Final progress report

YEAR 1

In the first year the aim of the project was to measure and compare the mutagenicity of commonly used platinum-based antineoplastic drugs in different cell culture based model systems. My earlier experiments used the DT40 chicken cell line, but for the present study I used a human cell line, TK6.

1.1. Testing of different human cell lines used as a model

In the first year of my project I started to work with three different human cell lines, namely NALM-6 (Human B cell precursor leukemia), DG-75 (Burkitt-lymphoma cell line) and TK6 (human lymphoblastoid cell line). Finally my choice fell on TK6 cell line. This cell line has normal karyotype, without known DNA repair defects. TK6 is a standard human cell line used for in vitro genotoxicity tests. The cell proliferation, colony formation and transfectional rate of TK6 is of high efficiency, these are suspensional cells so we can handle them easily, and the cell line has stable karyotype. TK6 was originally developed for the thymidine kinase (TK) gene mutation assay. Thanks to the TK6 Mutants Consortium (collaboration with Shunichi Takeda, Kyoto University, Kyoto), different genetically modified, DNA repair mutant TK6 cell lines –BRCA1 among others – are available to us.

1.2. Validation of the parameters of the cytotoxic treatments

Before starting the sensitivity tests and the cytotoxic treatments, I isolated single cell clones from the full population of TK6 cell line. I chose one single cell clone that I dedicated as starting clone. I isolated genomic DNA from the starting clone, that would be our reference sample. In the whole genome sequencing analysis every treated sample was compared to the starting clone. My aim was to determine the mutagenicity of selected chemotherapeutic agents that cause DNA damage or inhibit DNA repair. The platinum-based chemotherapy is widely used in the case of many types of cancer. The main mechanism of these drugs is well-known, the main target of platinum drugs is the DNA, they bind to N7-guanine, and then to a nearby guanine (G) or sometimes adenine (A). These drugs in 98 % of cases form GG- AG- intrastrand crosslinks. However platinum can cause interstrand- and DNA-protein crosslinks as well. I measured the platinum, namely cisplatin, carboplatin and oxaliplatin sensitivities of TK6 starting clone by cytotoxicity assay. I worked based on the previous results of chicken DT40 cell line sensitivities. In the cytotoxicity assays the treatments lasted for 1 hour, and the drugs were added as a simple 2 or 3-fold dilution series. The highest concentrations used were 100 uM for cisplatin, 750 uM for carboplatin and 25 uM for oxaliplatin. After 1 hour long treatment, I changed the media to fresh, pre-warmed, drug-free media, and I measured 3000 treated cells onto 96-well plates. The cells were allowed to recover for 72 hours. After the recovery phase I used PrestoBlue (Invitrogen) cell viability reagent to analyse the cell survival rate using a fluorescent plate reader (EnSpire). I determined the half maximal inhibitory concentration values (IC50) of these platinum drugs. I performed the long-term platinum treatments with 6 uM cisplatin, 120 uM carboplatin and 4 uM oxaliplatin dosage.

1.3. Cytotoxic treatments for the determination of mutagenesis by whole genome sequencing

In the knowledge of the IC50 values I started the cytotoxicity treatments with the WT TK6 human lymphoblastoid cell line. I treated starting single cell clone that I isolated before (1.2). I

exposured 10^6 cells with platinum drugs for 1 hour. After the treatments the cells were allowed to recover for a week. On the seventh day of the recovery phase I repeated the treatment. This long-term treatment lasted for four weeks, one treatment per week. The recovered four-times treated cells were cloned again. I selected six independent single cell clones from which I isolated genomic DNA according to the Puregene DNA Purification Kit. I used the DNA purification protocol for 1-2 million cells.
Every treated cell population was grown for 50 days. My aim was also to measure the spontaneous mutation of TK6 cell line as well, so I made a so-called „mock" treated sample. I grew the starting clone for 50 days in parallel with the treated cells and isolated single clones at the end of the mock treatment.
After the DNA isolation I checked the quantity and the quality of the genomic DNA preparations on Nanodrop ND-100 and Qubit instruments. I also checked the DNA samples on agarose gel electrophoresis and I sent them to a sequencing laboratory (Novogene). The mutation rates were determined by next-generation sequencing (Illumina HiSeq X Ten platform) of the whole genomes of the starting clones and treated clones.
In collaboration with Gergely Szakács's Research Group at the Institute of Enzymology we also treated a mouse cell line with platinum drugs to assay the mutagenic effect of platinum therapy.

