationships among DNA ligase inhibitors: Characterization of a selective uncompetitive DNA ligase I inhibitor

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ABSTRACT

In human cells, there are three genes that encode DNA ligase polypeptides with distinct but overlapping functions. Previously small molecule inhibitors of human DNA ligases were identified using a structure-based approach. Three of these inhibitors, L82, a DNA ligase I (LigI)-selective inhibitor, and L67, an inhibitor of LigI and DNA ligases III (LigIII), and L189, an inhibitor of all three human DNA ligases, have related structures that are composed of two 6-member aromatic rings separated by different linkers. Here we have performed a structure-activity analysis to identify determinants of activity and selectivity. The majority of the LigI-selective inhibitors had a pyridazine ring whereas the LigI/III- and LigIII-selective inhibitors did not. In addition, the aromatic rings in LigI-selective inhibitors had either arylhydrazone or acylhydrazone, but not vinyl linkers. Among the LigI-selective inhibitors, L82-G17 exhibited increased activity against and selectivity for LigI compared with L82. Notably. L82-G17 is an uncompetitive inhibitor of the third step of the ligation reaction, phosphodiester bond formation. Cells expressing LigI were more sensitive to L82-G17 than isogenic *LIG1* null cells. Furthermore, cells lacking nuclear LigIIIα, which can substitute for LigI in DNA replication, were also more sensitive to L82-G17 than isogenic parental cells. Together, our results demonstrate that L82-G17 is a LigI-selective inhibitor with utility as a probe of the catalytic activity and cellular functions of LigI and provide a framework for the future design of DNA ligase inhibitors.

1. Introduction

DNA ligation is required to generate an intact lagging strand during DNA replication as well as in almost every recombination and DNA repair event. In human cells, this reaction is carried out by the DNA ligases encoded by the three human *LIG* genes, *LIG1*, *LIG3* and *LIG4* [1]. Genetic analysis has revealed that there is considerable functional overlap among the DNA ligases encoded by the three *LIG* genes in nuclear DNA transactions [2–11]. A mitochondrial version of DNA ligase IIIα (LigIIIα) is generated by alternative translation initiation [12–15]. In addition, alternative splicing of the *LIG3* gene in male germ cells results in LigIIIβ, which has a different C-terminal region than LigIIIα [16].

The steady state level of LigI is frequently elevated in cancer cell lines and tumor samples [17,18]. This presumably reflects the hyperproliferative state of cancer cells since LigI is the predominant ligase involved in DNA replication [19–21]. Unexpectedly, many cancer cell lines exhibit both increased steady state levels of LigIIIα and reduced steady state levels of DNA ligase IV (LigIV), with these reciprocal changes indicative of alterations in the relative contribution of different DNA double-strand break repair pathways between non-malignant and cancer cells [18,22–25]. The dysregulation of DNA ligases in cancer cells together with the involvement of these enzymes in the repair of DNA damage caused by agents used in cancer chemotherapy and radiation therapy suggests that DNA ligase inhibitors may have utility as cancer therapeutics.

A set of small molecule LigI inhibitors were identified through an *in silico* structure-based screen, using the atomic resolution structure of LigI complexed with nicked DNA [18,26]. This screen yielded inhibitors that were selective for LigI (L82), inhibited both LigI and LigIII (L67)

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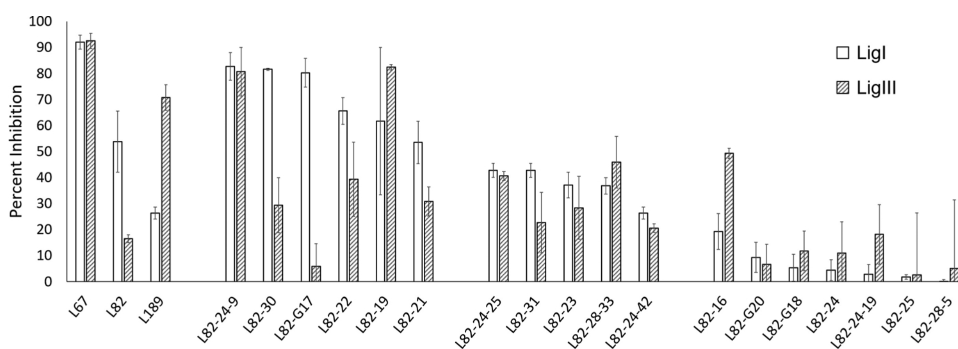

Fig. 1. Activity of compounds related to L67, L82 and L189. The effect of L67, L82, L189 and L82 derivatives (50 μM of each) on DNA joining by LigI (0.6 nM) and LigIII (1.7 nM) was measured in assays with the radiolabeled DNA substrate as described in Materials and Methods. Results are shown graphically with inhibition expressed as a percentage of ligation activity in assays with DMSO alone. The data shown, which is sorted based on activity against LigI, represents the results of at least three independent experiments.

and inhibited all three human DNA ligases (L189). As expected, subtoxic levels of the DNA ligase inhibitors enhanced the cytotoxicity of DNA damaging agents in cancer cell lines [18]. Surprisingly, non-malignant cell lines were not sensitized to DNA damage by the DNA ligase inhibitors under similar conditions, suggesting that there are alterations in genome maintenance pathways between non-malignant and cancer cells [18]. Further studies revealed that the repair of DNA double-strand breaks is abnormal in cancer cells with elevated levels of LigIIIα and PARP1 and that these cells are hypersensitive to inhibitors that target LigIIIα and PARP1 [22,23,25].

