Discovery of small-molecule inhibitors of Rad6 function and the Rad6-Rad18 interaction

Ph.D. Dissertation

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Introduction

Preserving genetic information during DNA replication is the most important task for dividing cells. However, cells constantly undergo multiple genotoxic abuses, which can result in genetic mutations that could cause cancer. Upon DNA damage, lesions can be left unrepaired from classical DNA repair mechanisms and these damages can stall the replication fork during the S-phase of the cell cycle. Drugs based on small molecules are generally chemical compounds with a molecular weight < 900 Da, and – due to their small size – they tend to easily translocate through the plasma membrane and interact with the cytoplasmic domain of cell surface receptors and intracellular signalling molecules.

Targeting specifically the post-translational modification of proliferating cell nuclear antigen (PCNA) by ubiquitin could serve as anti-cancer treatment own its own or could exhibit synergistic effects with other anti-cancer modalities and potentially restore drug sensitivity in patients displaying refractory responses to current therapies. Monoubiquitination serves as a trigger mechanism to bring in specialized DNA polymerases, called translesion synthesis (TLS) polymerases. These polymerases are involved in replication that is prone to errors, leading to mutagenesis and potential carcinogenic outcomes. This form of DNA repair, involving the use of TLS polymerases, is referred to as translesion DNA synthesis. we developed and optimised high-throughput assays to identify chemical modulators of the PCNA ubiquitination cascade. We identified a series of xanthenes as

first-in-class probes of the Rad6 function and the association of Rad6 and Rad18.

Goals and Objectives

Our study aimed to develop high-throughput *in vitro* assays that can assess the PCNA ubiquitination cascade and to screen for modulators of PCNA ubiquitination. Ubiquitination of PCNA activates DNA damage tolerance pathways, including mutagenic translesion DNA synthesis. The TLS pathway is initiated by monoubiquitination of PCNA, which swaps replicative DNA polymerases with error-prone TLS polymerases capable of replicating across DNA lesions. Inhibiting the PCNA ubiquitination cascade with small molecule inhibitors, either alone or in combination, could be a promising approach to halt cancer progression.

Experimental approach:

- Developing and optimising highly sensitive and reliable assays for the PCNA ubiquitination cascade based on amplified luminescent proximity homogeneous assay (Alpha) technology.
- Performing the screening of chemical libraries to discover modulators of the PCNA ubiquitination reaction.
- Characterising the hits from the primary screening to determine the target.

Materials and methods

Compounds source: The small-molecule libraries were obtained from the US National Cancer Institute (NCI) (Developmental Therapeutics Program (DTP) Diversity Set VI and Mechanistic Set IV).

Alpha assays: Alpha assays, which use luminescent oxygen channelling immunoassay, were conducted using Alpha beads obtained from PerkinElmer. There are two types of Alpha assays, AlphaScreen and AlphaLISA, differing only in the fluorophores used in the acceptor beads. AlphaScreen acceptor beads were used in the experiments with donor beads coated with streptavidin and acceptor beads coated with anti-FLAG antibody. The assays were performed in 96-well plates and read using a TecanSpark plate reader with precise temperature control.

PCNA ubiquitination: In 96-well white round-bottom polypropylene plates (Greiner), we reconstituted the *in vitro* PCNA ubiquitination reaction. Compounds were added to the samples and incubated for 15 minutes at 25°C before adding ATP to initiate the reaction cascade. The reaction was incubated for 2 hours at 25°C and then terminated for analysis using Alpha assays.

Uba1~ubiquitin thioester formation: FLAG-Uba1 and Biotin-ubiquitin were used in reaction buffer with compounds added separately, and samples were incubated with ATP for 30 min at 25°C. The reactions were diluted with 10x Alpha Buffer and incubated with donor and acceptor beads at 10 µg/ml, followed by reading the plates using a Tecan plate reader.

Rad6~ubiquitin thioester formation: We used a two-step process for Uba1 pre-charging, where His-Uba1 and biotin ubiquitin were incubated for 30 min at 25°C, followed by incubation of compounds with Flag-Rad6 for 15 min. The reactions were then mixed and diluted with Alpha Buffer and donor and acceptor beads and incubated in the dark for 4 hours before being read by Tecan plate reader.

