SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Roles of the transsulfuration pathways in melanoma BRAF V600E-targeted therapy resistance

by Klaudia Borbényi-Galambos

Supervisor: Prof. Péter Nagy, PhD, DSc

UNIVERSITY OF DEBRECEN

KÁLMÁN LAKI DOCTORAL SCHOOL

Debrecen, 2025

Roles of the transsulfuration pathways in melanoma BRAF V600E-targeted therapy resistance

by Klaudia Borbényi-Galambos, biologist

Supervisor: Prof. Péter Nagy, PhD, DSc

Kálmán Laki Doctoral School, University of Debrecen

Head of the Defense Committee:	Prof. László Csernoch, PhD, DSc
Reviewers:	Prof. Ildikó Bácskay, PhD
	Prof. Gergely Szakács, PhD
Members of the Defense Committee:	Prof. László Virág, PhD, DSc
	Prof. Miklós Geiszt, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 10:30 am on 17th of June 2025.

Table of contents

I. Introduction and aim of the thesis

Skin melanoma remains one of the most aggressive types of cancer, affecting more people each year including the young. Approximately 50% of skin melanoma patients carry the V600 activating mutation of *BRAF* oncogene, which is usually V600E or V600K mutations. This activating mutation in B-Raf protein leads to overactivation of the MAPK pathway resulting in uncontrolled cell proliferation. Although the available therapeutic agents successfully inhibit the growth of *BRAF* mutant melanomas, development of acquired resistance is mostly inevitable (*Robert et al, 2015, N Engl J Med*). Previous studies suggested a higher level of oxidative stress in resistant cells which could act as a potential vulnerability that could be targeted in the future to develop more efficient therapies.

Previous studies showed that the development of acquired resistance is linked to multiple changes in cellular signaling and cell metabolism. The two most common changes in cell signaling are the reactivation of the MAPK pathway by various receptor tyrosine kinases and the overactivation of the PI3K/Akt pathway to counteract MAPK inhibition (*Sun et al, 2014, Nature*). Regarding cellular metabolism, melanoma cells usually use aerob glycolysis to support uncontrolled proliferation which is further activated by the mutant B-Raf. Previous studies showed that by acquiring resistance, melanoma cells have elevated oxidative phosphorylation leading to increased production of reactive oxygen species (ROS) (*Corazao-Rozas et al, 2013, Oncotarget, Wang et al, 2018, Cell*). Cancer cells are usually characterized by increased levels of ROS which damage proteins and DNA leading to the rise of new mutations, and if the stress persist for a long period of time, to cell death. Increased oxidative stress leads to an elevation in antioxidant production as the neutralization of oxidants is inevitable to survive. Increased production along with elevated neutralization leads to altered redox environment with increased flux of ROS. This means that these cells are well-adapted to oxidative stress, but consequently, they are more susceptible to further increase of oxidant levels.

Cysteine plays a crucial role in maintaining redox homeostasis as this amino acid is used for the synthesis of redox active proteins, redox cofactors, glutathione and other sulfur-containing antioxidant molecules. Intracellular levels of cysteine are tightly regulated by its uptake, production and oxidative catabolism. As cysteine is a semi-essential amino acid, it can be taken up from the extracellular space in an oxidized form (cystine), and upon extracellular cystine limitation, cells can synthesize cysteine from serine and methionine-

derived homocysteine. Previous studies showed in various tumor types that cancer cells are sensitive to cystine transporter inhibition (*Zhu és mtsai, 2019, Cell Metab; Bonifacio és mtsai, 2021, Br J Cancer*). Depletion of cystine or inhibition of its transporter leads to the accumulation of lipid radicals and therefore to an iron-dependent type of cell death, ferroptosis.

Roles of sulfur-containing molecules in tumor progression is a heavily studied area. Hydrogen sulfide exerts its versatile biological functions by reacting with metalloprotein heme centers, by regulating protein function through persulfidation and by reacting with nitrogen monoxide. Persulfides (R-SSH) are closely linked biochemically to hydrogen sulfide via various reactions, including some enzyme-catalyzed reactions. Regarding the chemical properties of R-SSH species, despite being more nucleophilic in contrast to thiols (R-SH) they also engage in biochemical reactions as electrophiles. Physiological roles of persulfies include protection against oxidative stress, altering cellular signaling via posttranslational modifications and regulating cell metabolism, all playing vital roles in cancer cell biology (*Borbenyi-Galambos et al, 2024, Curr Op Chem Biol*).

Based on these observations our aim was to identify pathways involved in the development of resistance and to reveal the role of cysteine metabolism along with sulfides/persulfides in melanoma cells acquiring resistance. We assumed that the inhibition of the MAPK pathway and the consequent oxidative stress reported in resistant melanomas would strongly affect different metabolic pathways including the metabolism of sulfur-containing redox active molecules. We aimed to unfold the roles of cysteine and other sulfur-containing molecules by targeted metabolome analysis including a fluxomics approach. Apart from unraveling which pathways are essential in resistance development, our major goal was to identify potential weak points that could act as targets in the future to inhibit the onset of acquired resistance leading to an increase in progression free survival provided by the available therapies.