The DNA ligase inhibitors L82, L67 and L189 are similar in that they are each composed of two 6-member aromatic rings separated by different length linkers [18,26]. Here we have examined a series of related compounds in an attempt to identify determinants of activity and selectivity for LigI and LigIIIα. One of the compounds analyzed, L82-G17, is a selective, uncompetitive inhibitor of LigI. Furthermore, the activity of this compound in cell culture assays with genetically-defined cell lines indicates that it inhibits LigI function in cells.

2. Results

2.1. Biochemical activity of L82 derivatives

To gain insights into determinants of activity and selectivity of the structurally similar DNA ligase inhibitors, L67, L82 and L189 [18,26], we examined the activity of related derivatives that were either synthesized (L82-GXX) or purchased (L82-XX) on LigI, LigIII and T4 DNA ligase activity. DNA ligase IV/XRCC4 (LigIV/XRCC4) was not included in these assays as the purified enzyme, unlike LigI, LigIII and T4 DNA ligase, acts as a single turnover enzyme [27,28]. Any compounds that inhibited T4 DNA ligase, which lacks the DNA binding domain targeted in the structure-based screen [18], were presumed to be non-specific inhibitors and excluded (data not shown). The activities of the remaining compounds were compared with the previously described DNA ligase inhibitors, L82, L67 and L189 at 50 μM (Fig. 1). Among the L82 derivatives, there were 10 compounds that inhibited LigI and/or LigIII by at least 40%. In Fig. 2, the structures of L82, L67 and L189 (Fig. 2**A**) and the active (Fig. 2**B**) and inactive L82 derivatives (Fig. 2**C**) are shown with compounds grouped based on their activity against LigI and LigIII. Among the active compounds, 5 compounds preferentially inhibited LigI with two compounds, L82-30 and L82-G17 exhibiting increased selectivity for LigI compared with L82, two compounds preferentially inhibited LigIII and 3 compounds had similar activity against both LigI and LigIII (Figs. 1 and 2**B**).

There are three types of linkers between the two aromatic rings of all the DNA ligase inhibitors, vinyl (Fig. 3, upper panel), arylhydrazone (Fig. 3, middle panel), and acylhydrazone (Fig. 3, lower panel), linking the rings with 2, 3, and 4 atoms, respectively. None of the LigI-selective inhibitors has a vinyl linker. In addition, all the LigI-selective inhibitors except for L82-22 have a pyridazine ring whereas the LigI/LigIII and LigIII inhibitors do not. The repositioning of a hydroxyl group on the non-pyridazine ring from para to meta, and the removal of a nitro group (Fig. 2), appears to increase the selectivity of L82-G17 for LigI (Fig. 1). Active inhibitors with an arylhydrazone linker have at least one polar group, such as the phenol in L82-G17, at the meta positions (8 and 10, Fig. 3, middle panel). This also occurs in LigI-selective acylhydrazone class inhibitors L82-21 and L82-22, which have either a nitro or a hydroxyl group at their position 10 (Fig. 3, lower panel).

Comparing geometric shape coefficients [29,30], a value that represents a molecule's potential size based on the connections between atoms, the mean value for LigI-selective inhibitors (7.4) was significantly different ($p < 0.05$) than that of LigI/III inhibitors (9.5). The mean geometric shape coefficient of LigI-selective inhibitors was also significantly different than that of compounds that do not inhibit either LigI or LigIII. A further point of differentiation between LigI-selective and LigI/LigIII inhibitors is their calculated partition coefficient (LogP). The calculated LogP is significantly lower ($p < 0.01$) for LigI-selective inhibitors (2.53) than inhibitors of both LigI and LigIII (4.6).

2.2. L82-G17 is an uncompetitive inhibitor of LigI

We chose to focus on characterizing L82-G17 because of its higher potency and increased selectivity for LigI. L82-G17 is more related to L82 than L67 with Tanimoto similarity scores of 78% and 32%, respectively, calculated using the Maximum Common Substructure method [31,32]. As was observed with L82 [18], L82-G17 did not inhibit LigIV at 200 μM (Fig. 4**A**). Among the LigI inhibitors identified by computer-aided drug design, L82 was unique in that it appeared to act as an uncompetitive rather than a competitive inhibitor [18]. This prompted us to examine the effects of L82 (Fig. 4**B**) and L82-G17 (Fig. 4**C**) on the kinetics of ligation by LigI. Under these reaction conditions, the V_{max} and K_m values for DNA ligase were 0.9 pmol ligations per min and 1.4 μM, respectively. Notably, the addition of L82 increased K_m and decreased V_{max}, whereas L82-G17 reduced both K_m and V_{max}. The Lineweaver-Burk plots obtained with L82 (Fig. 4**D**) indicate that this compound is a mixed inhibitor. In contrast, the Lineweaver-Burk plots obtained with L82-G17 indicate that this compound is an uncompetitive inhibitor (Fig. 4**E**). It is likely that differences in experimental conditions underlie the discrepancy between the results of the kinetic analyses shown in Fig. 4 and the published study [18]. Here we used a shorter incubation time (5 min versus 30 min) and higher concentrations of protein and DNA substrate to more accurately measure initial and maximal reaction velocities of LigI.

Given the discordant results with L82 in the enzyme kinetic assays, we perfomed an electrophoretic mobility shift assay (EMSA) with a linear duplex containing a single non-ligatable nick that had been used previously to distinguish between uncompetitive and competitive inhibitors of LigI [26]. In accord with the published study [26], L82 increased the amount of LigI-DNA complex formed (Fig. 5**A**, compare lanes 2 and 4, and Fig. 5**B**) whereas the competitive inhbitor L67 prevented complex formation (Fig. 5**A,** compare lanes 2 and 5, and **B**), confirming that L82 is able to act as an uncompetitive inhibitor. As expected, L82-G17 also increased the amount of LigI-DNA complex formed in concentration-dependent manner (Fig. 5**A**, compare lanes 2

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