Rad18 autoubiquitination: His-Uba1 was charged with biotinylated ubiquitin by adding ATP, followed by 30 min incubation. FLAG-Rad18 and Rad6-Rad18 complex were added and incubated with compounds. Both reactions were mixed and incubated for 1 h, followed by termination and dilution in Alpha Buffer with donor and acceptor beads. The samples were then incubated and read by Tecan plate reader.

Rad6–Rad18 interaction: Rad6–Rad18 complex was formed by pre-incubating Flag-Rad6 and His-Rad18 together in a reaction buffer for 30 minutes, after which a compound was added, and the sample was incubated for another 15 minutes. The samples were then diluted and incubated with donor and acceptor beads before being read by Tecan plate reader.

Mms2–Ubc13~ubiquitin thioester formation: First Flag-Uba1 and GST-ubiquitin were incubated with ATP, and then, compounds were incubated with Mms2-Ubc13. The two reactions were mixed followed by incubation, and detection was carried out through silver staining.

Microscale thermophoresis: Microscale thermophoresis was used to examine small molecule-protein binding. A NanoTemper Monolith instrument was used to perform the assay. The

compound was diluted 16 times and then incubated with GFP-Rad6 or GFP-Rad18 for 15 minutes. Sixteen capillaries were filled with each dilution and loaded into the instrument to determine the *K*d from the titration data using the native software.

Rad6–Ubr1 pull-down: GST-Rad6 and FLAG-Ubr1 were mixed with NSC 9037 and incubated for 15 minutes at 25°C, then loaded onto a column with glutathione-agarose beads for affinity chromatography. Elution was done with 20 mM glutathione and visualised by silver staining.

Rad6–Ubr1 interaction alpha assay: Equimolar GST-Rad6 and FLAG-Ubr1 (100 nM) were mixed and incubated with the xanthene compounds for 15 min at 25°C in reaction buffer, followed by dilution by a factor of 10× in Alpha buffer, with donor and acceptor beads at 10 µg/ml. After 4-h incubation at 25°C in the dark, the plates were read by the plate reader.

Cell culture: HeLa cells were cultured in a growth medium consisting of Dulbecco's modified Eagle's medium (DMEM from Sigma-Aldrich) with 10% fetal bovine serum (FBS from Gibco) in a humified cell culture incubator at 37°C with 5% CO_2.

Cell survival: HeLa cells were seeded onto 96-well plates and incubated with compounds or DMSO. After 24 hours, resazurin was added and fluorescence was measured 4 hours later using a plate reader with an excitation wavelength of 570 nm and an emission wavelength of 585 nm.

Computational docking of compounds to Rad6: Autodock 4.2 software was used to perfrom docking of the Rad6B protein. human Rad6B protein was retrieved from a database and used as a target, 1,000 dockings were carried out in two grid volumes

to obtain an ensemble of 20,000 Rad6-NSC 9037 complexes, which were then clustered and ranked based on binding free energies. From binding free energies, we derived Ki values and determined specific residues of contact for refined binding poses.

QUANTIFICATION AND STATISTICAL ANALYSIS: To increase confidence in the hits, various statistical parameters were considered: Signal-to-noise (S/N), Signal-to-background (S/B), z-prime, z-robust, strictly standardised mean difference (SSMD), Signal window values and Half-maximal inhibitory concentration IC_{50}.

GraphPad Prism 8 and Microsoft Excel were used to calculate all the IC50 and curve fitting calculations. An Excel sheet template was created for the automatic calculation of S/N, S/B, SSMD, Z factor, and signal to window values.

Results

The PCNA ubiquitination reconstituted system with biotin-ubiquitin, hUba1, FLAG-tagged PCNA, RFC, Rad6-Rad18 dimer and nicked circular pUC19 plasmid, were used for the development of a quantitative high-throughput assay for PCNA ubiquitination based on a PerkinElmer's Alphascreen/AlphaLisa system. We have also developed and optimised in vitro ALPHA-based assays for the intermediatory steps involved in the process (Uba1-ubiquitin thioester, Rad6-ubiquitin thioester, Rad6- Rad18 interaction, and Rad18 auto-ubiquitination)

Development and optimisation of Rad6–Rad18 interaction assay: An Alpha system-based assay was developed to investigate the interaction between Rad6 and Rad18 and to

identify small molecules that may disrupt or inhibit this interaction. We evaluated the assay's performance by altering the concentration of each protein individually, while keeping the other constant, and found that it yielded satisfactory results across a range of concentrations. We ultimately selected a concentration of 100 nM for both proteins.