II. Methods

1. Model systems

1.1. *In vitro* models

To investigate the acute effects of the drugs along with resistance development, we used BRAF V600E mutant melanoma cell lines A375 and SK-MEL-28. To gain resistant lines, cells were treated in increasing doses of vemurafenib or the combination of dabrafenib and trametinib. Cell proliferation was constantly checked, and cells were considered resistant when their proliferation rate reached the therapy-naïve cells'. Resistant cells were further cultured in the presence of the inhibitors. Proliferation of cells under various treatments was checked using Sulforhodamine B assay.

1.2. *In vivo* models

To test the effects of MAPK inhibition under *in vivo* conditions, cell line- and patient-derived xenograft murine models were established by subcutaneous inoculation of cultured cells or human tumor samples to immunodeficient mice. Tumor volumes were estimated by measuring the length and width of tumors using a digital vernier caliper. Before the start of the treatments, mice were randomized into subgroups. At the end of the experiment, mice were sacrificed, and tumors were removed. To investigate the effects of dabrafenib-trametinib treatment on sulfur metabolism, a targeted metabolomics approach was used utilizing frozen tumor tissue samples. Vascularization of tumors was measured by immunohistochemistry.

1.3. Patient samples

To examine the role of cystathionine γ-lyase (CSE) in melanoma, melanoma patients' samples were collected from the Biobank of the National Institute of Oncology. Levels of CSE were measured from patients' samples removed before and after dabrafenib-trametinib therapy was applied using immunohistochemistry.

2. Gene expression analysis

To investigate the acute effects of dabrafenib-trametinib treatment along with the development of acquired resistance on gene expression, mRNA levels were measured using reverse transcriptase quantitative polymerase chain reaction and protein levels were measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western blot analysis.

3. Genetically modified strains

To unravel the roles of CSE in persister cells receiving targeted therapy, we generated a CSE overexpressing A375 cell line using the Sleeping beauty transposon system. Moreover, using CRISPR/Cas9 technology, we generated A375 and SK-MEL-28 cells lines lacking CSE. To investigate the effects of altered CSE levels, cell metabolism was examined by targeted metabolome analysis and cell proliferation was measured by Sulforhodamine B assay. To examine the roles of persulfidation in persister cells under targeted therapy, we generated a persulfide dioxygenase (ETHE1) silenced A375 cell line using lentiviral system based on RNA interference. After that, effects of ETHE1 gene silencing were investigated by measuring proteins and metabolites involved in sulfur metabolism and examining changes in cell proliferation under dabrafenib-trametinib treatment.

4. Investigating the importance of cystine uptake

To examine the importance of cystine uptake in melanoma cells undergoing targeted therapy, we measured cystine uptake of cells by measuring the levels of cystine in the culture media using a mass spectrometry approach. Moreover, we measured cell viability under cystine deprived conditions. In an additional experiment, we cultured cells with dabrafenib-trametinib with or without the inhibitor of cystine transporter, erastin. Cell proliferation after two months of treatment was measured using Sulforhodamine B assay.

5. Measuring intracellular metabolites

5.1. Measurement of sulfane sulfur

To measure intracellular sulfane sulfur live-cell light microscopy approach was used with *Sulfane Sulfur Probe 4* fluorescent dye.

5.2. Measuring amino acids

To measure differences in amino acid levels upon dabrafenib-trametinib treatment, the commercially available EZ:faast amino acid analysis kit was used according to the manufacturer's instructions. After derivatization, amino acid levels were measured using mass spectrometry. Measured amounts were normalized to total protein content of cell lysates.

5.3. Measurement of low molecular weight sulfur-containing metabolites

Measurements were based on the methods previously described by Akaike et al (*Akaike et al, 2017, Nature Communications*). Briefly, cells or frozen tissue samples were lysed in methanol and metabolites were alkylated by β-(4-hydroxyphenyl)ethyl iodoacetamide. After derivatization of analytes by high performance liquid chromatography, measurements were carried out by mass spectrometry. Measured amounts were normalized to total protein content of cell lysates.

5.4. Measuring thiols using monobromobimane alkylating agent

To measure hydrogen sulfide and thiosulfate, thiols were alkylated with monobromobimane, and after sample preparation fluorescently labeled analytes were detected. Quantification was carried out with a calibration curve using standard solutions. Measured amounts were normalized to total protein content of cell lysates.