1.4. Experiments for the direct detection of platinum-induced DNA damage

To explain the mutagenic effect of different platinum drugs, and correlate their mutagenicity with the chemical effect of the drugs, I performed experiments aimed at directly detecting treatment-induced DNA damage.
To measure DNA damage in the individual eukaryotic cells I performed alkaline comet assays, a technique that is also known as single cell gel electrophoresis. This assay is suitable for detecting single- and double strand breaks in the level of the individual cell. I treated chicken DT40 and human TK6 cells with platinum drugs for 1 hour. After the treatments the cells were allowed to recover for 24 hours. Then cells were collected, embedded into low-melting agarose and electrophorised. Finally I stained the cells with propidium-iodide and I analysed them with fluorescent microscopy (Leica DM IL LED). The experiments were evaluated with CometScore program. I detected the highest number of the DNA strand breaks after cisplatin treatment.
I also performed western blot analysis to detect the relative level of γH2AX protein after platinum treatments. When DNA damage occurs, it may form double stranded breaks, which is followed by the phosphorylation of the histone H2AX. ATM and ATR kinases are involved in the phosphorylation process. This newly phosphorylated protein is called γH2AX, which is a biomarker for DNA breaks. We were able to show that the DNA break formation after platinum induced DNA damage depends on the concentration of the treatment and also the length of the recovery time. α–tubulin was used to normalize three independent biological repeats. When comparing cisplatin, carboplatin and oxaliplatin treatments at equitoxic concentrations, I detected the highest relative level of γH2AX protein after cisplatin treatment. I also checked the γH2AX foci after platinum treatments with subnuclear immunofluorescent (IF) technique. This assay is suitable for the detection and visualization of γH2AX. The results of the IF analysis confirmed the western blot results: I detected the highest number of foci after cisplatin treatment.
I can state that equitoxic cisplatin treatment caused higher level of DNA damage compared to the other two tested platinum drugs, carboplatin and oxaliplatin. In the background of these results it is known that cisplatin causes the most severe common side effects for patients, for example ototoxicity (hearing loss), nephrotoxicity (kidney toxicity), nausea and vomiting. All of these facts can mean that cisplatin is likely the most toxic platinum drug compared to carbo-

and oxaliplatin.

YEAR 2

In the second year on the one hand we analysed the whole genome sequence data from cells treated with cytotoxic agents, on the other hand I developed cyclophosphamide metabolic activation techniques which can be used to analyse the mutagenic effect of cyclophosphamide in vitro.

2.1. Analysis of whole genome sequence data from cells treated with cytotoxic agents

We analysed the cytotoxic treatment induced mutational spectra obtained from whole genome sequencing in section 1.3 of the project. This analysis provided important data on the mutagenic effect of the tested platinum-based agents. After platinum drug long-term treatments, I generated single-cell clones from the cisplatin, carboplatin and oxaliplatin-treated cells. In the case of DT40 cells, we sequenced five independent clones, while in the case of TK6 we examined three clones. The chicken DT40 and human TK6 cell lines have relatively stable genotypes with low spontaneous mutation rates, which makes them well suited to mutagenesis studies. These cell lines are suitable for modeling the mutational processes that take place in human cancer cells. The mutation rates were determined by next-generation sequencing (Illumina HiSeq X Ten and BGISeq platform) of the whole genomes of treated cell clones. The mutagenic spectra of the tested agents were analysed using the IsoMut mutation detection tool. We found that cisplatin was considerably mutagenic and induced both single nucleotide variations and short insertions / deletions. The detailed analysis of the sequence context of the mutations showed that most occur at cisplatin-induced intrastrand DNA crosslinks, typically at GG or AG dinucleotides. We found that at equitoxic concentration, carboplatin and oxaliplatin treatments caused a lower number of mutations than cisplatin, although induced similar mutational spectra. Cisplatin also caused more DNA damage upon equitoxic treatments than carbo- and oxaliplatin. The cause of the higher mutagenic effect and DNA damage level of cisplatin treatment may lie in differences of drug mechanism, suggesting that carboplatin and oxaliplatin kill the cells by a slightly different mechanism than cisplatin.
In conclusion, this genomic study demonstrated greater mutagenicity of cisplatin compared to carboplatin and oxaliplatin in two cell lines. We showed that platinum drugs exert direct as well as indirect mutagenic effects, of which the direct mutagenic effects correlate with the amount of DNA damage caused by the treatments but not with cytotoxicity. Our results can contribute to a careful appraisal of the benefits versus the short-term and long-term side effects of platinum-containing chemotherapeutics to guide therapeutic choices.
The manuscript containing these results was submitted to the journal Mutagenesis, the manuscript is currently under revision.