Certain xanthenes and a related acridine derivative inhibit PCNA ubiquitination: We screened NCI DTP Mechanistic Sets and NCI DTP Diversity Set small molecule libraries and discovered a set of xanthenes inhibiting PCNA ubiquitination.

Dose-response analysis for different compounds in PCNA ubiquitination: The dose-response analysis was carried out for PCNA ubiquitination with all eight compounds to get the IC50 for each compound using ALPHA assay.

Dose-response analysis for Rad6~ubiquitin thioester formation: We found that several the xanthene-3-ones tested – NSC 9037, NSC 80693, NSC 157411, and NSC 119888, as well as the acridine NSC 71947 – inhibit the formation of the Rad6~ubiquitin thioester conjugate, while fluorescein, NSC 119891, and NSC 348718 do not inhibit this activity.

Dose-response analysis for Rad18 autoubiquitination: To validate the inhibition of Rad6, we conducted a Rad18 autoubiquitination assay that is reliant on Rad6 activity. Our results revealed that the NSC 9037, NSC 80693, and NSC 157411 compounds effectively hindered the process of Rad18 autoubiquitination.

Dose-response analysis for Uba1~ubiquitin thioester formation: We tested these compounds in the Uba1~ubiquitin

thioester formation assay, we found that some of these compounds (NSC 157411 and NSC 119888) appeared less specific to Rad6 inhibition because of their inhibition of Uba1~ubiquitin thioester formation.

Dose–response for the Rad6–Rad18 interaction in the presence of compounds: We found that some xanthene-3-ones inhibited the Rad6-Rad18 interaction in the assay, with NSC 9037, NSC 80693, and NSC 157411 being the most effective at disrupting the interaction. Other xanthene-3-ones, a non-ketone xanthene, and an acridine did not have a significant impact on the formation of the Rad6-Rad18 complex.

Rad6-Ubr1 ALPHA assay: Rad6-Ubr1 interaction assay in the presence of compounds at a final concentration of 50 μM were carried out. All the compounds showed weak to no inhibition of Rad6-Ubr1 interaction.

Confirmation of target-protein binding using microscale thermophoresis (MST): We tested NSC 9037 on MST. The dissociation constant (*K*d) of NSC 9037 binding to Rad6 was determined to be 3.79 ± 2.94 μM (mean ± standard deviation) through our calculations.

Dose-response analysis of cell survival: Cell viability was affected differently by the tested compounds. Fluorescein had no effect on cell survival, whereas the remaining compounds showed varying degrees of impact, ranging from mild effects that peaked at a partially inhibited state to complete cytotoxicity at relatively low concentrations.

Identification of possible compound-binding sites: The computational docking data suggests binding sites of certain

compounds are located far from the catalytic cysteine residue of human Rad6B, which is essential in thioester formation with ubiquitin. Inhibition of Rad6~ubiquitin thioester formation could occur through an allosteric mechanism involving significant conformational changes. The compounds may also cause alterations in the nearby molecular structure, leading to changes in the distribution of partial atomic charges that would decrease the affinity of Rad6 for Rad6BD, which coincides with Rad6's noncovalent binding site for ubiquitin.

Summary

Numerous types of genotoxic stress can harm cells, causing mutations that may contribute to cancer. Despite many years of research, cancer remains a major health concern, and traditional treatments such as radiation, chemotherapy, and surgery have limitations, including the challenge of distinguishing between cancerous and healthy cells, resulting in significant toxicity and side effects. Cells use different methods, including base-excision repair, nucleotide excision repair, homologous recombination, and non-homologous end joining, to fix DNA damage. However, damage can sometimes remain during replication and stall the replication fork, triggering PCNA monoubiquitination at lysine residue K164 by Rad6 and Rad18 enzymes. This process recruits specialized DNA polymerases called translesion synthesis (TLS) polymerases, which are involved in replication prone to errors, leading to mutagenesis and potential carcinogenic outcomes. This type of DNA repair is known as translesion DNA synthesis.

We have developed and optimized assays to efficiently identify small-molecule modulators that target the PCNA ubiquitination cascade. Through this process, we have discovered a set of xanthenes that act as first-in-class inhibitor for the function of Rad6 and the association of Rad6 and Rad18. This discovery is significant because it highlights a novel inhibitory activity for small molecules within the PCNA ubiquitination cascade. These xanthenes have the potential to be utilized as therapeutic agents for cancer treatment. Xanthenes are organic molecules with a unique chemical structure that includes a benzene ring, a six-carbon ring with alternating double bonds, and a carbonyl group, as well as a side chain composed of two carbon atoms. These compounds have various uses in fields such as pigments, dyes, and fluorescent markers, as well as in biology, material science, and electronics due to their exceptional characteristics such as high reactivity, fluorescence, and the capacity to form stable complexes with metal ions.