6. Measurement of protein persulfidation

Protein persulfidation was measured by using two different approaches. The first method was also based on the work of Akaike et al. Briefly, proteins were alkylated by β-(4-hydroxyphenyl)ethyl iodoacetamide, derivatization was carried out using high performance liquid chromatography followed by measuring with mass spectrometry. The other method was carried out by alkylating with EZ-Link iodoacetyl-PEG2-biotin followed by separation of the persulfide fraction using magnetic beads. The persulfide fraction was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by silver staining.

Protein content of the total lysate was measured by Coomassie staining. To identify persulfidated proteins, bands were cut out from the gel and after in-gel digestion, a proteomics approach was used. Moreover, validation of identified proteins was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western blot analysis.

III. New results of the thesis

1. Counteracting oxidative stress in cells under targeted therapy

1.1. Oxidative stress due to MAPK inhibition

To understand the molecular background of acquiring resistance, we generated a cell line resistant to the B-Raf V600E inhibitor dabrafenib and the MEK1/2 inhibitor trametinib. During our work, we examined the acute effects of dabrafenib-trametinib treatment in the drug-tolerant, persister cells that survive therapy, and also monitored changes upon acquired resistance. Detected changes were compared to therapy-naïve control cells.

Previous studies showed that melanoma cells resistant to targeted therapy are characterized by increased levels of oxidative stress. In line with this, we found that upon dabrafenib-trametinib treatment protein oxidation was more prominent, and upon additional oxidative stress the irreversibly oxidized forms of peroxiredoxins were accumulated. Moreover, under excessive oxidative stress conditions, we saw an increase in poly-ADP-ribosylation due to dabrafenib-trametinib treatment, which is a marker of oxidative DNA damage. These results confirmed that dabrafenib-trametinib treatment caused oxidative stress. Increased endogenous oxidative stress can be induced by elevated cytochrome P450 expression because these code monooxygenases that produce ROS while neutralizing xenobiotics.

1.2. Changes in enzyme levels in the redox enzyme systems

Counteracting oxidative stress is essential to ensure cell survival, because long-term stress can impair the DNA, lipids and proteins leading to an iron-dependent cell death form, called ferroptosis. We found that melanoma cells treated with dabrafenib and trametinib overexpressed several proteins involved in protection against oxidative stress including catalase, glutathione peroxidase 1 and 4, superoxide dismutase 2 and thioredoxin reductase 1 to ensure persister cell survival. The last two enzymes were also found to be overexpressed in resistant cells. To operate these antioxidant systems, cells need reducing power in the form of NADPH which is mostly produced through the pentose phosphate pathway by glucose-6-phosphate dehydrogenase. In accordance with our previous observation, overexpression of glucose-6-phosphate dehydrogenase along with 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKB) 4, which induce pentose phosphate pathway activity, were found upon dabrafenib-trametinib treatment.

2. Changes in cellular metabolism upon MAPK inhibition

2.1. Effects of dabrafenib-trametinib treatment on energy metabolism in cells

By investigating pathways involved in energy production we found that glycolytic activity and the glycolytic capacity were decreased upon dabrafenib-trametinib treatment. This could be reasoned by B-Raf inhibition, as B-Raf directly activates glycolysis under therapy-naïve conditions. Another explanation could be that PFKFB 3 and 4 levels were altered upon dabrafenib-trametinib treatment, and these are responsible for regulating the metabolic flux between glycolysis and the pentose phosphate pathway. Upon dabrafenib-trametinib treatment, the level of PFKFB4 was increased, while PFKFB3 was decreased which directly suggest decreased glycolytic activity and an increased flux towards the pentose phosphate pathway. This is in line with the increased ROS levels and the elevated expression of redox enzymes as these require NADPH to counteract oxidative stress.

By measuring mitochondrial respiration, we found that upon dabrafenib-trametinib treatment the reverse respiratory capacity of cells was increased, while in resistant cells both the respiratory capacity and the basal respiration were higher than in therapy-naïve cells. Elevated mitochondrial respiration can be another source of increased ROS production along with increased cytochrome P450 activity. To support increased respiration, cells need electron donors to support the electron transport chain. In line with the increased respiration, we found that upon dabrafenib-trametinib treatment expression of proteins responsible for NADH and $FADH_2$ production was elevated. Several of these are involved in the citric acid cycle.

2.2. Changes in glutamine metabolism

As the citric acid cycle is connected to the metabolism of several amino acids, we performed targeted amino acid analyses and examined the effects of dabrafenib-trametinib treatment. We found that upon dabrafenib-trametinib treatment, levels of glutamine, asparagine and aspartate increased and in resistant cells elevated levels of aspartate were found. In the culture media, we found that asparagine levels increased upon dabrafenib-trametinib treatment, whereas in the case of resistant cells both asparagine and aspartate levels were increased. Moreover, we found that both dabrafenib-trametinib treated and resistant cells took up more glutamine from the extracellular space than therapy-naïve ones. This is in line with previous observations that melanomas resistant to MAPK inhibitors have an increased glutamine demand.

