The mode of action of acrolein and other abiotic stressors and their role in environmental, agricultural and food safety (K123752)

1. The level and redox status of glutathione (GSH) is a good indicator for the rate of oxidative stress and eco-toxicological injury in plant cells and subcellular organelles. The effect of acrolein and the parallel applied ferroptosis inducer erastin can also be easily followed by the monitoring of cellular concentration and redox status of GSH. Thus the determination of GSH and its redox status has special importance. A variety of spectrophotometric and HPLC methods are available to measure GSH. The spectrophotometric DTNB-GSH recycling assay is specific due to the application of glutathione reductase, it is rather quick and easy to perform, not surprising that it is rather popular. As a first step of our project we make an attempt to compare the DTNB-GSH recycling assay and the more sophisticated, but also more difficult monochlorobimane (mBCl)-HPLC method to choose the one that best suits for our eco-toxicological, agricultural safety and plant stress investigations. We found that the acidification by sulphosalicylic acid (SSA) used for the stabilization of samples for DTNB-GSH recycling assay gives lower efficiency to the method than the formation of mBCl-GSH fluorescent conjugate. The measurable GSH contents were lower in the case of DTNB-GSH recycling assay than in the case of GSH-mBCl conjugates determined by HPLC with fluorescence detection. The auto-oxidation could almost fully be prevented by the presence of mBCl in the organelle isolation buffer. Furthermore, this way the reduced GSH content of organelles could be determined much more precisely. At the same time, it is worth to note that the application of mBCl significantly elevates the cost of GSH determination, especially in case of cell organelles. In spite of the higher cost we preferred the (mBCl)-HPLC in our experiments. On the base of our results the following method was applied for the determination of GSH from our samples.

Publication: P Hajdinák, Á Czobor, T Lőrincz, A Szarka The problem of glutathione determination: a comparative study on the measurement of glutathione from plant cells Periodica Polytechnica Chemical Engineering 63 (1), 1-10 (2018)

2.1. Plants in their natural environments are exposed to a variety of biotic and abiotic stresses, including pathogens, drought, heavy metals, extreme temperature, salt and high light. Under these stress conditions, reactive oxygen species (ROS) derived from molecular oxygen can accumulate in plant cells. Excess amount of ROS formation can lead to programmed cell death (PCD). Recently ferroptosis, a new form of cell death was described that is iron dependent and exclusively caused by the accumulation of lipid-based peroxides. It seems it can be dedicated uniquely to ROS generation. The presence of ferroptosis in plants was also described recently. Similar to the human cells, heat stress induced ferroptosis-like cell death (FCD) in plant cells was accompanied by the accumulation of lipid ROS, and depletion of GSH.

The overexpression of enzymes responsible for detoxification of toxic lipid metabolites leads to ferroptosis resistance supporting the possible role of these reactive aldehydes in ferroptotic cell death. Furthermore, it was found that acrolein, one of the lipid peroxide derived reactive carbonyl species, generated under environmental stress caused the depletion of the GSH pool in BY-2 tobacco cells. This effect gradually lowered the ascorbate level and enhanced the ROS level in the cell, followed by cell death at the end. All these observations are substantially similar to the results found in the case of heat treatment induced FCD in *Arabidopsis thaliana*. 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 for a mediator role in ferroptosis.

Thus, the effect of both the known ferroptosis inductor RSL3 and acrolein was investigated on the cell viability of *Arabidopsis thaliana* to elucidate the possible involvement of ferroptosis in acrolein induced cell death and the possible involvement of acrolein in the ferroptotic pathway. Both compounds caused the significant decrease of cell viability. The cytotoxic effect of acrolein could be mitigated by pre-treatment of the cells with the 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 in plant cells.

Acrolein is also a well-known activator of both caspase-1-like and caspase-3-like proteases, therefore the effect of the cell-permeable caspase inhibitor Z-VAD-FMK was also investigated on cell viability. Surprisingly the observed decrease of cell viability due to acrolein treatment was only mitigated, but the reduction of cell viability due to RSL3 treatment was totally abolished by Z-VAD-FMK. 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.

ROS generation, especially lipid ROS production play crucial role in the initiation of ferroptosis. The H_2DCFDA detectable ROS formation was significantly increased by addition of acrolein. Parallel, enhanced lipid peroxidation could also be observed. 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.

Thus the main aim of our research project has been reached.