2.2. Testing cyclophosphamide metabolic activation strategies

In the first period of the second year I set up some experimental strategies to activate the prodrug cyclophosphamide (CPA). CPA is a member of the nitrogen-mustard type drugs, an alkylating chemotherapeutical agent. This drug is used to treat various types of cancer, for example Burkitt lymphoma, leukemia, myeloma, lung and breast cancer. Besides, this drug also has immunesuppressive effects. CPA treatment increases the risk of development of secondary tumors, so the knowledge of the mutagenic effect of CPA is important and relevant in the field of oncology. CPA is a prodrug which is activated in the liver by the enzymes of cytochrome

P450 family, mainly by CYP2B6 in the human system. The active forms of CPA are acrolein and phosphoramide mustard (PM), of which PM has an anticancer effect. PM can interact with the DNA, cause monoadducts and interstrand crosslinks. My aim was to measure the mutagenic effect of cyclophosphamide in a cell line based model system. An activation and metabolisation strategy was neccessary for my experiments, because our lymphoblastoid cell lines do not express any of the cytochrome P450 enzymes. I have tried to develop different CPA activation methods. In our first approach we isolated liver extracts from rat and prepared sterile S9 lysates. I preincubated the S9 lysates with CPA, and the presumably active CPA was tested. I treated DT40 lymphoblastoid chicken cell line with rat-liver S9 extract-activated CPA and also with the inactivated, „intact" prodrug form of CPA. After 1-hour long treatments we performed colony formation assays and we compared the sensitivities of the the activated form to the prodrug CPA. According to our results, the S9 extract activation method was successful, we were able to metabolize the CPA via rat-liver isolated S9 extract. For our second approach, we aimed to generate a genome-edited knock-in DT40 cell line that expresses the CYP enzyme responsible for CPA metabolism. It was known from the literature that CYP2C18 takes part the activation process of CPA in the chicken system. So I isolated total RNA from chicken liver and made full-length cDNA by reverse transcription with oligo d(T)18 primer. From the cDNA-pool I amplified the relevant CYP2C18 enzyme sequence with PCR, and cloned the CYP2C18 cDNA into an expression plasmid (pLox-IRES-GFP) that was transfected into DT40 cells by stable transfection. I tested the successfully generated CYP2C18 knock-in DT40 cell line in several ways. I checked the CYP2C18 mRNA expression by RT-qPCR and compared it to the liver CYP2C18 mRNA level. I detected 11,25-fold higher expression level in our knock-in cell line compared to the expression level in the chicken liver. We have also measured and compared the CPA sensitivity of the CYP2C18-expressing and the control cell line using colony survival assays. This metabolic activation method has therefore proved to be effective, and it will be used by the research group for further mutagenesis measurement projects.

2.3. Automated cytotoxiciy assays with platinum drugs

I was involved in the validation of the automated cytotoxicity assays with Hamilton Robotics liquid handling equipment. This high-throughput technique gave us the opportunity to investigate the susceptibility of distinct DNA repair defect cell lines to different cytostatics.
The results showed that there is a good correlation between the toxicity of cisplatin and carboplatin on a range of HR and checkpoint mutant cell lines, but cisplatin achieves the same level of toxicity at much lower concentrations. The effects of oxaliplatin did not correlate perfectly with those of cisplatin.
The reliability of the cytotoxicity assay is highlighted by the excellent concordance of sensitivities to drugs with common mechanisms and by the lack of sensitivity to drugs that do not cause DNA damage or target DNA repair, such as paclitaxel and hydroxyurea. The different pattern of sensitivities to oxaliplatin as compared to cisplatin and carboplatin supports findings that oxaliplatin kills cells through additional mechanisms independent of the DNA damage response.
The results were used to support the findings of a larger study in the research group that correlated homologous recombination deficiency induced mutational signatures with sensitivity to PARP inhibitors and cytotoxic agents. The results, with my co-authorship, were published in 2019 (Póti et al, Genome Biology). The established methods would also be useful for my later studies with the BRCA mutant cell lines generated in year 3.

YEAR 3

In the third year I managed to introduce certain BRCA1 germline mutations to DT40 lymphoblastoid cell line with CRISPR genome editing technique.