By investigating expressions of proteins involved in the conversion of glutamine and glutamate, we found that upon dabrafenib-trametinib treatment expression of the glutamate producing protein was increased, while in resistant cells we found decreased expression of the glutamate consuming protein. These findings together with the increased glutamine demand suggest that melanoma cells under dabrafenib-trametinib treatment have an increased glutamate demand. Apart from producing alfa-ketoglutarate to feed the citric acid cycle, glutamate can be utilized to glutathione synthesis and cystine uptake, both playing crucial roles in maintaining redox homeostasis. We assumed that all three processes could be important in melanoma cell survival under dabrafenib-trametinib treatment.

3. Roles of cysteine metabolism in acquiring resistance

3.1. Increased cystine demand

The role of cysteine in maintaining redox homeostasis is essential to protect cells from oxidative stress as it is a substrate for the synthesis of several cofactors, sulfur-containing antioxidant molecules and proteins involved in redox balance. A previous study suggested that melanoma cells resistant to targeted therapy are more vulnerable to the transcriptional inhibition of the cystine transporter (*Wang et al, 2018, Cell*). In line with this we found that perister and resistant melanoma cells took up more cystine and resistant cells were more sensitive to cystine and selenite deprivation. Moreover, we found that co-treating cells with the inhibitor of the cystine transporter and MAPK inhibitors delayed the onset of acquired resistance. Cystine-derived cysteine along with glutamate are used for glutathione synthesis which plays a crucial role in maintaining redox homeostasis. Moreover, glutathione can be also utilized by glutathione S-transferases to neutralize certain xenobiotics. Increased levels of glutathione in resistant cells along with overexpression of glutathione S-transferase pi in both dabrafenib-trametinib-treated and resistant cells infer that glutathione has a dual role in acquiring resistance 1) by protecting cells against oxidative stress and 2) by neutralizing the drugs.

3.2. Reprogramming cysteine metabolism

Upon entering the cells, cystine is rapidly reduced to cysteine by the thioredoxin pathway and utilized by many different routes. Despite the increased cystine demand, we found decreased intracellular levels of cysteine and an increase in its oxidized/reduced forms upon dabrafenib-trametinib treatment. This may be explained by 1) the increased utilization of this amino acid or 2) its more prominent oxidative catabolism. Interestingly, we found that despite the increased cysteine demand, oxidative catabolism of cysteine was upregulated upon dabrafenib-trametinib treatment, which together with the increased uptake of cystine resulted in increased cystine/cysteine ratio.

Upon extracellular cystine limitation, cysteine can be synthesized from serine and methionine-derived homocysteine via the transsulfuration pathway. By measuring the levels of transsulfuration enzymes and further investigating their reactions using fluxomics approach, we found that under *in vitro* conditions melanoma cells did not synthesize measurable amounts of cysteine. This is not surprising as cystine is abundant in the culture media and it is easier to take it up from the extracellular space than to synthesize it through multiple reaction steps. However, in another study we showed in an embryonic kidney cell line using fluxomics approach that under lower cysteine availability the canonical, cysteine producing function of the transsulfuration pathway was upregulated. Moreover, we found that knocking out thioredoxin-related protein of 14 kDa (TRP14) further increased the canonical activity of the transsulfuration pathway under lower cystine availability.

Upon dabrafenib-trametinib treatment, we found that one of the transsulfuration enyzmes, cystathionine γ-lyase (CSE), was increased, which, based on fluxomics results, is responsible for the excess production of reactive sulfur species and not cysteine. Increased CSE expression was coupled with decreased cystathionine β-synthase (CBS) levels upon dabrafenib-trametinib treatment. Whereas in resistant cells, these enzyme levels were similar to therapy-naïve control cells.

3.3. Production of low molecular weight cysteine persulfide

By measuring the levels of sulfur-containing metabolites we found that upon dabrafenib-trametinib treatment, levels of sulfane sulfur, hydrogen sulfide, cysteine persulfide and glutathione persulfide were increased, whereas in resistant cells levels only the levels of glutathione persulfide remained high. These results were confirmed in other *in vitro* model systems. Moreover, by using genetically modified cell lines, we found that CSE is

responsible for the production of low molecular weight cysteine persulfide by directly using cystine as substrate. This observation was strengthened by showing that CSE overexpression resulted in decreased cystine (substrate) and increased cysteine persulfide (product) levels. In cells lacking CSE, cysteine persulfide levels were low even under dabrafenib-trametinib treatment, which gives credence that dabrafenib-trametinib treatment induced increase in cysteine persulfide levels is caused by elevated CSE function.