2.2. We investigated the role of ferroptosis in acrolein induced cell death using HT-1080 N-RAS mutant fibrosarcoma cells. According to our results acrolein at the concentration of 100 µM showed marked cytotoxicity after 24 hours of exposure. This acrolein dose reduced cell viability measured by the MTT assay to practically 0%. The degree of cytotoxicity dropped abruptly at lower acrolein concentrations: at 90 µM cell viability increased to ~50%, while 80 µM was barely toxic. To investigate the involvement of ferroptosis we examined the effect of the specific ferroptosis inhibitors ferrostatin-1 and liproxsatatin-1 on acrolein induced loss of cell viability. Neither inhibitors were able to significantly counter cytotoxicity. Other inhibitors of specific cell death pathways were also ineffective including

necrostatin-1 (necroptosis) and z-VAD-fmk (apoptosis). However significant protective effect was absorbed by using the antioxidant glutathione, while the iron-chelator deferoxamine could moderately alleviate acrolein induced cell death.

2.3. Cyclophosphamide is a highly efficient anti-cancer drug and immunosuppressive agent. It is converted into two bioactive toxic compounds such as phosphoramide mustard and acrolein. In our study, 33 individuals with different autoimmune diseases were treated with cyclophosphamide according to standard protocols. 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 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.

2.4. 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.

2.5. The possible role of carbonyl stress and acrolein in translational science was discussed in a review.

Publications:

P Hajdinák, Á Czobor, A Szarka The potential role of acrolein in plant ferroptosis-like cell death Plos one 14 (12), e0227278 (2019)

P Hajdinák, M Szabó, E Kiss, L Veress, L Wunderlich, A Szarka Genetic Polymorphism of GSTP-1 Affects Cyclophosphamide Treatment of Autoimmune Diseases Molecules 25 (7), 1542 (2020)

K Makk-Merczel, A Szarka The role of carbonyl stress in the development of diabetic complications Orvosi hetilap 160 (40), 1567 (2019)

F Sarnyai, T Szekerczés, M Csala, B Sümegi, A Szarka, Z Schaff, J Mandl Bgp-15 protects mitochondria in acute, acetaminophen overdose induced liver injury Pathology & Oncology Research, 1-7 (2019)

3. Plant Uncoupling Proteins (UCPs) are proved to take part in the fine-tuning of mitochondrial ROS generation. It has emerged that 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 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 orally presented at the 14th POG (Plant Oxygen Group) conference in München.

Publication: Á Czobor, P Hajdinák, B Németh, B Piros, Á Németh, A Szarka Comparison of the response of alternative oxidase and uncoupling proteins to bacterial elicitor induced oxidative burst Plos one 14 (1), e0210592 (2019)

4.1. 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. However, either of the specific inhibitors of ferroptosis (ferrostatin-1 and liproxstatin-1) could not 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 HT1080 cells failed to mitigate the cytotoxic effect of pharmacologic ascorbate further strengthened this assumption. Furthermore, at lower concentrations (0.1–0.5 mM) ascorbate could avoid the effects of ferroptosis inducers. Our results indicate that pharmacologic ascorbate induced cytotoxicity and ferroptosis – albeit phenotypically they show similar traits – are governed by different mechanisms.

4.2. In the forthcomming study, a mathematical model was built containing the main elements of the regulatory network in KRAS mutant cancer cells in which we predicted that the addition of both pharmacologic ascorbate and chloroquine is able to block both KRAS and mTOR pathways: in this case, no GLUT1 expression is observed, mean- while autophagy, essential for KRAS mutant cancer cells, is blocked.

4.3. A comprehensive review and a book chapter was written on the regulation of the level of ascorbate in both plant and animal cells, tissues. The beneficial and potentially harmful effects of ascorbate in humans and plants are discussed. Later, as a continuation of the previous review, another was written and published on the role of pharmacological ascorbate in cell death. Finally, the topic was closed by a review on the dual functions and the role in cell fate decision of iron, the generation of reactive oxygen species and ascorbate.

4.4. Multiple factors suggest a potential link between the ferroptotic and JNK pathways; (i) both processes are ROS mediated; (ii) both can be inhibited by lipid peroxide scavengers; (iii) RAS mutations may play a role in the initiation of both pathways. We aimed to investigate the possible link between ferroptosis and the JNK pathway. Interestingly, JNK inhibitor co-treatment could enhance the cancer cytotoxic effect of the ferroptosis inducers in NRAS and KRAS mutation-harboring cells (HT-1080 and MIA PaCa-2). Since cancer's cytotoxic effect from the JNK inhibitors could only be suspended by the ferroptosis inhibitors, and that sole JNK-inhibitor treatment did not affect cell viability, it seems that the JNK inhibitors "just" amplify the effect of the ferroptosis inducers. This cancer cell death amplifying effect of the JNK inhibitors could not be observed in other oxidative stress-driven cell deaths. Hence, it seems it is specific to ferroptosis. Finally, our results suggest that GSH content/depletion could be an important candidate for switching the anti-cancer effect of JNK inhibitors.

