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Synthetic modified pyrrolo[1,4] benzodiazepine molecules demonstrate selective anticancer activity by targeting the human ligase 1 enzyme: An *in silico* and *in vitro* mechanistic study

Mohammad Shameem [a], Ravi Kumar [b], Shagun Krishna [a], Chandan Kumar [a], Mohammad Imran Siddiqi [a], Bijoy Kundu [b,*], Dibyendu Banerjee [a,*]

[a] Molecular and Structural Biology Division, CSIR-Central Drug Research Institute, B.S. 10/1, Janakipuram Extension, Sitapur Road, Lucknow 226031, India
[b] Medicinal and Process Chemistry Division, CSIR-Central Drug Research Institute, B.S. 10/1, Janakipuram Extension, Sitapur Road, Lucknow 226031, India

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ABSTRACT

Human DNA ligase1 (hLig1) is the major replicative enzyme in proliferating mammalian cells that join Okazaki fragments of the lagging strand during DNA replication. Interruptions in the process of ligation cause DNA damage to accumulate, resulting in cytotoxicity and cell death. In the present study we demonstrate that pyrrolo[1,4] benzodiazepine (PBD) derivatives exhibit anticancer properties by targeting the nick sealing activity of hLig1 as opposed to the DNA interaction activity known for such compounds. Our *in silico* and *in vitro* assays demonstrate the binding of these molecules with amino acid residues present in the DNA binding domain (DBD) of the hLig1 enzyme. Two of these hLig1 inhibitors S010–015 and S010–018 demonstrated selective anti-proliferative activity against DLD-1 (colon cancer) and HepG2 (hepatic cancer) cells in a dose dependant manner. The molecules also reduced cell viability and colony formation at concentrations of $\leqslant$20 μM in DLD-1 and HepG2 cells and induced apoptotic cell death. In yet another significant finding, the molecules reduced the migration of cancer cells in wound healing experiments, indicating their anti-metastatic property. In summary, we report the anticancer activity of PBD derivatives against DLD-1 and HepG2 cells and propose a new molecular target for their activity.

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1. Introduction

DNA ligases join breaks in DNA by forming phosphodiester bonds between adjacent 5′-phosphoryl and 3′-hydroxyl termini at single strand breaks in duplex DNA molecules. DNA ligases are required for DNA replication, repair and recombination processes [1–5]. The joining of interruptions in the phosphodiester backbone of duplex DNA is critical for maintaining genomic integrity. In humans, there are three known ATP dependant DNA ligases, viz., Ligase 1, Ligase 3 and Ligase 4. Human DNA ligase 1 plays a major role during replication by processing the ligation of Okazaki fragments. It is also involved in the Base Excision Repair (BER) pathway where it joins single strand breaks in DNA produced by DNA glycosylases [6]. During the ligation process, hLig1 reacts with ATP to form a covalent enzyme-adenylate intermediate. Subsequently, AMP is transferred to the 5′ phosphate terminus of DNA at a strand break and catalyzes phosphodiester bond formation [7,8]. DNA ligation is the necessary process required by all cells during replication. Defects in DNA ligation can cause increased genomic instability and hypersensitivity to DNA damaging agents leading to cellular lethality [9–11]. However, genomic instability can also give rise to uncontrolled cell division leading to cancers. Therefore alterations in one or more of the mechanisms that maintain genome stability occur at some stages during the development of most cancers. Previous studies have shown elevated hLig1 levels in tumor cells compared to normal cells [12]. Thus, hLig1 may be a viable therapeutic target for the development of novel anticancer agents.

PBD molecules structurally comprise a 5-7-6 tricyclic skeleton with an aromatic ring-A, a pyrrolidine ring-C and a seven-membered ring-B (Supplementary Fig. 1A). The naturally occurring PBD have attracted significant attention because of their broad spectrum biological activities such as antibacterial, antileishmanial, herbicidal and anticancer agents [13–15]. Representative examples of PBD include Anthramycin, Tomaymycin, Sibiromycin, Neothramycins and DC-81 (Supplementary Fig. 1B). The anticancer

* Corresponding authors. Tel.: +91 522 2772450x4729; fax: +91 522 2771941 (B. Kundu). Tel.: +91 522 2772450x4443; fax: +91 522 2771941 (D. Banerjee).
E-mail addresses: b_kundu@cdri.res.in (B. Kundu), d.banerjee@cdri.res.in (D. Banerjee).

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activity of PBD molecules has been traditionally attributed to the covalent binding of guanine residues from double-stranded DNA to the C11 position and exocyclic N2 amine, thus leading to distortion of the double-stranded structure by fitting into the minor groove of B-form of DNA [16]. However, when we embarked on the anticancer screening of some PBD derivatives with varying substitutions on the two aromatic rings either fused with or originating at C-11 position of the 7-membered ring, we found that they could interact with the hLig1 enzyme as suggested by the *in silico* screening and showed no significant binding with DNA at their effective concentrations.

In this study we report for the first time the identification of two new molecularly targeted PBD derivatives (S010–015 and S010–018) that can bind with and inhibit the hLig1 enzymatic activity leading to decreased cell viability, decreased cell migration and increased apoptosis selectively in HepG2 (hepatic) and DLD-1 (colon) cancer cells.