3.4. Investigating sulfur metabolism *in vivo*

In the following, we were interested in whether the dabrafenib-trametinib treatment induced changes in sulfur metabolism observed *in vitro* can also be detected under *in vivo* conditions. To examine this, we established cell line- and patient-derived xenograft murine models. We treated mice with dabrafenib and trametinib, removed the tumors and examined the drug effect using targeted metabolome analysis. Using these xenograft models, we were able to confirm our *in vitro* results: levels of hydrogen sulfide and persulfides increased which protect persister melanoma cells from drug induced oxidative damage. Moreover, these experiments showed that CBS activity was downregulated upon dabrafenib-trametinib treatment *in vivo* in line with our previous *in vitro* observations.

4. Roles of persulfidation in protecting melanoma cells under therapy

4.1. Protection role of protein persulfidation

Increased sulfide and persulfide levels protect cells from oxidative stress and lipid peroxidation induced ferroptosis. Moreover, persulfidation of protein cysteine residues as a posttranslational modification, is an important mediator of signaling events and protects proteins from the formation of irreversible oxidative modifications. Using two different methods, we showed that dabrafenib-trametinib treatment resulted in elevated protein persulfidation. In addition, protein sulfenic acid (protein-SOH) and -perthiosulfenic acid (protein-SSOH) modifications were also increased, which further demonstrates the increased oxidative stress upon dabrafenib-trametinib treatment and directly shows that protein persulfidation protects thiols from overoxidation induced degradation.

From the persulfidated protein fraction, we were able to identify proteins involved in stress response including protein disulfide isomerases, peroxiredoxins and catalase. Elevated levels of persulfidated peroxiredoxin 2 were confirmed by immunoblotting technique. These data together show that elevated oxidative stress upon dabrafenib-trametinib

treatment is counteracted by increased levels of low molecular weight persulfides and protein persulfidation.

Persulfide dioxygenase (ETHE1) is one of the key enzymes in persulfide degradation. To investigate the role of persulfidation in melanoma cells under dabrafenib-trametinib therapy, we silenced ETHE1 in melanoma cells. Gene silencing resulted in increased glutathione persulfide and protein persulfide levels. Most importantly, ETHE1 silenced cells became resistant earlier to dabrafenib-trametinib therapy than normal melanoma cells underlying the importance of elevated persulfide levels in persister cells survival and therefore in acquired resistance.

4.2. Fueling the electron transport chain

Hydrogen sulfide and persulfide levels are tightly regulated by their degradation through the mitochondrial sulfide catabolic pathway which is crucial in maintaining their physiological concentrations. By measuring the end-product and the expression of the enzymes in this pathway, we found that dabrafenib-trametinib induced overactivation of sulfide and persulfide catabolism in all three model systems. These results show that not only the production but also the degradation of sulfide and persulfide species were elevated resulting in an increased flux. As hydrogen sulfide donates electrons to the electron transport chain via its catabolism, sulfide catabolism can stimulate mitochondrial respiration. Therefore, in addition to increased NADH and $FADH_2$ production the increased flux of hydrogen sulfide can also stimulate mitochondrial respiration and oxidative phosphorylation upon dabrafenib-trametinib treatment.

5. Dual inhibition of cystathionine γ-lyase and the MAPK pathway delays the onset of drug resistance

5.1. Investigating the role of cystathionine γ-lyase by gene editing

Apart from revealing the molecular background of therapy resistance, the most important goal of the work presented here was to find potential weak points of persister cells and target them to delay the onset of acquired resistance. We found elevated levels of CSE upon dabrafenib-trametinib treatment in melanoma patients' samples which strengthens our *in vitro* observations. CSE could act as a potential target as it has a commercially available, relatively selective inhibitor D,L-propargylglycine (PAG). Moreover, upon dabrafenib-trametinib treatment induced elevation in CSE levels, the other transsulfuration enzyme,

CBS, which could produce sulfide and persulfide species was downregulated. Therefore, we hypothesized that with downregulated CBS, melanoma cells would be more sensitive to CSE inhibition because they cannot cover their increased persulfide demand upon DT treatment.

By using a CSE overexpressing melanoma cells line, we found that the increase in CSE levels and therefore in low molecular weight cysteine persulfide production was beneficial for melanoma cells under dabrafenib-trametinib therapy, which is in line with our observation using the ETHE1 silenced cell line. In another experiment, using melanoma cells lacking CSE, we found that in the absence of CSE the onset of acquired resistance was delayed. These results highlight the importance of CSE in persister cell survival and suggest that dual inhibition of CSE and the MAPK pathway could lead to a better therapeutic response.

