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Identification and characterization of human Rad51 inhibitors by screening of an existing drug library

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ABSTRACT

Homologous Recombination (HR) plays an essential role in cellular proliferation and in maintaining genomic stability by repairing DNA double-stranded breaks that appear during replication. Rad51, a key protein of HR in eukaryotes, can have an elevated expression level in tumor cells, which correlates with their resistance to anticancer therapies. Therefore, targeted inhibition of Rad51 through inhibitor may improve the tumor response to these therapies. In order to identify small molecules that inhibit Rad51 activity, we screened the Prestwick Library (1120 molecules) for their effect on the strand exchange reaction catalyzed by Rad51. We found that Chicago Sky Blue (CSB) is a potent inhibitor of Rad51, showing IC_{50} values in the low nanomolar range (400 nM). Biochemical analysis demonstrated that the inhibitory mechanism probably occurs by disrupting the Rad51 association with the single-stranded DNA, which prevents the nucleoprotein filament formation, the first step of the protein activity. Structure Activity Relationship analysis with a number of compounds that shared structure homology with CSB was also performed. The sensitivity of Rad51 inhibition to CSB modifications suggests specific interactions between the molecule and Rad51 nucleofilament. CSB and some of its analogs open up new perspectives in the search for agents capable of potentiating chemo- and radio-therapy treatments for cancer. Moreover, these compounds may be excellent tools to analyze Rad51 cellular functions. Our study also highlights how CSB and its analogs, which are frequently used in colorants, stains and markers, could be responsible of unwanted side effects by perturbing the DNA repair process.

1. Introduction

Homologous Recombination (HR) is a ubiquitous process important for DNA repair, generation of genetic diversity, and the proper segregation of chromosomes during meiosis. The defining mechanistic step of HR, exchange of base-paired partners between a single-stranded DNA (ssDNA) and its homolog within double-stranded DNA (dsDNA) is promoted by a superfamily of proteins, called recombinases [1] including bacterial RecA [2], archaeal RadA [3] and eukaryotic Rad51 [4] and DMC1 [5]. Strand exchange is essentially a DNA rearrangement event catalyzed by a DNA-bound recombinase (nucleoprotein) filament [6], coupled to ATP binding and hydrolysis [7,8].

Consistent with the importance of recombinases in the HR pathway, several studies strongly suggest the involvement of the Rad51 activity in tumorigenesis or tumor progression [9–12]. Moreover, a high level of HR induced by overexpression of Rad51 has also been frequently described in many human cancers, including breast, prostate, bladder, soft tissue sarcoma, and lung (reviewed in [13]). This Rad51 overexpression appears to contribute to chemo- and radio-resistance of these human cancers, which explains that reducing Rad51 amount, following antisense or ribozyme treatment, increases the effectiveness of cancer treatment [14–16]. Given the involvement of Rad51 in such severe diseases, it represents an important target for anticancer therapy and the development of small molecule inhibitors capable of inhibiting Rad51 activities is warranted. Thence, very recently, few compounds have been identified as inhibitors of Rad51 and the great majority of them have been selected by high-throughput screening from chemical libraries [17–20]. However, the development of a new drug is a hard and expensive process, including the fact that new drugs have to undergo a challenging approval process by the Food and Drug Administration (FDA) in order to make sure

Abbreviations: HR, Homologous Recombination; ssDNA, single-stranded DNA; dbDNA, double-stranded DNA; CSB, Chicago Sky Blue 6B..

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that the drug is safe for consumption. We postulated that repurposing of already FDA approved drugs may decrease the time and effort in bringing drugs with novel anticancer activity from the bench to the bedside.

To this end, we have investigated the inhibitory effect of drugs from the Prestwick Chemical Library (Prestwick Chemical, Illkirch, France) on the Rad51 strand exchange activity. This library consists of 1120 small molecules composed of 85% FDA-approved, off-patent drugs with a wide range of functions and mechanisms of action and with well-characterized pharmacological and toxicological properties and 15% bioactive alkaloids or related substances. The Prestwick Chemical Library presents the greatest possible degree of drug-likeliness. The active compounds were selected for their high chemical and pharmacological diversity as well as for their known bioavailability and safety in humans.

As described here, using an in vitro activity assay, we demonstrated that the compound Chicago Sky Blue (CSB) is a potent inhibitor of strand exchange activity catalyzed by human Rad51 (Rad51). Also known as Niagara sky blue or direct blue 1, this compound is a large organic acid, structurally related to glutamate that is a potent and efficient competitive inhibitor of vesicular glutamate uptake [21]. This molecule and some of its analogs are also known to be used as dyes both in clinical and biochemical areas [22–24]. These compounds may serve as tool for dissecting the molecular mechanism of Rad51 and as aids in the development of anticancer treatment.

2. Materials and methods

2.1. Reagents and materials

Most of the chemical compounds were purchased from Sigma-Aldrich (MO, USA), except where indicated otherwise. Disposable fiber-optic streptavidin-coated tips were purchased from FortéBio Inc. (CA, USA).