3.1. Introduction of BRCA1 germline mutations to a DT40 lymphoblastoid cell line with CRISPR genome editing technique

BRCA1 (the breast and ovarian cancer type 1 susceptibility protein) is important for the maintenance of genome integrity and stability. It has dedicated roles in the DNA damage response, including cell cycle checkpoint control and DNA repair. Germline mutations of the human BRCA1 gene are responsible for most familial cases of breast and ovarian cancer. BRCA1 encodes a protein of 1863 amino acids. It has three domains, the highly conserved RING domain and tandem BRCT domains, and coiled-coil domain. In the RING domain Cys61Gly and Cys64Gly, in BRCT domain Met1775Arg, and in coiled-coil domain Leu1407Pro and Met1411Thr are clinically important frequent inherited 'founder' mutations. In this study my aim was to characterise the phenotypical properties of the germline BRCA1 mutations in isogenic DT40 cell lines. We aim to find out whether the different domain mutants elicit similar or different mutagenic phenotypes, and thereby also find out how well the mutation spectra of cancer genomes can be used to make predictions about BRCA1 loss of function.
Using CRISPR technology, I have introduced specific modifications into the genome of my experimental cell line, DT40. At first I analysed the differencies and the similarities of the human and the chicken BRCA1 gene. I found that the regions with the germline SNPs are in perfect consensus between the human and chicken genes.
I have designed small guide RNAs (sgRNAs) with the CRISPOR software to introduce the above mentioned five germline BRCA1 mutations with the CRISPR-Cas9 system. After the annealing of the sgRNA strands, I cloned all sgRNAs into a pX458 plasmid vector through BbsI restriction sites. This plasmid contains the Cas9 sequence and an EGFP gene cassette, which allows us to sort out the GFP positive cells following the transient transfection of sgRNAs into DT40 cells. To control the efficient working of CRISPR-Cas9, I checked the GFP positive cells with the T7 assay. T7 endonuclease I recognizes structural variations on non-perfectly matched DNA, and cleaves there. With this assay we can determine genome targeting efficiency by digesting annealed PCR products with T7 Endonuclease I. I was able to find functional sgRNAs for the Cys61Gly and Cys64Gly mutations in the RING domain, Met1775Arg mutation in the BRCT domain, and for the Leu1407Pro and Met1411Thr mutations in the coiled-coil domain. I transfected the mutation-carrier template DNA strands with the sgRNAs at the same time. 55-65 independent single cell clones grew-up after the transfection. The clones were tested with allelespecific qPCR and High Resolution Melting (HRM) qPCR techniques using control samples (wt, mutant and heterozygous) cloned into pCR.2_TOPO plasmids. Finally I filtered out 4-5 candidate mutant clones that had different melting points compared to the wild type according to the melting temperature of the qPCR products. So far we were not able to identify any desired Cys61Gly, Cys64Gly and Met1775Arg mutant cell clone. This workflow is expected to be completed in January 2021.
As an another method of targeted genome editing is transfection of plasmid-based homology-directed repair (HDR) templates with the sgRNAs at the same time. I designed and successfully built-up pBluescript-based HDR templates for Cys61Gly, Cys64Gly, Leu1407Pro, Met1411Thr and Met1775Arg mutations. In this way I could screen the survival cell clones after transfection easily with antibiotic selection. With this approach so far I managed to create the coiled-coil domain mutant BRCA1 cell lines (Leu1407Pro, Met1411Thr). These mutations

disrupt the protein-protein interaction of BRCA1 and PALB2, so BRCA2 cannot be recruited and homologous recombination is likely to fail. My aim was to describe the phenotypic characteristics of the established cell lines, which I achieved by analyzing cytotoxicity tests with a PARP inhibitor, olaparib. Homologous recombination deficient cell lines are known to be highly sensitive to PARP inhibitors, including olaparib. Cytotoxicity and sensitivity tests with olaparib showed that the SNP mutant DT40 cells were slightly more sensitive to the drug compared to the parental, baseline WT cell line. This observation suggests that the mutations interfere with the homologous recombination function of BRCA1, and further suggests that I succeeded in mutating the wild type allele of the parental cell line in the heterozygous clones. Consequently, the resulting mutation-bearing cell lines may further provide useful information in mapping the therapeutic potential of BRCA1-associated cancer and in selecting potentially appropriate therapeutic options.
So far we were not able to identify any desired Cys61Gly, Cys64Gly and Met1775Arg mutant cell clone. This workflow is expected to be completed in January.

