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Biochemical and cell biological assays to identify and characterize DNA helicase inhibitors

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ABSTRACT

The growing number of DNA helicases implicated in hereditary disorders and cancer indicates that this particular class of enzymes plays key roles in genomic stability and cellular homeostasis. Indeed, a large body of work has provided molecular and cellular evidence that helicases act upon a variety of nucleic acid substrates and interact with numerous proteins to enact their functions in replication, DNA repair, recombination, and transcription. Understanding how helicases operate in unique and overlapping pathways is a great challenge to researchers. In this review, we describe a series of experimental approaches and methodologies to identify and characterize DNA helicase inhibitors which collectively provide an alternative and useful strategy to explore their biological significance in cell-based systems. These procedures were used in the discovery of biologically active compounds that inhibited the DNA unwinding function catalyzed by the human WRN helicase-nuclease defective in the premature aging disorder Werner syndrome. We describe *in vitro* and *in vivo* experimental approaches to characterize helicase inhibitors with WRN as the model, anticipating that these approaches may be extrapolated to other DNA helicases, particularly those implicated in DNA repair and/or the replication stress response.

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1. Introduction

Seminal studies from the Ashworth [1] and Helleday [2] laboratories first published in 2005 described small molecules (<500 Da) effective in sensitizing mutant cancer cells, defective in the tumor suppressors BRCA1 or BRCA2, to clinically relevant anti-cancer agents. These studies led to the discovery of poly(ADP)ribose polymerase (PARP) inhibitors that show promise in the clinic for treatment of BRCA1- or BRCA2-negative cancers. Aside from their own success, the PARP inhibitors provide a roadmap for investigation of other DNA repair targets using small molecule screens as the prevailing experimental strategy for initial identification of lead compounds. It is now apparent that DNA repair represents a broad class of proteins which may be valuable for targeting in anti-cancer schemes. While this field is still in its early stages at the translational level, progress continues to be made to identify new targets in DNA repair pathways, particularly homologous recombination (HR) repair where the PARP inhibitor story first got started.

A recent review by Huang and Mazin provides a detailed perspective of small molecule inhibitor screens to identify druggable targets of the HR pathway [3]. This is a very useful review as it discusses proof-of-concept examples for compound library screens adapted to cell-based assays which illustrated the utility of small molecule modulators as research tools and also potential drug candidates to modulate the DNA damaging effects of classical chemotherapy drugs. HR repair is elicited in rapidly dividing cancer cells undergoing prolific DNA synthesis to cope with double-strand breaks due to replication-blocking lesions that occur endogenously or double-strand breaks that are induced directly or indirectly by chemotherapeutic drugs or ionizing radiation; therefore, it is conjectured that HR targets might be a focal area for cancer therapy. Hot on the heels of PARP inhibitors, small molecules that affect the major strand recombinase RAD51 is attracting interest. In addition, the double-strand DNA translocate RAD54, which stimulates DNA strand exchange activity of RAD51 among other activities including Holliday Junction branch-migration and remodeling of protein-DNA complexes, is also a candidate for small molecule modulation.

Our own group has addressed the potential value of targeting molecular motor DNA unwinding enzymes known as helicases for anti-cancer therapies [4–6]. Helicases catalytically disrupt hydrogen bonds between bases in structured nucleic acids, and have important functions in virtually all aspects of nucleic acid metabolism [7]. Because DNA helicases play a unique and early role in a number of DNA damage response and DNA repair pathways especially in dividing cells, we hypothesize that they represent a useful target to exploit synthetic lethal relationships with other DNA damaging agents and/or in specific mutant backgrounds. In a 2013 review, the Frick laboratory provided an overview of the helicase inhibitors described to date with an emphasis on published work that used screens to identify compounds that modulate the human RecQ helicases or the RNA helicase elongation initiation factor 4A [8]. In the current review for this special Methods collection on DNA helicases, we have focused on the actual experimental approaches and assays we employed to perform a small molecule screen for inhibitors of the Werner syndrome helicase implicated in the premature aging disorder Werner syndrome [9–11]. Furthermore, we provide the reader guidance on important experimental approaches that address issues relating to potency, specificity, and reversibility of helicase inhibitors *in vitro*. In addition, the review places a major emphasis on cell-based assays to characterize the biological effects of WRN helicase inhibitors and synthetic lethal approaches we have used in laboratory experiments. The review is written in plain language so that it may be helpful to many experimental biologists, even those who are relatively new to the helicase field. The review is divided into two over-arching sections: 1) biochemical screen for WRN inhibitors and related *in vitro* assays; 2) biological assays with the WRN helicases inhibitors and human cells. In keeping with the theme of *Methods*, we have focused on rapidly developing techniques and strategies to characterize DNA helicases using small molecules as novel tools for basic science investigation and potential development into translational therapies, particularly in the anti-cancer field.

2. Biochemical small molecule helicase inhibitor screens

Screening and characterization of biologically active small molecules that modulate the DNA unwinding function of a target helicase represents a unique approach to studying helicase function in human cells [4,5,8]. We have used this approach to investigate the molecular and cellular functions of the WRN helicase-nuclease defective in the premature aging disorder Werner syndrome. These studies were initially guided by an *in vitro* radiometric-based helicase assay using the purified recombinant WRN protein in which approximately 500 compounds from the National Cancer Institute Diversity Set were screened [10]. One compound that we identified to inhibit WRN with relatively high potency compared to other compounds in the NCI library was 1-(propoxymethyl)-maleimide, designated NSC 19630 ($IC_{50} \sim 20$ μM). Having determined potency for WRN helicase inhibition, the specificity of compounds which tested positively for helicase inhibition *in vitro* was assessed by evaluating their effects on other DNA helicases. In parallel, DNA binding, ATPase, and WRN exonuclease assays were performed to further characterize compounds which selectively inhibited WRN helicase activity. In addition, selected WRN helicase inhibitory compounds were assayed for displacement of the fluorescently active DNA intercalating compound Thiazole Orange to assess the relative ability of each respective compound from the NCI Diversity Set to bind the DNA substrate used for WRN helicase assays. This effort helped to eliminate those compounds whose effect on WRN helicase activity was mediated by its direct interaction with the DNA helicase substrate and therefore considered to be non-specific in nature. Further testing of structures similar to NSC 19630 led to the identification of a more potent WRN helicase inhibitor designated NSC 617145 [9]. In the following sections, we will describe the procedures for these assays used to identify and characterize the WRN helicase inhibitors NSC 19630 [10] and NSC 617145 [9], and highlight some salient points which are useful to keep in mind when designing experiments and carrying out biochemical assays.

2.1. Semi-high-throughput helicase activity screen

Semi-high-throughput screening of a large number of small molecules for inhibition of helicase activity requires a DNA substrate (either radiolabeled or fluorescently labeled) that is relevant for measuring helicase activity, purified helicase protein devoid of contaminating nuclease activity, reaction salts optimal for helicase activity, a source of energy (typically ATP) for the helicase enzyme, and the library of small molecules in solution (typically dissolved in DMSO). Reactions are typically 20 μl with 0.5 nM DNA substrate used. A good preliminary experiment prior to screening is to test