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Depletion of tyrosyl DNA phosphodiesterase 2 activity enhances etoposide-mediated double-strand break formation and cell killing

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ABSTRACT

DNA topoisomerase 2 (Top2) poisons, including common anticancer drugs etoposide and doxorubicin kill cancer cells by stabilizing covalent Top2-tyrosyl-DNA 5′-phosphodiester adducts and DNA double-strand breaks (DSBs). Proteolytic degradation of the covalently attached Top2 leaves a 5′-tyrosylated blocked termini which is removed by tyrosyl DNA phosphodiesterase 2 (TDP2), prior to DSB repair through non-homologous end joining (NHEJ). Thus, TDP2 confers resistance of tumor cells to Top2-poisons by repairing such covalent DNA-protein adducts, and its pharmacological inhibition could enhance the efficacy of Top2-poisons. We discovered NSC111041, a selective inhibitor of TDP2, by optimizing a high throughput screening (HTS) assay for TDP2's 5′-tyrosyl phosphodiesterase activity and subsequent validation studies. We found that NSC111041 inhibits TDP2's binding to DNA without getting intercalated into DNA and enhanced etoposide's cytotoxicity synergistically in TDP2-expressing cells but not in TDP2 depleted cells. Furthermore, NSC111041 enhanced formation of etoposide-induced γ-H2AX foci presumably by affecting DSB repair. Immuno-histochemical analysis showed higher TDP2 expression in a sub-set of different type of tumor tissues. These findings underscore the feasibility of clinical use of suitable TDP2 inhibitors in adjuvant therapy with Top2-poisons for a sub-set of cancer patients with high TDP2 expression.

1. Introduction

DNA Type I and Type II topoisomerases (Top1 and Top2) transiently modify DNA topology to allow DNA transcription, replication, and chromosomal recombination and segregation [1–3]. Top1 activity produces a transient Top1-DNA 3′-tyrosyl phosphodiester bond and single strand break, to change the linking number by one. Top2 generates transient DNA double-strand break (DSB) with Top2-DNA-5′-tyrosyl phosphodiester covalent linkage, followed by strand passage, and re-ligation of the double-strand break, thus changing the linking number by two, in end-restricted DNA [1–3]. Top2-poisons prevent re-ligation of the transient DNA DSB, leading to formation of DSBs with stable Top2-DNA adducts, which undergo proteolytic cleavage to leave a peptide-DNA adduct [1–3]. Multiple Top2-poisons, in particular, etoposide and doxorubicin are approved by the Food and Drug Administration for cancer therapy; a few others are in active clinical trials for various types of

Abbreviations: DSB, double strand break; TDP1, tyrosyl-DNA phosphodiesterase1; TDP2, tyrosyl-DNA phosphodiesterase2; Top1, topoisomerase 1; Top2, topoisomerase 2; T5PNP, thymidine 5′-monophosphate p-nitrophenyl ester sodium salt.

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cancer [4,5]. While the Top2 poisons are effective, they suffer from limited tumor selectivity. Their side effects due to toxicity in normal cells which like cancer cells require Top2 for survival and growth, limit administrable dose. Furthermore, the cancer cells can acquire resistance against Top2-poisons [6–8]. One possible way to overcome Top2-poison resistance and improve therapeutic efficacy is through inhibiting the repair of Top2-DNA complex and associated DSBs. However, the molecular mechanism for repairing Top2-DNA cleavable complexes is poorly understood. Recently, a human 5′-tyrosine phosphodiesterase, named, tyrosyl DNA phosphodiesterase 2 (TDP2) which excises Top2-DNA adducts, was identified [9,10]. Knockdown of TDP2 showed increased sensitivity of cancer cells to the Top2-poison etoposide, but not to Top1 specific poison, camptothecin (CPT) [9–11]. Microarray analysis in lung cancer tissues showed that TDP2 expression is higher in cancer compared to the normal control [11]. TDP2 null mice are viable and do not show any obvious phenotype [12]; however, they are hypersensitive to Top2-poisons [13]. Hence, specific TDP2 inhibitors could significantly increase the efficacy of Top2-poisons by reducing the optimum dose in cancer treatment, thereby diminishing non-specific systemic toxicity. TDP2 inhibitors should also be invaluable tools for studying DNA DSB repair mechanisms. Furthermore, TDP2 has recently been shown to have additional function beyond DNA repair. For example, it was shown to be critical for replication of RNA viruses in host cells including picornaviruses that cause myocarditis, aseptic meningitis, hepatitis, encephalitis, and common cold [14]. TDP2 was also identified as a regulator of HPV episome stability and TDP2 depletion resulted in a net loss of episomes in cells [15]. Thus, the TDP2 inhibitors could have broad clinical applications in oncology and virology.

