

**Discovery of small-molecule inhibitors of Uba1 and
development of step-specific assays for PCNA
ubiquitination**

Ph.D. Thesis Summary

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Introduction

The discovery of small-molecule inhibitors targeting different post-translational modifications has been a hot topic since the last decade. In general, post-translational modifications are important events that signal the fate of myriad proteins in the cell. Here, we have focused on a specialized post-translational modification, *i.e.* ubiquitination of proliferating cell nuclear antigen (PCNA), where 76-amino-acid residue ubiquitin gets covalently attached to PCNA, which recruits translesion DNA synthesis (TLS) polymerases which are inherently error-prone and lead to mutagenesis and carcinogenesis. Our project is focussed on the discovery of small-molecule inhibitors of PCNA ubiquitination so that TLS inhibition can become a part of cancer treatment. The small-molecule inhibitors of PCNA ubiquitination could synergize with other anti-cancer treatments and reverse drug tolerance in patients who are refractile to present treatments.

Upon DNA damage, lesions can be left unrepaired during the classical DNA repair mechanisms and these damages can stall the replication fork during the S phase of the cell cycle. Here, the specialized translesion DNA polymerases come into the play which are specialized to bypass these lesions, a process known as translesion DNA synthesis (TLS). Further, TLS gets activated by the monoubiquitination of PCNA at the stalled replication fork which involves Uba1 (UBE1) as E1 ubiquitin-activating enzyme, Rad6 as a ubiquitin-conjugation enzyme complex with Rad18 as E3 ubiquitin ligase and PCNA which is the ultimate substrate of ubiquitination.

There can be different ways of targeting error-prone translesion DNA synthesis either by inhibition of activity of an individual protein involved in PCNA ubiquitination, by direct binding, disruption of a specific regulatory domain/function and/or by blocking regulatory protein-protein interactions. In order to target TLS we have developed a high throughput step-specific screening assay to discover small-molecule inhibitor of PCNA ubiquitination, ultimately epigallocatechin gallate (EGCG) an inhibitor of PCNA ubiquitination targeting Uba1 protein in the ubiquitination cascade was discovered.

Aims

The main goal of the project was to develop various high-throughput step-specific PCNA ubiquitination assays and discover modulators of the PCNA ubiquitination reaction cascade. PCNA ubiquitination is a key early event in DNA damage tolerance processes, it recruits TLS polymerases at stalled replication fork. The TLS polymerases are inherently error-prone and lead to mutagenesis and carcinogenesis. So, PCNA ubiquitination is a potential therapeutic target in combination with currently known cancer therapeutics.

The following aims were envisaged:

- To develop a robust, reliable and sensitive high-throughput assay to quantitatively measure PCNA ubiquitination, based on the amplified luminescent proximity homogeneous assay (Alpha) technology.
- Carry out screening of chemical libraries to discover modulators of the PCNA ubiquitination reaction.
- Hit characterization and structure-activity relationship (SAR) studies.

- To develop secondary assays based on the Alpha system for secondary screening to reveal the specific step and protein targeted in the reaction.

Materials and Methods

Compounds source: The chemical library was purchased from Avicor Ltd. The screening hit EGCG and its 11 analogs were purchased commercially from Avicor, Selleck Chemicals and Adooq Biosciences. EGCG and its analogs were dissolved in DMSO with final concentration of 50 mM for *in vivo* experiments and 10 mM for *in vitro* experiments.

PCNA Ubiquitination: The PCNA loading and ubiquitination cascade was reconstituted in 96-well white round-bottom polypropylene plates (Greiner). Compounds were added to the samples, with pre-incubation for 15 min at 25°C before the addition of ATP (to initiate the reaction cascade), followed by incubation for 2 h at 25°C. The reactions were then terminated and analyzed for Alpha assays or for western blot analyses or for silver staining.

Uba1~Ubiquitin thioester assay: FLAG-Uba1 was charged with biotin-ubiquitin by adding ATP. The reaction was incubated for 30 minutes at 25°C and stopped by adding 20 mM of EDTA and Acceptor and Donor beads in a buffer were added with 10x dilution followed by incubation for 4 h at 25°C and for Western blot and silver staining Laemmli sample buffer was added into the reaction.