5.2. Pharmacological inhibition of cystathionine γ-lyase

Our *in vitro* experiments showed that dual inhibition of CSE and the MAPK pathway delayed the onset of acquired resistance in all three model systems used. By measuring phosphorylated Akt levels, we found decreased levels of the active Akt in melanoma cells treated with the combination of CSE inhibitor PAG and MAPK inhibitors, which suggests a decreased activation of the PI3K/Akt pathway. This is crucial, as overactivation of this signaling pathway is important in developing resistance to MAPK inhibitors. This observation could partly explain why co-treatment with PAG and MAPK inhibitors delays the onset of acquired resistance. Although the exact molecular background behind these observations remains to be elucidated, we believe that elevated CSE activity leads to increased cysteine persulfide levels and therefore protects persister melanoma cells from drug-induced oxidative stress. Moreover, as a posttranslational modification, elevated persulfidation can result in altered activation of signaling pathways, including the PI3K/Akt pathway, which is important in the development of acquired resistance, but these need further elucidation. It is important to highlight that the applied concentrations of PAG alone did not alter proliferation of melanoma cells and tumor growth of xenograft tumors under therapy-naïve conditions.

Dual inhibition of CSE and the MAPK pathway in xenograft mice delayed the onset of acquired resistance and increased progression free survival. These results show that the addition of CSE inhibition to MAPK inhibitor therapy could increase progression free

survival of melanoma patients and open new avenues in drug development to increase therapeutic efficacy.

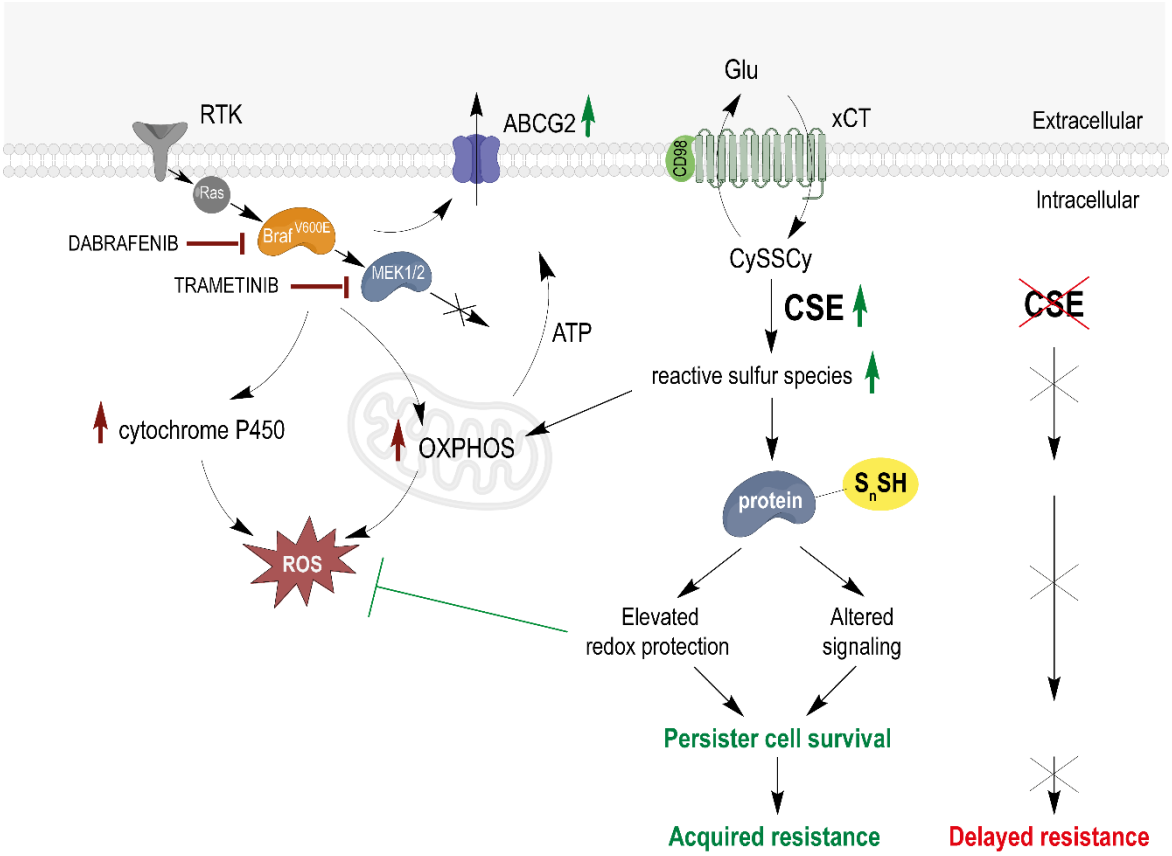

Figure 1. | The most important findings of the thesis are summarized in this figure. *Upon B-Raf V600E and MEK1/2 inhibition, oxidative phosphorylation and cytochrome P450 expression are elevated leading to increased production of reactive oxygen species (ROS). To counteract oxidative stress, cells take up more cystine (CySSCy) to produce reactive sulfur species via cystathionine γ-lyase (CSE), thereby further stimulating mitochondrial respiration. Elevated levels of reactive sulfur species lead to increased protein persulfidation, which protects proteins from oxidative stress and alters cell signaling pathways leading to persister cell survival and therefore to acquired resistance. Dual inhibition of CSE and the MAPK pathway delayed the onset of acquired resistance.*

6. Summary of the new results in thesis statements

- By characterizing our *in vitro* models, we found increased expression of genes that play a vital role in acquiring resistance upon dabrafenib-trametinib treatment, including the ABCG2 transporter, glutathione S-transferase pi and cytochrome P450, the latter potentially contributing to the elevated oxidative stress caused by the therapy.

