Detailed, final research report - The potential role of ferroptosis in citotoxicity induced by high dose ascorbate and acetaminophen treatment- KH129593

Our present project was based on our initial study in which, we showed that ferroptosis, is involved in the cytotoxicity of hepatocytes treated with high dose of acetaminophen (APAP). As the continuation of the original topic our aim was to clarify the relationship of the various cell death pathways (necroptosis, apoptosis, autophagy, ferroptosis) which can be related to APAP induced cytotoxicity. We would have liked to achieve this goal by utilising a more advanced cell culture method compared to our previous murine cellular model, thus 2D and 3D cell culture have been established from human bipotent progenitor – termed HepaRG – and HepG2 hepatocellular carcinoma cell lines. The use of immortal cell lines enabled us to compare the effects of APAP treatment to other known initiators of various types of cell death, to elucidate the relationship of ferroptosis and other types of cell death pathways. **Finally, we would have liked to investigate whether ferroptosis is involved in the cell death initiated by pharmacologic ascorbate.**

According to our research plan HepG2 cells were maintained both in 2D and 3D cultures. The expression of Cyp2E1 was determined as a marker for drug metabolism ability of the cell lines. The expression of Cyp2E1 in the 3D cultures was 6-10-fold elevated compared to the expression of Cyp2E1 in the 2D cultures. There were no differences in the applied two different 3D culturing methods. Cell viability was assessed in a wide concentration range of APAP (1-80 mM) in the 2D HepG2 cell culture. The EC_{50} was found to be 10 mM. After establishing a stable cell bank of HepaRG cells in our laboratory, we performed experiments on HepaRG through monitoring the expression of Cyp2E1 during different culture conditions. Cyp2E1 expression in undifferentiated HepaRG cells increased by 140-fold compared to 2D cultured HepG2 cells, while the differentiation process caused a further 10-fold increase in Cyp2E1 expression. Interestingly the expression was not affected by APAP treatment in all of the investigated cell lines under neither investigated cell culture condition (2D or 3D).

The observed decline of cell viability could be suspended by Z-VAD-FMK treatment suggesting the involvement of apoptosis in the APAP induced cell death in 2D HepG2 cells. The involvement of apoptosis in the death of 2D HepG2 was reinforced by the significant 15-fold increase of caspase-3 activity due to 24h of high dose APAP treatment. The late apoptosis marker, the cleavage of PARP-1 (cleaved fragment: 89kDa), could also be detected by western blot due to high dose of APAP treatment in HepG2 cells. According to the low expression of Cyp2E1 no difference could be observed in the DCF, DHE detectable ROS production nor in the BODIPY detectable lipid ROS production in 2D HepG2 cells. Similarly, 10 mM APAP treatment was accompanied by only a moderate decrease in cellular GSH . Significant decrease in cellular GSH was only detected at a much higher (25mM) APAP concentration.

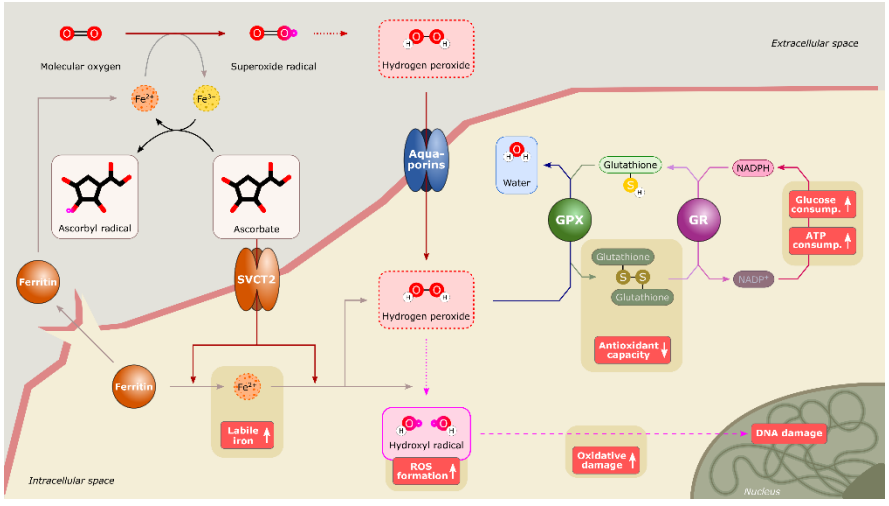
Interestingly, the decrease of cell viability of the differentiated HepaRG cell line due to APAP treatment lagged behind to our expectations. We hypothesize a matrix effect caused by the biliary epithelial-like cell population present in the HepaRG culture in a proportion of about 50%. As this population is less sensitive to APAP treatment, we assume that in parallel with the death of hepatocytes, epithelial-like cells survive due to their low levels of Cyp2E1 expression. The different APAP sensitivities of the two cell types were clearly demonstrated by propidium iodide cell death marker staining. This presumption was further strengthened by flow cytometry: hepatocytes and biliary epithel-like cells may be distinguished based on their different nuclear staining, however, a more specific staining method for