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List of Publications

MTMT number: MTMT number: 10074814

1. Mandatory peer-reviewed international publications for the fulfilment of the doctoral process and on which this thesis is based:

1. Gabriel Fenteany*, Gaurav Sharma*, Paras Gaur, Attila Borics, Edit Wéber, Ernő Kiss, and Lajos Haracska (2022). A series of xanthenes inhibiting Rad6 function and Rad6–Rad18 interaction in the PCNA ubiquitination cascade. iScience https://doi.org/10.1016/j.isci.2022.104053 (*shared first authors) IF: 5.74

2. *Fenteany, G., *Gaur, P., Sharma, G., Pintér, L., Kiss, E., & Haracska, L. (2020). Robust high-throughput assays to assess discrete steps in ubiquitination and related cascades. BMC Molecular and Cell Biology, 21(1). https://doi.org/10.1186/s12860-020-00262-5 (*shared first authors) IF: 3.227

2. Other scientific work

1. Straub days 2022 (Poster presentation)

2. EMBO chemical biology workshop, Heidelberg, September 2022 (Poster presentation). Title: High-throughput screening for small molecule inhibitors of the PCNA ubiquitination cascade

3. HCEMM PhD-Postdoc symposium, Eger, Hungary, November 2022 (Poster presentation). Title: Discovery of small-molecule inhibitors of Rad6 function and the Rad6-Rad18 interaction

4. Attended 10th CEGSDM (Central European Genome Stability and DNA Repair Meeting) in Bratislava, Slovakia, 26th-27th September 2019

Declaration

I declare that the data used in the thesis written by Gaurav Sharma reflect the contribution of the doctoral candidate to the article: “Gabriel Fenteany*, **Gaurav Sharma***, Paras Gaur, Attila Borics, Edit Wéber, Ernő Kiss, and Lajos Haracska (2022). A series of xanthenes inhibiting Rad6 function and Rad6–Rad18 interaction in the PCNA ubiquitination cascade. iScience https://doi.org/10.1016/j.isci.2022.104053 (*shared first authors) **IF: 5.74**” and “*Fenteany, G., *Gaur, P., **Sharma, G**., Pintér, L., Kiss, E., & Haracska, L. (2020). Robust high-throughput assays to assess discrete steps in ubiquitination and related cascades. BMC Molecular and Cell Biology, 21(1). https://doi.org/10.1186/s12860-020-00262-5 (Co-author) **IF:3.227**” The results reported in the Ph.D. thesis and the publication were not used to acquire any Ph.D. degree previously. I further declare that the candidate has made a

significant contribution to the creation of the above-mentioned publication.

Szeged, 06 March 2023

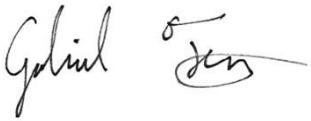
Lajos Haracska Ph.D., D.Sc.

Co-authors Declaration

I declare that the data used in the thesis written by Gaurav Sharma reflect the contribution of the doctoral candidate to the article: “Gabriel Fenteany*, **Gaurav Sharma***, Paras Gaur, Attila Borics, Edit Wéber, Ernő Kiss, and Lajos Haracska (2022). A series of xanthenes inhibiting Rad6 function and Rad6–Rad18 interaction in the PCNA ubiquitination cascade. iScience https://doi.org/10.1016/j.isci.2022.104053 **(*shared first authors) IF: 5.74**” and “*Fenteany, G., *Gaur, P., **Sharma, G**., Pintér, L., Kiss, E., & Haracska, L. (2020). Robust high-throughput assays to assess discrete steps in ubiquitination and related cascades. BMC Molecular and Cell Biology, 21(1). https://doi.org/10.1186/s12860-020-00262-5 **(Co-author) IF:3.227**”

The results reported in the Ph.D. thesis and the publications were not used to acquire any Ph.D. degree previously. I further declare that the candidate has made a significant contribution to the creation of the above-mentioned publications.

Szeged, 06 March 2023



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