2.2. Preparation of Rad51 protein

Rad51 protein was purified as previously described [25]. Human Rad51 gene was inserted at the NdeI site of the pET15b expression vector (Novagen, Merck, Germany) and expressed in the Escherichia coli JM109 (DE3) strain that also carried an expression vector for the minor tRNAs (Codon(+)RIL®, Novagen). The protein was purified on Nickel–nitrilotriacetic acid (Ni–NTA) agarose (Invitrogen, CA, USA). The hexahistidine tag was then removed from the Rad51 portion by incubation with 10 units of thrombin protease (GE Healthcare, UK) per mg of Rad51 during 18 h. The tag-free protein was further purified by chromatography on a MonoQ column (Amersham Biosciences, NJ, USA). Finally, the Rad51-containing fractions were dialysed against storage buffer (20 mM Tris–HCl, pH 8, 0.25 mM ethylenediaminetetraacetic acid (EDTA), 20% glycerol, 5 mM dithiothreitol (DTT) and 200 mM KCl) and kept at −80 °C. Protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard protein.

2.3. Oligonucleotides

The 58-ssDNA* sequence was 5′-TCC TTT TGA TAA GAG GTC ATT TTT GCG GAT GGC TTA GAG CTT AAT TGC TGA ATC TGG T-3′. The 5' end of this oligonucleotide was labeled with DY-782. The 33-dsDNA was obtained by hybridation of the two oligonucleotides at equimolar amounts: 5′-GCC ATC CGC AAA AAT GAC CTC TTA TCA AAA GGA-3′ and 5′-TCC TTT TGA TAA GAG GTC ATT TTT GCG GAT GGC-3′. These oligonucleotides and the polydT (58 nucleotides, unlabeled and labeled with Biotin at the 5′ position) were purchased from Eurofins MWG operon (Germany). The 100-ssDNA** was purchased from Integrated DNA Technologies (IA, USA): 5′-GGG CGA ATT GGG CCC GAC GTC GCA TGC TCC TCT AGA CTC GAG GAA TTC GGT ACC CCG GGT TCG AAA TCG ATA AGC TTA CAG TCT CCA TTT AAA GGA CAA G-3′. The 5′ end of this oligonucleotide was labeled with IRD-700. All the DNA concentrations are expressed in nucleotide bases or base-pairs.

2.4. Strand exchange activity

Labeled 58-ssDNA* (1.16 μM) was incubated with 0.5 μM Rad51 in presence or absence of the indicated amounts of the corresponding molecule in 10 μL of standard reaction buffer containing 20 mM Tris–HCl (pH 8), 1 mM ATP, 1 mM DTT, 20 mM $MgCl_2$, 0.0075% Tween20 and 2% glycerol, at 37 °C for 20 min. The reaction was initiated by adding the 33-dsDNA (1.65 μM bp), which shared sequence homology with the 58-ssDNA*. After 1 h incubation at 37 °C, the reactions were stopped and deproteinized by the addition of 0.7% SDS, and 0.7 mg/mL proteinase K. The reaction mixtures were further incubated for 15 min at 37 °C. After adding 5-fold loading dye ((0.05% bromophenol blue, 8% glycerol, 1 mM EDTA), the reaction products were separated by electrophoresis on 15% polyacrylamide gel. The electrophoresis was carried out in 0.5 X TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA) at 100 V for 2 h. The labeled products (58-ssDNA* and 58/33-dsDNA*) were visualized and quantified by the detection of the DY-782 dye with the 800 nm infrared fluorescent detection channel of an Odyssey Infrared Imager (LI-COR Biosciences, NE, USA).

2.5. D-loop formation

Labeled 100-ssDNA** (1 μM) was incubated with 0.5 μM Rad51 in presence or absence of the indicated amounts of the corresponding molecule in 10 μL of standard reaction buffer containing 20 mM Tris–HCl (pH 8), 1 mM ATP, 1 mM DTT, 1 mM $CaCl_2$ at 37 °C for 20 min. The reaction was initiated by adding supercoiled pPB4.3 DNA (200 μM in bp). After incubation of 30 min at 37 °C, the reactions were stopped and deproteinized by a stop solution (10 mM Tris–HCl pH 8, 10 mM $MgCl_2$, 1% SDS, and 1 mg/mL proteinase K. The reaction mixtures were further incubated for 15 min at 37 °C. After adding 5-fold loading dye (0.05% bromophenol blue, 8% glycerol, 1 mM EDTA), the reaction products were separated by electrophoresis on 1% agarose gel. The electrophoresis was carried out in 0.5 X TAE buffer (20 mM Tris, 10 mM acetic acid and 1 mM EDTA) at 100 V for 2 h. The labeled products (100-ssDNA** and D-loop) were visualized and quantified by the detection of the IRD-700 dye with the 700 nm infrared fluorescent detection channel of an Odyssey Infrared Imager (LI-COR).

2.6. ssDNA association of Rad51

The binding of Rad51 to ssDNA was monitored and quantified by Bio-Layer Interferometry technology using the BLItz platform (FortéBio Inc.). The procedure includes four steps: (1) the baseline in buffer A containing 1× PBS, 10 mM $MgCl_2$ and 0.0075% Tween20 was recorded for 20 s; (2) loading and binding of Rad51 were measured for 40 s using 1.5 μM Rad51 and 1 mM ATP in buffer A; (3) the dissociation of Rad51 from ssDNA was recorded for 20 s in buffer A; (4) the ssDNA biosensor was regenerated using baths of 50 mM NaOH. Except for the step 2, which used 4 μL drop of the protein sample, the steps 1, 3 and 4 used 250 μL solutions in 0.5 mL tube. The measurement parameters were as follows: room temperature measurement and stirring speed of 2200 rpm. To investigate the effect of CSB and analogs to the ssDNA binding of