separation would be beneficial. A significant depletion in cellular GSH can be observed at 15mM and 20mM APAP.

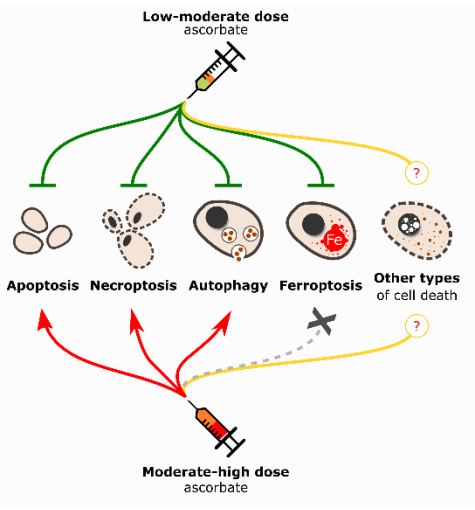
The cell viability was assessed at 10, 15, 20, 25 mM APAP treatment in the 2D HepaRG cell culture, and it declined to about 50% due to 25 mM of APAP treatment, while this APAP concentration resulted in about 30% viability on HepG2 cells. Although it is clear from our observations by microscopy that selective death of hepatocytes occurs at lower APAP doses, hence the different degree of cytotoxic effect of APAP on the two cell lines can be explained again by the matrix effect. Since the viability of differentiated HepaRG cells did not decrease below 50% even at 25 mM of APAP concentration according to MTT assay due to this matrix effect, MTT viability assay was not found to be suitable to detect the potential effect of cell death inhibitors. A more sensitive monitoring of the viability of hepatocytes could be achieved by the measurement of the enzyme activity ASAT (Aspartate aminotransferase). This way, the matrix effect of epithel-like cells could be excluded. The decline of cell viability of HepaRG due to APAP treatment could be mitigated by Z-VAD-FMK and dabrafenib (a necroptosis inhibitor) treatment, suggesting the possible involvement of apoptosis and/or necroptosis in the APAP induced cell death in HepaRG cultures.

Experiments are currently underway to further investigate the APAP-induced cell death types of HepaRG mixed cell culture with additional cell death inhibitors and to examine the marker proteins of each cell death species by western blot technique. Furthermore, flow cytometric measurements are used to obtain a finer picture of the APAP-response of the cell culture. **At the same time the preparation of a manuscript has also been started.**

Our second main goal was to investigate the potential involvement of ferroptosis in pharmacologic ascorbate induced cell death. Pharmacologic ascorbate induced cell death and ferroptosis share common features such as iron dependency, production of ROS, lipid peroxidation, caspase independency and the possible involvement of autophagy. These observations lead us to hypothesize that ferroptosis may also be involved in cancer cell death due to pharmacologic ascorbate treatment. Thus cell death of HT-1080 cell line was induced by ferroptosis inducers and pharmacologic ascorbate



then the mechanism of cell death was compared. The EC_{50} value of pharmacologic ascorbate on HT-1080 cell line was found to be 0.5 mM that is in the range of the most ascorbate sensitive cell lines. However, neither of the specific inhibitors of ferroptosis (ferrostatin-1 and liproxstatin-1) could elevate the viability of pharmacologic ascorbate treated cells suggesting that ferroptosis was not involved in the pharmacologic ascorbate induced cell death. α-tocopherol that could effectively