4.5. In vivo and in vitro toxicological model systems are highly required. An attempt was made to compare the hepatocarcinoma HepG2 and the stem cell-derived HepaRG cell lines both in 2D and 3D culture conditions to find the most suitable model.

Publications: T Lőrincz, M Holczer, O Kapuy, A Szarka The interrelationship of pharmacologic ascorbate induced cell death and ferroptosis Pathology & Oncology Research 25 (2), 669-679 (2019)

Kapuy O, Makk-Merczel K, Szarka A. Therapeutic Approach of KRAS Mutant Tumours by the Combination of Pharmacologic Ascorbate and Chloroquine. Biomolecules. 2021 Apr 28;11(5):652. doi: 10.3390/biom11050652.

G Bánhegyi, A Szarka, J Mandl Role of Ascorbate and Dehydroascorbic Acid in Metabolic Integration of the Cell Vitamin C, In: Chen, Qi; Vissers, Margreet C.M. (szerk.) Vitamin C : New Biochemical and Functional Insights Boca Raton (FL), Amerikai Egyesült Államok : CRC Press, (2020) pp. 99-112., 14 p.

SZ Tóth, T Lőrincz, A Szarka Concentration does matter: the beneficial and potentially harmful effects of ascorbate in humans and plants Antioxidants & redox signaling 29 (15), 1516-1533 (2018)

A Szarka, O Kapuy, T Lőrincz, G Banhegyi Vitamin C and cell death Antioxidants and Redox Signaling Volume 34, Number 11, 2021 831-844. https://doi.org/10.1089/ars.2019.7897 (2021)

Szarka A, Lőrincz T, Hajdinák P. Friend or Foe: The Relativity of (Anti)oxidative Agents and Pathways. Int J Mol Sci. 2022 May 6;23(9):5188. doi: 10.3390/ijms23095188.

Varga D, Hajdinák P, Makk-Merczel K, Szarka A. The Possible Connection of Two Dual Function Processes: The Relationship of Ferroptosis and the JNK Pathway. Int J Mol Sci. 2022 Sep 20;23(19):11004. doi: 10.3390/ijms231911004.

Lőrincz T, Deák V, Makk-Merczel K, Varga D, Hajdinák P, Szarka A. The Performance of HepG2 and HepaRG Systems through the Glass of Acetaminophen-Induced Toxicity. Life (Basel). 2021 Aug 21;11(8):856. doi: 10.3390/life11080856.

5. The lipid peroxide scavenger enzyme GPX4 and its cofactor glutathione play a crucial role in the recently described programmed cell death, ferroptosis. Since sGPX4 was found in multiple organelles and non-canonical import pathways are presumable we evaluated *in silico* methods for the prediction of the localization of a chimeric signal containing peptide CYP2B1 as well as a solely mitochondrial targeted CYP27A1.

Publication: T Lőrincz, A Szarka In silico Analysis on the Possible Role of Mitochondria in Ferroptosis Periodica Polytechnica Chemical Engineering 62 (4), 370–378-370–378 (2018)

6.1. 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. We gave an *in silico* analysis on the subcellular localization of different glucose transporters and summarized the collected knowledge on them.

We observed that the silencing of NRF2 resulted in a constant activation of AMPK leading to hyperactivation of autophagy during oxidative stress. Hence we conclude that NRF2 negatively regulates autophagy through delayed down-regulation of the expression of AMPK upon prolonged oxidative stress.

Publications: Lizák, B.; Szarka, A.; Kim, Y.; Choi, K.-s.; Németh, C.E.; Marcolongo, P.; Benedetti, A.; Bánhegyi, G.; Margittai, É. Glucose Transport and Transporters in the Endomembranes. *Int. J. Mol. Sci.* **2019**, *20*, 5898. https://doi.org/10.3390/ijms20235898

M Kosztelnik, A Kurucz, D Papp, E Jones, T Sigmond, J Barna, MH Traka, T Lorincz, A Szarka, G Banhegyi, T Vellai, T Korcsmaros, O Kapuy Suppression of *AMPK/aak-2* by NRF2/SKN-1 down-regulates autophagy during prolonged oxidative stress The FASEB Journal 33 (2), 2372-2387 (2019)