- We showed that the oxidative stress caused by the inhibition of the MAPK pathway in *BRAF* V600E mutant melanoma led to oxidative modifications of protein cysteine residues.

- We found that expressions of several antioxidant enzymes were elevated upon dabrafenib-trametinib treatment from which thioredoxin reductase 1 and superoxide dismutase 2 remained high in resistant cells.

- We showed that 6-phosphofructo-2-kinase (PFKFB) 3 levels were decreased upon dabrafenib-trametinib treatment, whereas PFKFB 4 levels increased. These regulate the flux between glycolysis and the pentose phosphate pathway.

- We found that elevated glutamine uptake upon dabrafenib-trametinib treatment was coupled with increased glutaminase expression in persister cells and decreased glutamine synthase levels in resistant cells.

- We showed that in line with the dabrafenib-trametinib treatment induced elevation in cystine uptake combining targeted therapy with erastin, which inhibits the cystine-glutamate antiporter, could delay the onset of acquired resistance.

- We found that upon the inhibition of B-Raf V600E, cystathionine γ-lyase (CSE) expression was elevated which was associated with increased levels of reactive sulfur species protection, whereas CBS levels decreased. In resistant cells, levels of CSE, CBS and reactive sulfur species were restored to therapy-naïve control levels, whereas glutathione persulfide levels remained high.

- We showed that cysteine levels were increased in cells under dabrafenib-trametinib treatment, whereas cysteine levels were decreased in both persister and resistant cells, which was associated with increased activity of the catabolic pathway in both cell lines.

- Using metabolomics and fluxomics approaches we found that in persister cells under dabrafenib-trametinib treatment increased levels of CSE led to elevated hydrogen sulfide and homolanthionine production using homocysteine as substrate.
- Using melanoma cells lines lacking or overexpressing CSE we showed that increased levels of CSE were responsible for cysteine persulfide production in persister cells under therapy by using cystine as substrate.
- We found that the oxidative stress caused by dabrafenib-trametinib treatment led to oxidation and persulfidation of protein cysteine residues. Moreover, we showed that persulfidation protected proteins from the formation of irreversible oxidative modifications.
- We showed that increased persulfidation is important for the protection of persister cells, as silencing the enzyme responsible for persulfide degradation led to elevated persulfide levels and silenced cells acquired resistance earlier to dabrafenib-trametinib treatment.
- We found elevated flux of the mitochondrial catabolism of hydrogen sulfide upon dabrafenib-trametinib treatment.
- Our findings regarding the changes in cysteine and hydrogen sulfide metabolism were confirmed in A375 cells under vemurafenib treatment, and in the SK-MEL-28 cell line, in A375 cell line-derived and in patient-derived xenografts under dabrafenib-trametinib treatment.
- We found elevated CSE levels upon dabrafenib-trametinib treatment in paired before and after treatment human samples.
- Using a CSE-overexpressing melanoma cell line, we showed that with elevated CSE levels cells acquire resistance earlier.
- Our *in vitro* experiments showed that genetical or pharmacological inhibition of CSE delayed the onset of acquired resistance.
- Using an animal model, we showed that combining B-Raf V600E and MEK inhibitors with a CSE inhibitor, the progression free survival of mice can be increased.

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/90/2025.PL
Subject: PhD Publication List

Candidate: Klaudia Borbényi-Galambos
Doctoral School: Kálmán Laki Doctoral School
MTMT ID: 10084191

List of publications related to the dissertation

1. **Galambos, K.**, Erdélyi, K., Ditrói, T., Jurányi, E. P., Szántó, N., Szatmári, R., Czikora, Á., Schmidt, E. E., Garai, D., Cserepes, M., Liszkay, G., Tóth, E., Tóvári, J., Nagy, P.: Realigned transsulfuration drives BRAF-V600E-targeted therapy resistance in melanoma.
   Cell Metab. [Epub ahead of print], 2025.
   DOI: http://dx.doi.org/10.1016/j.cmet.2025.01.021
   IF: 27.7 (2023)