Rad6-Ubiquitin thioester assay: Variable concentrations of FLAG-Rad6, with biotin-ubiquitin held at constant and variable concentrations of biotin-ubiquitin, with FLAG-Rad6 held constant, were each separately varied under identical reaction and assay conditions in a

buffer. Uba1 concentration was 50 nM in these experiments. The reaction was stopped by adding EDTA or Laemmli sample buffer for western blot analyses or silver staining, and Acceptor and Donor beads in a buffer were added with 10x dilution. The reaction was incubated for 4 h at 25°C.

Rad6-Rad18 interaction assay: FLAG-Rad6 and His-Rad18 equimolar concentrations were varied together under otherwise identical reaction and assay conditions. The reaction was incubated at 25°C for 30 min, then diluted by a factor of 10' in a buffer consisting of 25 mM HEPES, pH 7.5, 100 mM NaCl, 0.1% Tween 20 and 20 mM EDTA with donor and acceptor beads at 10 mg/ml. After 2 h of incubation at 25°C in the dark, the plates were read on the Tecan plate reader at 23 °C.

Alpha assay for Rad18 auto-ubiquitination: FLAG-Rad18 and biotin-ubiquitin concentrations were separately varied under identical reaction and assay conditions in a buffer. The reaction was incubated at 25°C for 1 h, then terminated and diluted by a factor of 10' in a buffer consisting of donor and acceptor beads. After 4 h of incubation at 25°C in the dark, the plates were read on the Tecan plate reader at 23°C.

Cell culture: For cellular experiments, HEK 293FT cells (a fast-growing SV40 large T antigen-transformed strain of HEK 293 cells designed for enhanced transgene expression in transient transfections) were grown in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified cell culture incubator at 37°C with 5% CO₂.

Cell survival assay: HEK 293FT cells were evaluated for survival following compound treatments by the Alamar Blue (resazurin) assay. Cells were plated onto 96-well plates at 4.8

$\times 10^4$ cells/well in 0.5% FBS-containing DMEM at 37 °C and 5% CO₂. After 24 h, compounds were added. Further, followed by 24 h of treatment, resazurin was added to a final concentration of 0.12 mM. Following 4 h of incubation, conversion to the resorufin product was measured (with excitation at 542 nm and emission at 590 nm) on a fluorescence plate reader.

Cell transfections: HEK 293FT cells (1.5×10^6) were plated onto 6-cm plates in 5 ml of DMEM with 10% FBS and allowed to grow for 24 h at 37 °C and 5% CO₂. For transfections, 6 μ g of plasmid DNA (an empty vector containing FLAG-tag sequence only, FLAG-Uba1, or FLAG-ubiquitin) and 10 μ l of Lipofectamine 2000 were used for each plate.

Cellular ubiquitination: HEK 293FT cells were plated in 6-well plates. After 24 h, EGCG was added; then 30 min later, MG132 was added to a concentration of 50 μ M, with incubation for another 30 min. Cells were harvested and whole-cell lysates were prepared in RIPA lysis buffer containing a protease inhibitor cocktail (SIGMAFAST from Sigma-Aldrich) and sonicated. Equivalent loadings of total proteins for each treatment, as determined by Bradford assay of each extracted sample, were subjected to electrophoresis on 8% SDS-polyacrylamide gels and western blot analysis, probed with anti-ubiquitin antibody, then stripped and re-probed with an anti- β -tubulin antibody.

Statistical analysis: In a high-throughput assay (HTS); large chemical library was screened, hits were identified as an active compound that showed inhibition of PCNA ubiquitination. Various statistical parameters were considered to have high confidence in hits.

Signal-to-noise (S/N), Signal-to-background (S/B), z-prime, z-robust, Strictly standardized mean difference (SSMD), Signal window values and Half-maximal inhibitory concentration IC_{50} .

To calculate S/N, S/B, SSMD, Z factor and signal to window values an excel sheet template was prepared which automatically calculated these values. All the Curve fitting, IC_{50} calculations were carried out using the GraphPad Prism 8.0–8.2 software.

Results

Development of a high-throughput screening assay to discover modulators of PCNA

ubiquitination: 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 PerkinElmers Alphascreen/AlphaLisa system.