elevate the viability of erastin and RSL3 treated HT-1080 cells failed to mitigate the cytotoxic effect of pharmacologic ascorbate, which further strengthened this assumption. Furthermore, at lower concentrations (0.1-0.5 mM) ascorbate could avoid the effects of ferroptosis inducers. Our results show that both pharmacologic ascorbate and RSL3 induce ROS and lipid peroxide (LOOX) formation. The co-treatment of RSL3 treated cells with low dose (0.1 mM) ascorbate significantly decreased the level of both ROS and lipid peroxide formation. The production of ROS, LOOX and the elevation of labile iron pool due to pharmacologic ascorbate treatment was concentration dependent. Ascorbate concentration as low as 0.25 mM induced detectable levels of ROS while cells treated with 0.1 mM ascorbate showed ROS levels similar to untreated cells. **Our results indicate that pharmacologic ascorbate induced cytotoxicity and ferroptosis – albeit phenotypically they show similar traits – are governed by different mechanisms. Our results were submitted to Pathology and Oncology Research for publication. The manuscript has been accepted.**

As a supplementation of the above detailed experimental work a comprehensive review was written on Vitamin C and cell deaths. We paid special attention to the hottest sole oxidative stress and lipid peroxidation driven cell death, ferroptosis. The high dose ascorbate induced cell death was considered to be apoptosis in the studies published before 2010. The caspase independency of high dose ascorbate induced cell death proposed the possible involvement of necroptosis and autophagy. Thus the possible role of all these cell death forms and their possible anti-cancer role was discussed. **Our manuscript was submitted to Antioxidants and Redox Signaling. The manuscript has been accepted.**

The drug metabolism of APAP and Cyclophosphamide (CYC) shows similar features. Both of them are activated during their metabolism by hepatocytes, both of them cause GSH depletion and cell death in the case of overdose. Thus the drug metabolism of CYC and the possible therapeutic aspects of the polymorphism of enzymes involved in its metabolism was investigated. Cyclophosphamide (CYC) is one of the most potent and reliable anti-cancer and immunosuppressive drugs. CYC is a prodrug, which is activated by biotransformation phase I enzymes. 70-80% of the administered CYC is transformed to 4-hydroxycyclophosphamide by hepatic cytochrome P450 (CYP) enzymes. Several CYP isoenzymes are involved in the 4-hydroxilation of CYC in humans, including CYP3A4, and CYP2B6. Biotransformation Phase II enzymes, such as glutathione S-transferases (GST) catalyse the neutralization of active intermediers of CYC by conjugation with glutathione, resulting in 4-glutathionylcyclophosphamide or diglutathionylphosphoramide mustard. The isoforms GSTM1, GSTP1, and GSTT1 are only involved in the production of 4- glutathionylcyclophosphamide. The direct detoxification is catalysed by CYP3A4 and CYP3A5 and results in the formation of 2-dechloroethylcyclophosphamide via side chain oxidation. The level of the bioactive phosphoramide mustard is determined by the rate of enzymatic activation and detoxification of CYC and its

metabolites. The enzymes involved in CYC metabolism are known to be highly polymorphic, and have alleles with decreased or missing activity. Thus, genetic differences may be responsible for the earlier observed large interindividual variations in both efficacy and toxicity of CYC treatment. In our study, 33 individuals with different autoimmune diseases were treated with cyclophosphamide according to standard protocols. The responses to the treatments were determined by measuring the alteration of several typical parameters characterizing the given autoimmune diseases over time. We concluded that about 45% of the patients responded to the treatment. Patients were genotyped for polymorphisms of the CYP3A4, CYP2B6, GSTM1, GSTT1, and GSTP1 genes and disease remission cases were compared to the individual polymorphic genotypes. It was found that the GSTP1 I105V allelic variation significantly associated with the cyclophosphamide treatment-dependent disease-remissions. At the same time the GSH content of the erythrocytes in the patients with I105V allelic variation did not change. It appears that the individuals carrying the Ile105Val SNP in at least one copy had a significantly higher response rate to the treatment. **Since this variant of GSTP1 can be characterized by lower conjugation capacity that results in an elongated and higher therapeutic dose of cyclophosphamide, our data suggest that the decreased activity of this variant of GSTP1 can be in the background of the more effective disease treatment. Our results were submitted to Molecules for publication. The manuscript has been accepted.**