2. Martí-Andrés, P., Finamor, I., Torres-Cuevas, I., Pérez, S., Rius-Pérez, S., Colino-Lage, H., Guerrero-Gómez, D., Morato, E., Marina, A., Michalska, P., León, R., Cheng, Q., Jurányi, E. P., **Galambos, K.**, Millán, I., Nagy, P., Miranda-Vizuete, A., Schmidt, E. E., Martínez-Ruiz, A., Arner, E. S. J., Sastre, J.: TRP14 is the rate-limiting enzyme for intracellular cystine reduction and regulates proteome cysteinylation.
   EMBO J. 43 (13), 2789-2812, 2024.
   DOI: http://dx.doi.org/10.1038/s44318-024-00117-1
   IF: 9.4 (2023)

3. **Galambos, K.**, Czikora, Á., Erdélyi, K., Nagy, P.: Versatile roles of cysteine persulfides in tumor biology.
   Curr. Opin. Chem. Biol. 79, 1-8, 2024.
   DOI: http://dx.doi.org/10.1016/j.cbpa.2024.102440
   IF: 6.9 (2023)

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

List of other publications

4. Combi, Z., Potor, L., Nagy, P., Sikura, K. É., Ditrói, T., Jurányi, E. P., **Galambos, K.**, Szerafin, T., Gergely, P., Whiteman, M., Torregrossa, R., Ding, Y., Beke, L., Hendrik, Z., Méhes, G., Balla, G., Balla, J.: Hydrogen sulfide as an anti-calcification stratagem in human aortic valve: altered biogenesis and mitochondrial metabolism of H2S lead to H2S deficiency in calcific aortic valve disease.
   Redox Biol. 60, 1-19, 2023.
   DOI: http://dx.doi.org/10.1016/j.redox.2023.102629
   IF: 10.7

5. Bogdándi, V., Ditrói, T., Bátai, I. Z., Sándor, Z., Minnion, M., Vasas, A., **Galambos, K.**, Buglyó, P., Pintér, E., Feelisch, M., Nagy, P.: Nitrosopersulfide (SSNO) Is a Unique Cysteine Polysulfidating Agent with Reduction-Resistant Bioactivity.
   Antioxid. Redox Signal. 33 (18), 1277-1294, 2020.
   DOI: http://dx.doi.org/10.1089/ars.2020.8049
   IF: 8.401

Total IF of journals (all publications): 63,101
Total IF of journals (publications related to the dissertation): 44

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

14 March, 2025

IV. Acknowledgements

First, I would like to express my sincere gratitude to my supervisor, **Prof. Péter Nagy PhD, DSc**, for helping me during my MSc and my PhD work with his invaluable guidance, feedback and support. I thank him for introducing me to the field of redox biology and for helping me gain experience in research.

I am extremely thankful for my co-worker, **Katalin Erdélyi PhD** for not only helping me with the experiments but also acting as my mentor throughout the past years. I have learned all the methodologies from her.

I am very grateful for **Noémi Szántó**, executive assistant of the Department of Molecular Immunology and Toxicology who helped me continuously to carry out the experiments, even if the workday got a little bit longer than usual.

I would like to say special thanks to **Eszter Petra Jurányi** for the execution of the analytical measurements and for her extra help during the revision of our paper.

I am thankful for **Tamás Ditrói PhD** for his analytical work, help and suggestions during my MSc and PhD years.

I am grateful for **Dorottya Garai PhD** for helping me with the animal experiments and for giving me guidance in the administrative work during the PhD.

I would like to thank **Ágnes Czikora PhD** and **Réka Zsuzsanna Szatmári** for contributing to my work with their exceptional methodological knowledge.

I am grateful for **Zsuzsanna Lénárt**, administrator of the Department of Molecular Immunology and Toxicology for ensuring the smooth operation of our research group.

I am extremely lucky to work in an exceptional research group in the past years where every member is an inspiration to me in my professional and private life alike: **Katalin Erdélyi PhD, Ágnes Czikora PhD, Dorottya Garai PhD, Noémi Szántó, Eszter Petra Jurányi, Réka Zsuzsanna Szatmári, Zsuzsanna Lénárt, Tamás Ditrói PhD, Éva Ditóriné Dóka PhD, Laura Somodi PhD, Annamária Hakl, Andrea Domán PhD, Zsuzsanna Gyöngy PhD, Anna Lopata PhD** and **Daniella Dörgő**.

I am thankful for **József Tóvári PhD** and **Mihály Tamás Cserepes PhD** for their help in the execution of the animal experiments.

I am grateful for **Erika Tóth MD** and **Gabriella Liszkay MD, PhD, DSc** for their contributions to the analysis of the human samples.

I would like to thank **Anita Hidvégi, Annamária Hakl** and **Emese Békné Kaplar** for their exceptional technical assistance.

I am thankful for the Gedeon Richter Talentum Foundation for their support during my PhD years.

Finally, I am extremely grateful to my family for encouraging and supporting me, especially my husband, **Márton Borbényi**.