In the present study, using an innovative and simple high-throughput screening assay we described for the first time that a novel selective inhibitor of TDP2 increased the efficacy of a Top2-poison. This study documents for the first time that there is significant inter-patient variation of TDP2 expression among cancer patients, implicating limited clinical success of Top2 poisons. Thus, patients with high TDP2 expression could be treated effectively with the Top2 poison-TDP2 inhibitor adjuvant therapy.

2. Materials and methods

2.1. Reagents

Unless specified otherwise, all chemicals and reagents are purchased from Sigma-Aldrich (St. Louis, MO) including the chromogenic substrate thymidine 5′-monophosphate p-nitrophenyl ester sodium salt (T5PNP). Cell culture reagents were purchased from Cellgro (Manassas, VA) and TDP1 protein was purchased from Topogen Inc. (Port Orange, Florida). Research involving human material that is reported in the manuscript has been performed with the approval of an appropriate ethics committee and the research carried out on humans is in compliance with the Helsinki Declaration [16]. The IRB reference number is 1992-048 (Georgetown University).

2.2. Development and optimization of the primary assay for TDP2

Purification of recombinant TDP2 and preparation of oligonucleotide substrate for validation assays were described previously [17,18]. For the primary screening assay we used T5PNP as the substrate as described before [17]. The high-throughput readiness of the assay has been statistically validated using the protocol described here. Briefly, EDTA (non-specific inhibitor) or DMSO was added to the assay buffer containing TDP2 (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM $MgCl_2$, 50 mM KCl, 100 μg/ml BSA, 130 nM purified TDP2) and incubated for 10 min at 22 °C. T5PNP (10 mM) was then added and incubated for 30 min at 37 °C. Formation of the product was measured at 415 nm absorbance [17]. The final reaction volume was 20 μl in 384-well format.

2.3. High-throughput screening for TDP2 inhibitors and dose kinetics

TDP2 recombinant protein, 20 μM inhibitor and 20 μl T5PNP, was added in each well in a 384-well plate format. The assay buffer and the sequence of addition of the reagents are described above. Small molecule libraries obtained from National Cancer Institute's Developmental Therapeutic Program (NCI-DTP), which includes Challenge Set (57 compounds), Diversity Set (1990 compounds), Mechanistic Set (879 compounds), and Natural Product Set (235 compounds) were screened using a laboratory automation workstation (Biomek NX^P, Beckman Coulter, Pasadena, CA). The hits obtained from the primary screen were checked for dose dependency in the T5PNP assay. Briefly, 0–15 μM of different hit compounds were incubated individually with 130 nM purified TDP2 and 10 mM T5PNP.

2.4. Chemical characterization and re-synthesis of hit compounds

The candidate hit compounds were collected individually from the NCI Developmental Therapeutics Program (DTP) and were sent to proteomics and metabolomics shared resource (PMSR) facility in Lombardi Comprehensive Cancer Center for identity confirmation. The final hit compound was re-synthesized by Toronto Research Chemical, Canada for use in follow-up studies.

2.5. Secondary validation of the primary screen

For the secondary validation of the primary hits, purified TDP2 protein (45–50 pM) was individually incubated with ^{32}P-labeled double-stranded 5'-phosphotyrosyl oligonucleotide substrate (40–50 nM) in the presence of different inhibitors for 7 min at 37 °C in an assay buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 50 mM KCl and 100 μg/ml BSA) in 20 μl. The reaction was stopped by inactivating the enzyme at 80 °C for 5 min, and then mixed with 20 μl loading buffer containing 1× DNA dye (blue-orange loading dye; Promega, Madison, WI) and 45% formamide and heated at 95 °C for 5 min and analyzed by sequencing gel electrophoresis (Model S2, Life Technologies, Rockville, MD) at 50 °C containing 20% polyacrylamide and 7 M urea. Radioactivity in the incised oligonucleotides was quantified by exposing the gel to X-ray films and measuring the band intensities in Chemigenius Bioimaging System and quantification software (Syngene Inc., San Diego, CA) [17,18].

2.6. Inhibition of 5′-tyrosine DNA phosphodiesterase activity in cell extracts

MDA-MB-231 and A549 cell extracts (1.1 μg), prepared as described earlier [17], were incubated with 1 nM ^{32}P-labeled double-stranded 5′-phosphotyrosyl oligonucleotide substrate (40–50 nM) in the presence of inhibitor for 15 min at 37 °C. The order of addition was similar to that of primary assay as described earlier. The substrate and product were separated and quantified in a sequencing gel [18].

2.7. Effect of the inhibitor on APE1 and TDP1 activities

The inhibitor was tested for selectivity by testing for its inhibitory activity against AP-endonuclease 1 (APE1) and tyrosyl DNA phosphodiesterase 1 (3′-tyrosine phosphodiesterase; TDP1). TDP1 (200-400 pM) and APE1 (50 pM) were individually incubated