Optimization and miniaturization of the PCNA-ubiquitination-based Alpha assay:

Based on the sum of all the optimization experiments for both the PCNA ubiquitination reaction and the Alpha detection step, the final conditions were chosen. The PCNA ubiquitination reaction with 10 nM RFC, 10 nM Uba1, 100 nM Rad6–Rad18 dimer, 50 nM FLAG-PCNA, 250 nM biotin-ubiquitin and 2 nM nicked pUC19 was initiated by addition of 2 mM ATP in a reaction volume of 20 μ l in 96-well plates. The reaction was incubated for 2 h at 25 °C and after that diluted by a factor of 10⁷ in Alpha buffer containing donor and acceptor beads (10 mg/ml each) and 20 mM EDTA, with incubation of 4 h in the dark at 25 °C, followed by the Alpha detection.

EGCG inhibits PCNA ubiquitination: We screened a chemical library from which we identified EGCG as a potential bioactive hit that inhibits PCNA ubiquitination.

Dose-response analysis for different compounds in PCNA ubiquitination: The dose-response experiments were performed with EGCG and its analogs using western blot analysis. Dose-response experiments revealed that EGCG inhibits PCNA ubiquitination with a half-maximal inhibitory concentration (IC_{50}) of 228 nM.

EGCG blocks Uba1-ubiquitin thioester formation: To identify target proteins involved in the ubiquitination pathway, we investigated each small step involved in ubiquitination. We, therefore, looked at a specific step in the ubiquitination cascade and found that EGCG directly inhibits the formation of Uba1-ubiquitin thioester formation by directly targeting the Uba1 protein. We found out that EGCG directly targets Uba1 protein. These results were further supported by 1H NMR, STD NMR and TrNOE experiments.

Dose-response analysis for Uba1~ubiquitin thioester formation: The dose-response for the relative percentage of Uba1~ubiquitin thioester formation with increasing concentrations of each compound was calculated by gel silver staining. The calculated IC_{50} of EGCG is 1.6 μ M.

Uba1 or ubiquitin protects the cells from cytotoxic effects of EGCG and EGCG reduces the global ubiquitination in cells: HEK 293 cells overexpressing FLAG-tagged Uba1 or FLAG-tagged ubiquitin exhibited reduced sensitivity to EGCG compared to cells transfected with the FLAG-tag-containing empty vector alone. EGCG treatment inhibited the accumulation of ubiquitin-conjugated protein species in HEK 293 cells

Structure activity relationship (SAR) studies: SAR with EGCG analogs reveals distinct groupings of congeners by structure, activity profile and mechanisms of action.

Robust high-throughput assays to assess discrete steps in PCNA ubiquitination: We developed a high-throughput Alpha-based step-specific PCNA ubiquitination assay that balances high sensitivity with low consumption of reagents, as well as analogous assays to probe Uba1~ubiquitin thioester formation, Rad6~ubiquitin thioester formation, Rad6-Rad18 interaction and Rad18 auto-ubiquitination.

Summary

Cells are continuously exposed to various DNA damage some of the damages get repaired by classical DNA damage repair mechanisms while some are left unrepaired. The unrepaired DNA damages can stall the progressive replication fork because classical DNA polymerases are unable to process the lesions. In this scenario, PCNA undergoes monoubiquitination at K164 residue and it works as a molecular switch that switches classical DNA polymerases with specialized translesion DNA polymerases (TLS polymerases) which are specialized to bypass the lesion. The TLS polymerases are inherently error-prone. Our project is focussed on the discovery of small-molecule inhibitors of PCNA ubiquitination so that TLS inhibition can become a part of cancer treatment. The small-molecule inhibitors of the TLS pathway can be a great option in combination with current cancer therapeutics.

We have developed robust high-throughput step-specific assays for PCNA monoubiquitination, and by screening the chemical libraries we have identified a green tea polyphenol epigallocatechin gallate (EGCG) as a potent inhibitor of PCNA ubiquitination. EGCG targets Uba1 and blocks Uba1~ubiquitin thioester formation. We studied EGCG and