APAP induces hepatotoxicity involves activation of c-Jun amino-terminal kinase (JNK), mitochondrial damage and ER stress. BGP-15, a hydroximic acid derivative, has been reported to have hepatoprotective effects in APAP overdose induced liver damage. Effect of BGP-15 was further investigated on mitochondria in APAP-overdose induced acute liver injury in mice. We found that BGP-15 efficiently preserved mitochondrial morphology, and it caused a marked decrease in the number of damaged mitochondria. Attenuation of mitochondrial damage by BGP-15 is supported by immunohistochemistry as the TOM20 label and the co-localized autophagy markers detected in the livers of APAP-treated mice were markedly reduced upon BGP-15 administration. **This effect, along with the observed prevention of JNK activation likely contribute to the mitochondrial protective action of BGP-15. Our results were submitted to Pathology and Oncology Research for publication. The manuscript has been accepted.**

The relationship between the potentially developing complications of the 451 million people affected by diabetes and hyperglycaemia can be based on the enhanced generation of advanced glycation endproducts and the more intensive oxidative and carbonyl stress. Advanced glycation endproducts generated partly due to the carbonyl stress play important role in the pathogenesis of diabetic complications such as elevated arterial thickness, vascular permeability, enhanced angiogenesis or the more rigid vessels induced nephropathy, neuropathy, retinopathy. Furthermore, the elevated thrombocyte aggregation, the reduced fibrinolysis induced elevated coagulation, and the atherosclerosis or the mitochondrial dysfunction. The most potent target of both the non-oxidative and oxidative generation of advanced glycation endproducts can be the scavenging of α, β-unsaturated aldehydes. **The endogen dipeptide L-carnosine was expected to mitigate the complications due to carbonyl stress.** However, its clinical significance was limited by the serum carnosinases and by the consequent low serum stability and bioavailability. The carnosinase resistance of the molecule can be achieved by the change of the carboxyl group of the molecule to hydroxyl group, the modified molecule is called carnosinole. At the same time the biosafety and the carbonyl

stress scavenging activity of the molecule could be preserved. **Although clinical studies could not be performed in the last six months, on the base of the in vitro and in vivo results carnosinole seems to be a promising compound to mitigate and prevent the diabetic complications. Our review study was published in the Medical Journal.**

The similar features of acrolein induced cell death and heat stress induced ferroptosis raised the hypothesis that this molecule can be another ferroptosis inducer. Furthermore, it is also a good candidate a mediator role in ferroptosis. The cytotoxic effect of acrolein could be mitigated by the pre-treatment of *A. thaliana* cells with well-known ferroptosis inhibitors such as ferrostatin-1, deferoxamine, α-tocopherol, and GSH. This observation clearly indicates that ferroptosis is involved in acrolein induced cell death. The similar inhibitory profile of the known ferroptosis inducer RSL3 further confirmed that ferroptosis is, at least partly, responsible for the acrolein induced cell death in plant cells. It was further strengthened by the clear positive effect of the cell permeable iron chelator deferoxamine on the cell viability of acrolein treated cells. The reactive carbonyl species scavenger dipeptide, carnosine could successfully scavenge acrolein and mitigate cell death earlier. In our experiments it could also moderately elevate the cell viability in acrolein treated cells and even more significantly in RSL3 treated cell. According to these results we suppose that both cell death inducers can act on a similar way. **The protective effect of carnosine in RSL3 treated cells also raised the possible involvement of reactive carbonyl species (acrolein) in the RSL3 induced (ferroptosis-like) cell death (FCD)**. Both acrolein and RSL3 treatment resulted in enhanced caspase-3-like protease activity, that could be significantly mitigated by GSH or ferrostatin-1 pre-treatment. All these observations demonstrate, that caspase-like activity is clearly involved in RSL3 and heat stress induced FCD in plant cells. Acrolein induced ROS generation and lipid ROS formation could be significantly mitigated by pre-treating the cells with ferroptosis inhibitors, the acrolein scavenger Carnosine and the cell-permeable caspase inhibitor Z-VAD-FMK. These observations further strengthen the role of FCD in acrolein induced cytotoxicity and the possible role of caspase-like proteases in FCD. **Therefore, on the contrary to the caspase independent ferroptosis in human cells it is found that caspase-like activity can be involved in plant FCD. Our results were submitted to PLOSOne for publication. The manuscript has been accepted.**

