During the projects we have invested efforts into the development of new covalent binders and novel approaches in the field of covalent inhibition. From this research work ten papers were accepted and published, and we have submitted two manuscripts that was requested to be revised. Moreover, we are aiming to submit three more papers that are under preparation. The main results achieved are as follows:

1. A systematically designed heterocyclic electrophilic library from a broad scope of five- and six-membered nitrogen containing heterocycles (pyridines, pyrimidines, pyrazines, pyrazoles, imidazoles, oxazoles, thiazoles) and six electrophilic warheads (Cl, Br, I, CN, vinyl, ethynyl groups) has been generated (86-membered) and characterized (reactivity, cysteine-selectivity). The library was investigated on several protein targets, in particular on MurA, MAO-A, MAO-B, thrombin, the immunoproteasome, HDAC8, Histamine H3 and H4 receptors and on the KRas G12C oncogenic mutant. In most cases valuable starting points have been found for further development. Lead-like compounds against MurA, MAO-A, MAO-B, the immunoproteasome, HDAC8 and KRas G12C have been synthesized. MurA inhibitors did not show considerable activity, thus X-Ray crystallographic investigation was started for finding the appropriate growing vectors. In the case of MAO-A, MAO-B and the immunoproteasome the synthetic efforts have been unsuccessful, this project have been put on hold. For HDAC8 and KRas G12C the lead-like compounds showed promising activity, further development is still ongoing. Two publications were accepted from this work. During the COVID-19 pandemia participating in a large international collaboration we have identified heterocyclic electrophiles binding to the 3CL main protease of COVID-12. This is the basis of a future research project based on the heterocyclic covalent fragments.

Keeley, A.; Ábrányi-Balogh, P.; Hrast, M.; Imre, T.; Ilas, J.; Gobec, S.; Keserű, G. M. Heterocyclic covalent warheads as new MurA inhibitors. Arch Pharm Chem Life Sci. 2018;351:e1800184 1–7. https://doi.org/10.1002/ardp.201800184

Keeley, A.; Ábrányi-Balogh, P.; Keserű, G. M. Design and characterization of a heterocyclic covalent fragment library for the discovery of cysteine targeted covalent inhibitors. Med. Chem. Commun.2018, 10, 263-267.

Wágner, G.; Mocking, T. A. M.; Kooistra, A. J.; Slynko, I.; Ábrányi-Balogh, P.; Keserű, Gy. M.; Wijtmans, M.; Vischer, H. F.; de Esch, I. J. P.; Leurs, R. Covalent inhibition of the histamine H3 receptor, Molecules, 2019, 24, 4541-4560.

Douangamath, A.; Fearon, D.; Gehrtz, P.; Krojer, T.; Lukacik, P.; Owen, C. D.; Resnick, E.; Strain-Damerell, C.; Aimon, A.; Ábrányi-Balogh, P.; Brandaõ-Neto, J.; Carbery, A.; Davison, G.; Dias, A.; Downes, T. D.; Dunnett, L.; Fairhead, M.; Firth, J. D.; Jones, S. P.; Keeley, A.; Keserű, G. M.; Klein, H. F.; Martin, M. P.; Noble, M. E. M.; O'Brien, P.; Powell, A.; Reddi, R.; Skyner, R.; Snee, M.; Waring, M. J.; Wild, C.; London, N.; von Delft, F.; Walsh, M. A. Crystallographic and electrophilic fragment screening of the SARS-CoV-2 main protease. Nat. Commun. 2020, accepted. bioRxiv 2020.05.27.118117; doi: https://doi.org/10.1101/2020.05.27.118117.

2. We have filtered our formerly generated 137-membered library for OH-reactive compounds, and we could identify Tyr or Thr reactive and/or selective compounds. We did not identify any serine-selective fragment. As no serine-selective compounds were identified, we have decided to target the cysteine of JAK3 instead of JAK1 and JAK2.

3. A 300-membered diverse covalent fragment library has been screened on the DJ1 protein in a glyoxalase assay, and several cysteine-selective starting points have been identified. The X-Ray crystallography of DJ1 protein together with

CFL55 and SzR07 compounds was successful, the Cys106 labelling has been proven, thus the synthesis and biological testing of a library of isoquinoline and quinoline-derivatives was attempted, and 30 new analogues have been investigated. Deeper biological testing is ongoing, the manuscript is under preparation.

3. A set of 28 covalent fragments with the same non-covalent core, but with different warheads has been developed for mapping the accessibility and tractability of cysteines in relevant targets. This library has been screened on MurA, MAO-A, HDAC-8, the immunoproteasome, KRas G12C and several kinases, in particular RSK2, ERK2, BTK, MELK, MAP2K6 and JAK3. We have concluded that the cysteines in the target proteins discriminate between the electrophilic warheads. We have chosen JAK3 and MELK to design lead-like covalent inhibitors. Biochemical assay proved the high affinity, while MSMS and NMR measurements proved the covalent binding. The published manuscript shows the retrospective identification of efficient JAK3 inihibitors, and this is the first presentation of a covalent MELK inhibitor.

Petri, L.; Egyed, A.; Bajusz, D.; Imre, T.; Hetényi, A.; Martinek, T.; Ábrányi-Balogh, P.; Keserű, G. M. An electrophilic warhead library for mapping the reactivity and accessibility of tractable cysteines in protein kinases. Eur. J. Med. Chem. 2020, 207, 112836-112845.