its 10 analogs on Uba1~ubiquitin thioester assay, PCNA ubiquitination assay, cell viability assay, overexpression of Uba1/ubiquitin assay (to see whether overexpression protects the cells from cytotoxic effects of EGCG) and global ubiquitination of cells. All the assays supported the fact that EGCG targets Uba1 and overexpression of either Uba1 or ubiquitin protects the cells. The structure-activity relationship (SAR) studies of EGCG and its analogs for all the assays suggested that all molecules have different mechanisms of action. As a result, we classified EGCG analogs in four simple classes as catechin gallate series, simple catechin series, myricetin series and simple gallate series. Each series of the bioactive small molecule identified represents a different first-in-class type of chemical modulator of ubiquitination, a hierarchical cascade of reactions that has drawn great interest from both the basic research and drug discovery communities. In the future, improved specificity and selectivity may arise from the assessment of further natural, semisynthetic, or synthetic congeners of the pharmacophore types delineated in the present study, yielding valuable therapeutic drug leads and tools from these different structural and mechanistic sets of molecules.

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List of Publications

MTMT number: 10069362

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

- *Fenteany, G., ***Gaur, P.**, Hegedűs, L., Dudás, K., Kiss, E., Wéber, E., Hackler, L., Martinek, T., Puskás, L.G., and Haracska, L (2019). Multilevel structure–activity profiling reveals multiple green tea compound families that each modulate ubiquitin-activating enzyme and ubiquitination by a distinct mechanism. *Scientific Reports*, 9(1). <https://doi.org/10.1038/s41598-019-48888-6> (*shared first authors) **IF: 4.525**
- *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

- Talk entitled “Small molecule inhibitors of PCNA ubiquitination and post replication repair” at the 10th CEGSDM (Central European Genome Stability and DNA Repair Meeting) in Bratislava, Slovakia, 26th-27th September 2019
- Talk entitled “PCNA Orchestrates the DNA Damage Tolerance Pathway” at the Annual Conference of the Doctoral School of Biology and UNKP Conference (2018), Szeged, Hungary, 28th -30th May 2018

- Poster entitled “Targeting Translesion DNA Synthesis for Cancer Therapeutics” at the 9th CEGSDM (Central European Genome Stability and DNA Repair Meeting) in Warsaw, Poland, 13th-14th September 2018
- Poster entitled “Fighting Fatal Errors: *Targeting Translesion DNA synthesis to Kill Cancer*” at the GINOP conference held at the University of Debrecen, Debrecen, Hungary on 14th -15th June 2018
- Poster entitled “*Using Artificial Amino Acid to study the interaction between Polymerase η and PCNA*” at the Central European Genome Stability Meeting Research Centre for Natural Sciences, Budapest, Hungary on 29th-30th September 2017
- Poster entitled “*Artificial Amino Acid and its application in Protein-Protein interactions*” at the Straub-days conference held at BRC, Szeged, Hungary on 24th-25th May 2017
- Attended International conference on “Cellular and molecular mechanisms of disease processes” at the Department of Biotechnology, the University of Kashmir from 13th-16th April 2014

Declaration

I declare that the data used in the thesis written by Paras Gaur reflect the contribution of the doctoral candidate to the article: “*Fenteany, G., ***Gaur, P.**, Hegedűs, L., Dudás, K., Kiss, E., Wéber, E., Hackler, L., Martinek, T., Puskás, L.G., and Haracska, L (2019). Multilevel structure–activity profiling reveals multiple green tea compound families that each modulate ubiquitin-activating enzyme and ubiquitination by a distinct mechanism. *Scientific Reports*, 9(1). <https://doi.org/10.1038/s41598-019-48888-6> (***shared first authors**) **IF: 4.525**” 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> (***shared first authors**) **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, 04 June 2020

Lajos Haracska Ph.D., D.Sc.

Co-authors Declaration

I declare that the data used in the thesis written by Paras Gaur reflect the contribution of the doctoral candidate to the article: “*Fenteany, G., ***Gaur, P.**, Hegedűs, L., Dudás, K., Kiss, E., Wéber, E., Hackler, L., Martinek, T., Puskás, L.G., and Haracska, L (2019). Multilevel structure–activity profiling reveals multiple green tea compound families that each modulate ubiquitin-activating enzyme and ubiquitination by a distinct mechanism. *Scientific Reports*, 9(1). <https://doi.org/10.1038/s41598-019-48888-6> (***shared first authors**) **IF: 4.525**” 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> (***shared first authors**) **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, 04 June 2020

Gabriel Fenteany, Ph.D.