2. Materials and methods

2.1. Chemicals and cell Lines

Fluorescent (cyanin3) and 6-FAM labelled oligos were purchased from IDT, USA. Cell Culture media (DMEM, RPMI, EMEM, and Ham's F12), antibiotics and fetal bovine serum (FBS) were purchased from Invitrogen life technologies USA. Annexin-V/FITC kit and propidium iodide (PI) were procured from BD biosciences, USA. Cell lines, ethidium bromide (EtBr), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) and DMSO, were purchased from Sigma–Aldrich, USA. Primary antibodies (hLig1 and beta actin) and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

2.2. Synthesis of the Inhibitor molecules

Modified PBD derivatives were synthesized as described previously [17].

2.3. In silico molecular docking of hLig1 Inhibitors

The PBD derivatives described [17] were drawn using Sketch module of Sybyl7.1. The compound structures for docking were prepared by geometry optimization using a combination of the MMFF94 force field of the SYBYL molecular modeling package with Powell energy minimization algorithm, Gasteiger–Huckel charges, and 0.001 kcal/(mol Å) energy gradient convergence criterion. For the receptor structure preparation, the crystal structure of hLig1 was obtained from RCSB Protein Data Bank (PDB ID-1X9N) [18]. To prepare this structure for docking studies, the DNA was removed from the structure, hydrogen atoms were added and the structure was subjected to energy minimization using Sybyl7.1 (Sybyl7.1, Tripos, Inc., 2005) with the default values. The resulting structure was used for docking all the compounds into the DNA binding site of hLig1 using FlexX module of Sybyl7.1. The docking method executed in FlexX was focused around an incremental construction algorithm that first parts the compound into its fundamental fragments and naturally chooses the base part by the utilization of a pattern recognition technique called pose clustering and positions it into an active site emulated by the incremental building of the remaining portion onto the active site. The conformational adaptability of the ligand was incorporated by creating various conformations for each fragment, and position of the ligand was scored to calculate the free energy of binding of the ligand with the protein [19]. Chimera was used for visualization of the structures [20].

2.4. Purification of hLig1 protein

Human DNA ligase1 protein was purified as described earlier [21]. Briefly, hLig1 protein was expressed in the *Escherichia coli* strain BL21 (DE3) RP cells grown in 2XYT media. Protein was purified over phosphor–cellulose, followed by gel filtration chromatography (GE Healthcare), dialyzed against storage buffer (25 mM Tris–Cl pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 4 mM DTT, protease inhibitors), concentrated, quantified and used for further experiments.

The DBD of hLig1 was expressed and purified same as described previously [18]. The DBD was purified by the nickel affinity chromatography (QIAGEN) and size exclusion chromatography using Superdex 200 10/300 (GE healthcare). The protein was concentrated with a 10 kDa Amicon centrifugal device (Millipore) and stored in buffer containing (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM DTT, 10% glycerol, 0.1 mM EDTA).

2.5. DNA ligation assay

DNA joining assay was performed as described previously [22]. A fluorescent (cyanin3) labeled 27-mer (5′-/Cy3/GTACTATCTCACTGA CATACATAGACA-3′) oligo was annealed with 52-mer (5′-GTACGTC GATCGATTGGTAGATCAGTGTCTATGTATGTCAGTGAGATAGTAC-3′), and 25-mer (5′-CTGATCTACCAATCGATCGACGTAC-3′) to form a complimentary double stranded nicked substrate for the ligase enzymes. The reaction mixture (20 μl) contained 1 pmol of labeled DNA substrate and 0.2 pmol of purified hLig1 in a ligation buffer containing Tris–Cl (50 mM, pH 7.5), $MgCl_2$ (10 mM), BSA (0.25 mg/ml), NaCl (100 mM), and ATP (500 μM). The double-stranded nicked DNA was incubated with the purified ligase in the absence or presence of inhibitors at 37 °C for 30 min. Reactions were stopped by adding 10 μl of stop buffer (90% formamide and 10% of 50 mM EDTA). The ligated and unligated DNA molecules were separated in a denaturing gel, containing 7 M urea and 12% acrylamide and bands were detected by using image quant LAS 4010 (GE Life Sciences).

2.6. Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as described earlier [22]. Briefly, a fluorescent labeled 27-mer oligo with a dideoxy modified 3′ end (5′-/6-F AM/GTACTATCTCACTGACATACATAGAC/ddc/-3′) and 25-mer oligo (5′-CTGATCTACCAATCGATCGACGTAC-3′) were annealed with a complimentary 52-mer oligo (5′-GTACGTCGATCGATTGGTAGATCA GGGTCTATGTATG TCAGTGAGATAGTAC-3′). This annealed nicked oligo with non-ligatable end was used as a substrate for ligase 1 protein. This allowed the hLig1 to bind with DNA but it did not release because ligation could not be completed. DNA substrate (1 pmol) was incubated with hLig1 (8 pmol) in the presence or absence of inhibitor in a ligation buffer containing Tris–Cl (50 mM, pH 7.5), $MgCl_2$ (10 mM), BSA (0.25 mg/ml), NaCl (100 mM), and ATP (500 μM) in a reaction volume of 20 μl for 2 h at 4 °C. After the addition of 10 μl of native gel buffer (Tris-Cl (pH 7.5) 50 mM), 20% glycerol, 0.05% bromophenol blue), samples were separated by 6.0% native PAGE, and bands were detected by image quant LAS4010 (GE Life Sciences).

2.7. Fluorescence spectroscopy

Fluorescence spectroscopy of hLig1 and its DNA binding domain (DBD) was performed at the fixed excitation wavelength of 280 nm and emission spectra was recorded from 300 to 400 nm with 5–10 nm slit width on a Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies). Inhibitors (50 μM) were added to the hLig1 protein (400 pmol) and DBD protein (400 pmol) in a volume of

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