3.2. Development of cisplatin and olaparib resistant DT40 cell lines

BRCA1 germline SNP-mutation carrier cell lines would serve us as the basis for our cisplatin- and olaparib-resistance evolution studies. I was not able to start this part of the work because the BRCA1 mutant cell lines are not completely ready. Due to the COVID-19 epidemic situation, I was unable to perform laboratory work for 3 months because of childcare reasons, which greatly influenced the progress of the research.

Presentation of results during the PD-OTKA grant:

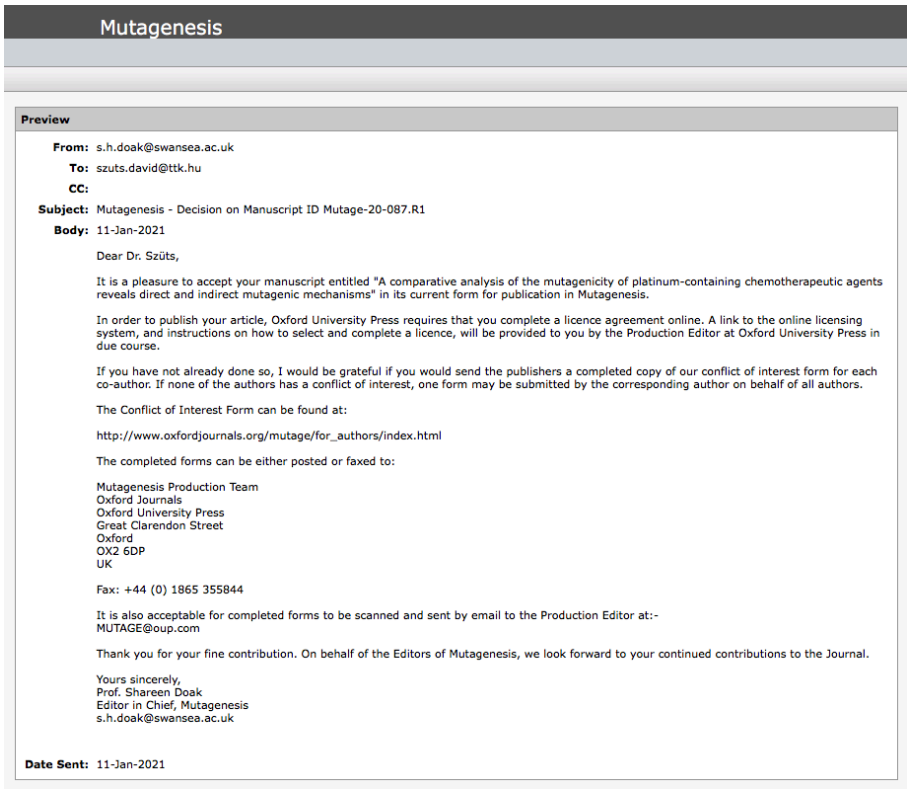
Publications:

Póti Á, Gyergyák H, Németh E, Rusz O, Tóth S, Kovácsházi C, Chen D, **Szikriszt B**, Spisák S, Takeda S, Szakács G, Szallasi Z, Richardson AL, Szüts D. Correlation of homologous recombination deficiency induced mutational signatures with sensitivity to PARP inhibitors and cytotoxic agents. Genome Biol. 2019 Nov 14;20(1):240. doi: 10.1186/s13059-019-1867-0. PMID: 31727117; PMCID: PMC6857305.

Bernadett Szikriszt, Ádám Póti, Eszter Németh, Nnennaya Kanu, Charles Swanton, Dávid Szüts. Cisplatin is more mutagenic than carboplatin or oxaliplatin at equitoxic concentrations. Pre-print server for biology: bioRxiv, doi: https://doi.org/10.1101/2020.08.11.245969. This preprint contains an early version of the next item.

Bernadett Szikriszt, Ádám Póti, Eszter Németh, Nnennaya Kanu, Charles Swanton and Dávid Szüts. A comparative analysis of the mutagenicity of platinum-containing chemotherapeutic agents reveals direct and indirect mutagenic mechanisms. Accepted at the journal Mutagenesis, on 11 January 2021.

The decision on the manuscript:



Mutagenesis

Preview

From: s.h.doak@swansea.ac.uk
To: szuts.david@ttk.hu
CC:
Subject: Mutagenesis - Decision on Manuscript ID Mutage-20-087.R1
Body: 11-Jan-2021

Dear Dr. Szüts,

It is a pleasure to accept your manuscript entitled "A comparative analysis of the mutagenicity of platinum-containing chemotherapeutic agents reveals direct and indirect mutagenic mechanisms" in its current form for publication in Mutagenesis.