Petri, L.; Ábrányi-Balogh, P.; Imre, T.; Pálfy, Gy.; Perczel, A.; Knez, D.; Hrast, M.; Gobec, M.; Sosic, I.; Nyíri, K.; Vértessy, B. G.; Jänsch, N.; Desczyk, C.; Meyer-Almes, F.-J.; Ogris, I.; Golic Grdadolnik, S.; Iacovino, L. G.; Binda, C.; Gobec, S.; Keserű, Gy. M. Assessment of tractable cysteines by covalent fragments screening, Bioorg. Chem. 2020, under revision

5. Five covalent fragments were tested for the inhibition of the intrinsically disordered protein-protein interaction of calpain and calpastatin in the cooperation of the research group of Peter Tompa at VUB. It was proven that even small covalent fragments are able to significantly inhibit the PP interaction. The development of larger lead-like compounds have been performed, and a publication has been accepted from this work. In addition, based on our former experiences we have screened a covalent fragment library against the intrinsically disordered protein tau (K18 derivative) responsible for Alzheimer's and Pick's disease. We have successfully identified covalently binding compounds by MS, MS/MS and NMR-based methods. The design and synthesis of lead-like analogues have been performed, and the compounds successfully inhibited partially the aggregation of tau. The development of further compounds and the publication is still ongoing.

Nguyen, H.; Ábrányi-Balogh, P.; Petri, L.; Mészáros, A.; Pauwels, K.; Vandenbussche, G.; Keserű, Gy.; Tompa, P. Targeting an Intrinsically Disordered Protein by Covalent Modification. Meth. Mol. Biol. 2020, 2141:835-854. doi: 10.1007/978-1-0716-0524-0_43.

6. We have identified reversible inhibitors against MurA and MAO-A from the screening of our 137-membered diverse covalent fragment library. Moreover, in the mapping library the reversibility of the cyanoacrylamide functional group has been experimentally proven, and it has shown significant activity against kinase targets.

Ábrányi-Balogh, P.; Petri, L.; Scarpino, A.; Imre, T.; Hrast, M.; Mitrović, A.; Pečar Fonovič, U.; Németh, K.; Barreteau, H.; Roper, D. I.; Horváti, K.; Ferenczy, G. G.; Kos, J.; Ilaš, J.; Keserű, G. M.; Gobec, S. A road map for optimizing the warheads of targeted covalent inhibitors. Eur. J. Med. Chem. 2018, 160, 94-107.

Petri, L.; Egyed, A.; Bajusz, D.; Imre, T.; Hetényi, A.; Martinek, T.; Ábrányi-Balogh, P.; Keserű, G. M. An electrophilic warhead library for mapping the reactivity and accessibility of tractable cysteines in protein kinases. Eur. J. Med. Chem. 2020, 207, 112836-112845.

7. The reactivity profile of small-molecule cysteine surrogates has been investigated and compared to each other. We have claimed that the different surrogates discriminate very differently between the same covalent fragments, and GSH is the most effective one that is suggested to use.

Petri, L.; Ábrányi-Balogh, P.; Varga, P. R.; Imre, T.; Keserű, Gy. M. Comparative reactivity analysis of small-molecule thiol surrogates, Bioorg. Med. Chem., 2020, 28, 115357.

8. Targeting antibodies as target proteins we have investigated in cooperation with the group of Vijay Chudasama at the University College of London the covalent binding of isothiocyanates. Considering the binding reaction at different pHs and the effect of the non-covalent scaffold on the ITC warhead, we have optimized the labelling of the antibody herceptin. The isothiocyanate-labelling of antibodies was extended also to trastuzumab and ontuzumab Fab structural moiety. A new fluorescein analogue was synthesized based on the optimized labelling protocol. Moreover, using click reactions on azido-dyes and alkynyl substituted synthesized covalent fragments, trastuzumab was labelled with various organic dyes capable for confocal, super-resolutional and two-photon microscopy. In addition, we have successfully modified the cytostatic drug mertansine to be clickable on trastuztumab. The evaluation of the labelled antibodies have been performed by flow-cytometry and the imaging of labelled cellular samples as well, as cytotoxicity investigations. Finally, the development of new warheads and new reconjugation and labelling methods have been started, from five agents one promising compound has been chosen for futher development.

Petri, L.; Szijj, P. A.; Kelemen, Á.; Imre, T.; Gömöry, Á.; Lee, M. T. W.; Hegerdűs, K.; Ábrányi-Balogh, P.; Chudasama, V.; Keserű, G. M. Cysteine specific bioconjugation with benzyl isothiocyanates, RSC Adv., 2020, 10, 14928-14936.

Further publications regarding the development of covalent inhibitors:

We have published a review paper after invitation to a highly rated journal of drug discovery:

Keeley, A.; Petri, L.; Ábrányi-Balogh, P.; Keserű, Gy. Covalent fragment libraries in drug discovery. Drug. Discov. Today 2020, 25, 983-996.

A computational protocol for the screening of covalent inhibitors:

Scarpino, A.; Petri, L.; Damijan, K.; Imre, T.; Ábrányi-Balogh, P.; Ferenczy, G.; Gobec, S.; Keserű, G. M. WIDOCK: a Reactive Docking Protocol for Virtual Screening of Covalent Inhibitors. J. Comp. Aid. Mol. Des. 2020, under revision