Glucose is a basic nutrient in most creatures; its transport through biological membranes is an absolute requirement of life. Glucose transport through intracellular membranes has not been elucidated yet; however, glucose is formed in the lumen of various organelles. Despite the obvious necessity, the mechanism of glucose transport and the molecular nature of mediating proteins in the endomembranes have been hardly elucidated for the last few years. However, recent studies revealed the intracellular localization and functional features of some glucose transporters; the aim of our paper was to summarize the collected knowledge. **We also gave an *in silico* analysis on the subcellular localization of different glucose transporters. Our review and in silico results were submitted to International Journal of Molecular Sciences for publication. The manuscript has been accepted.**

Cancer cells show altered glucose metabolism. Any difference in the behaviour of cancer cells compared to the normal ones gives the opportunity to find them and to fight against them. Otto Heinrich Warburg found that cancer cells of different origin could be characterized by an order of magnitude higher glucose consumption than normal cells. Warburg effect has been considered a

potential therapeutic target to fight against cancer progression. The coexistence of oxidative respiration and glycolysis, also called hybrid metabolic state, contributes to adaptation of cancer cells to the various stress factors in tumour microenvironments. Thus the examination of the regulation of Warburg effect is crucial to identify the weak point of tumour progression. A mathematical model was built containing the main elements of the regulatory network in KRAS-mutant cancer cells. To study its dynamical characteristics, it was converted to a set of nonlinear ordinary differential equations and analysed using the techniques of dynamical system theory. PKM2 (pyruvate kinase isozyme M2) has been assumed to be the key switch in the stress response mechanism. Albeit the knock-down of either PKM2 or c-Myc downregulates GLUT1 the KRAS pathway remains still active and has a positive effect on mTOR (mammalian target of rapamycin) pathway that keeps autophagy inhibited. We predicted that addition of both pharmacologic ascorbate and rapamycin is able to block both mTOR and KRAS pathways: in this case no GLUT1 expression is observed, meanwhile autophagy gets significantly active. **Corresponding to our systems biological analysis this combined pharmacologic ascorbate and rapamycin treatment in KRAS mutant cancer cells might be a therapeutic approach in anti-cancer therapies. Our results were submitted to Biomolecules for publication.**

Plant UCPs (uncoupling proteins) are proved to take part in the fine-tuning of mitochondrial ROS generation. It has emerged that the mitochondrion can be an important early source of intracellular ROS during plant-pathogen interaction thus plant UCPs must also play key role in this redox fine-tuning during the early phase of plant–pathogen interaction. On the contrary of this well-established assumption, the expression of plant UCPs and their activity has not been investigated in elicitor induced oxidative burst. Thus, the level of plant UCPs both at RNA and protein level and their activity was investigated and compared to AOX (alternative oxidase) as a reference in Arabidopsis thaliana cells due to bacterial harpin treatments. Similar to the expression and activity of AOX, the transcript level of UCP4, UCP5 and the UCP activity increased due to harpin treatment and the consequential oxidative burst. **The quite rapid activation of UCP due to harpin treatment gives another possibility to fine tune the redox balance of plant cell, furthermore explains the earlier observed rapid decrease of mitochondrial membrane potential and consequent decrease of ATP synthesis after harpin treatment. Our results were submitted to PLOSOne for publication. The manuscript has been accepted.** Our results were also orally presented at the 14th POG (Plant Oxygen Group) conference in München.