In order to publish your article, Oxford University Press requires that you complete a licence agreement online. A link to the online licensing system, and instructions on how to select and complete a licence, will be provided to you by the Production Editor at Oxford University Press in due course.

If you have not already done so, I would be grateful if you would send the publishers a completed copy of our conflict of interest form for each co-author. If none of the authors has a conflict of interest, one form may be submitted by the corresponding author on behalf of all authors.

The Conflict of Interest Form can be found at:

http://www.oxfordjournals.org/mutage/for_authors/index.html

The completed forms can be either posted or faxed to:

Mutagenesis Production Team
Oxford Journals
Oxford University Press
Great Clarendon Street
Oxford
OX2 6DP
UK

Fax: +44 (0) 1865 355844

It is also acceptable for completed forms to be scanned and sent by email to the Production Editor at:-
MUTAGE@oup.com

Thank you for your fine contribution. On behalf of the Editors of Mutagenesis, we look forward to your continued contributions to the Journal.

Yours sincerely,
Prof. Shareen Doak
Editor in Chief, Mutagenesis
s.h.doak@swansea.ac.uk

Date Sent: 11-Jan-2021

<u>Oral presentations:</u>

(1) My results were incorporated in a poster presentation at the *'FEBS 3+ From molecules to living systems*' international conference on 2-5 September 2018. 12. 13 in Siófok, Hungary.

Poster title: „A comparative study of the mutagenic effect of platinum-based chemotherapeutic agents in cell line based model system"
Authors: Bernadett Szikriszt, Ádám Póti, Dávid Szüts

(2) We showed our results in an oral presentation at the 'Onkoplatform' Hungarian mini conference on 17-19 October 2018 in Mátraháza.
Title of the presentation: „A platina-tartalmú kemoterápiás szerek mutagenezisének összehasonlító vizsgálata"
Authors: Bernadett Szikriszt, Ádám Póti, Dávid Szüts

(3) I attended at the „Hungarian Molecular Life Science 2019" conference on 29-31 March, 2019 in Eger as a speaker.
Title of my presentation: „Analysis of the mutagenic effect of platinum-based chemotherapeutic agents in cell line based model system"
Authors: Bernadett Szikriszt, Ádám Póti, Eszter Németh, Dávid Szüts

(4) We showed our results at the „10th Central European Genome Stability and Dynamics Meeting" international conference on 26-27 September 2019 in Bratislava, Slovakia.
Poster title: „Cisplatin is more mutagenic than carboplatin or oxaliplatin at equitoxic concentrations"
Authors: Bernadett Szikriszt, Ádám Póti, Eszter Németh, Dávid Szüts

(5) We showed our results in an oral presentation at the 'Onkoplatform' Hungarian mini conference on 20-22 November 2019 in Mátraháza.

Title of the presentation: „A platina-tartalmú kemoterápiás szerek mutagenikus hatásának vizsgálata”
Author: Bernadett Szikriszt

Last, but not least I should mention that we are constantly looking after talented BSc and MSc students to promote the research topics of the Genome stability research group. During the three year of my research work, one B.Sc. and two M.Sc. students took part in different topics of the project under my supervision, and wrote their thesis with an extremely successful defense.

Szabó Kinga (M.Sc.): A ciklofoszfamid metabolikus aktivációja és mutagenikus hatásának vizsgálata (2018). Budapesti Műszaki és Gazdaságtudományi Egyetem, Vegyészmérnöki és Biomérnöki Kar, Gyógyszervegyész-mérnöki Szak.

Farkas Flóra (B.Sc.): Ciklofoszfamid metabolizációjának vizsgálata CYP2C18 génbevitel segítségével csirke DT40 limfoblasztóma sejtvonalban (2019). Budapesti Műszaki és Gazdaságtudományi Egyetem, Vegyészmérnöki és Biomérnöki Kar, Egészségvédelmi specializációs biomérnök Szak.

Vadkerti Zsófia (M.Sc.): Gyakori öröklődő BRCA1 mutációt hordozó DT40 sejtvonalak létrehozása CRISPR technika segítségével (2020). Eötvös Lóránd Tudományegyetem, Természettudományi Kar, Biológiai Intézet, Bitechnológia mesterszak, Gyógyszer-biotechnológia specializáció.