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Glutathione-dependent cell deaths

Ph.D. theses

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Background

Programmed cell death

Cell death processes can be categorised into two groups: programmed cell death which after initiation is characterised by the activation of an intrinsic molecular mechanism and cell deaths which lack any molecular mechanism, also referred to as necrosis. The prototypical example of programmed cell death is apoptosis which is considered as an active (both energy-dependent and as a working apparatus) process. During apoptosis, the cell is disassembled into its' components in order to evade the release of pro-inflammatory agents into the surrounding. As programmed cell death are regulated processes their emergence is surrounded by great scientific attention as regulated mechanisms also raise the possibility of modulation.

As of now numerous programmed cell death pathways are known, like the ones which are the subject of the present dissertation: necroptosis, autophagy and ferroptosis (**Galluzzi *et al.***, **2012**).

Necroptosis

Necroptosis is a programmed cell death process with phenotypical characteristics of necrosis. Its' activation is possible by the means of apoptosis inducers (e.g. death ligands as FasL or TNFα) when apoptosis is inhibited either genetically or chemically (e.g. caspase inhibitors as z-vad-fmk) (see more in-depth in the review of **Christofferson and Yuan**, **2010**), but intrinsic initiation (e.g. oxidative stress) is also possible.

One key element of the necroptotic pathway is the activated necrosome which is composed of the RIP1-RIP3 (receptor interacting protein 1-3) phosphorylated kinase complex (**Holler *et al.***, **2000**; **Degterev *et al.***, **2005**; **Cho *et al.***, **2009**) which also functions as a cell death switch between apoptosis and necroptosis (**Zhang *et al.***, **2009**).

The first described specific inhibitor of necroptosis is necrostatin-1 which inhibits the activation of the necrosome (**Degterev *et al.***, **2005**, **2008**; **Ofengeim and Yuan**, **2013**). Sequential downstream events are the binding of MLKL (mixed lineage kinase domain-like protein) (possible inhibition by necrosulfonamide (NSA)) which forms the necroptosome complex (**Sun *et al.***, **2012**) and association with PGAM5 (*phosphoglycerate mutase/protein phosphatase 5*) (**Wang *et al.***, **2012**). The complex containing PGAM5S (short) isoform can further serve as an oligomerisation scaffold for Drp1 (dynamin-related protein 1) which results in mitochondrial fission, a terminal step in necroptosis (**Wang *et al.***, **2012**). Mitochondrial fission occurs in

apoptosis and as well in necrosis, it promotes mitophagy although mitochondrial fragmentation increases ROS production, alters energy production and calcium flux.

Ferroptosis

Ferroptosis is assumed to be a programmed oxidative cell death which can be induced by specific inductor compounds (e.g. erastin, RSL3) and inhibited by specific inhibitors (e.g. ferrostatin-1, liproxstatin-1). Ferroptosis inducers are believed to have a genotype selective (oncogenic H/K/N Ras allele) tumor-specific cytotoxicity (**Dolma *et al.***, **2003**; **Dixon *et al.***, **2012**; **Friedmann Angeli *et al.***, **2014**).

The first inhibitor of ferroptosis, ferrostatin-1, was described in 2012 (**Dixon *et al.***, **2012**) which meant the denomination of the process as ferroptosis as a reference to the importance of iron. In 2014 the second inhibitor, liproxstatin-1 was found (**Friedmann Angeli *et al.***, **2014**). Other non-specific inhibitors are the anti-oxidant glutathione, the lipophilic antioxidant α-tocopherol with mild inhibitory effect and the iron chelator deferoxamine (**Yang and Stockwell**, **2008**; **Dixon *et al.***, **2012**).

The known ferroptosis inducer coumpunds (FIN) can be divided into two groups based on their mechanism of action:

- FIN I. induce GSH depletion (erastin-like effect),

- FIN II. affect GPX4 (RSL3-like effect).

Recently two other FIN II. member compounds were discovered: FIN56 and $FINO_2$; the former induces the specific elimination of GPX4 (**Shimada *et al.***, **2016**), while the latter is an organo-peroxide inducing iron oxidation and lipid peroxidation (**Gaschler *et al.***, **2018**). GPX4 seleno-protein is a glutathione-dependent lipid-peroxide eliminating enzyme.
FIN I. compounds induce GSH depletion by inhibiting the cystine-glutamate antiporter system x_c^-. Known direct inhibitors are erastin (**Dixon *et al.***, **2012**), FDA-approved reference antitumor compound for hepatocellular carcinoma sorafenib and sulfasalazine (**Louandre *et al.***, **2013**; **Dixon *et al.***, **2014**).
Irrelevant of the inductor the process of ferroptosis is characterised by increased ROS production, lipid peroxidation (LOOX) and the elevation of the labile iron pool (LIP).

Acetaminophen-induced hepatotoxicity

Acetaminophen (*N*-acetyl-*para*-aminophenol, paracetamol, APAP) is a widely used painkiller and antipyretic drug with no anti-inflammatory effect.

Our knowledge of its' mechanism of action is limited, it is not based on the inhibition of the cyclooxygenase (COX) pathway as most NSAIDs (nonsteroidal anti-inflammatory drug, e.g. salicylates), although recently it was shown to have a COX-specific inhibitory effect in the central nervous system (**Ghanem *et al.***, **2016**).

Acetaminophen administered *per os* is absorbed almost entirely through the gastrointestinal tract. After absorbtion ~25% of the dose is eliminated by the liver through biotransformation ('first-pass metabolism') (**Clements *et al.***, **1978**) which is followed by the biotransformation of more than 90% of the drug with only 2% being eliminated by urine unchanged (**Ghanem *et al.***, **2016**). APAP is primarily conjugated by transferases (glucuronidation, sulfation), however a small fraction (5-15%) is metabolised by CYP450s (mainly CYP2E1) **Larson**, **2007**; **Ghanem *et al.***, **2016**). As a consequence of APAP oxidation by CYP450s NAPQI (*N*-acetyl-*para*-benzoquinoniminde) is formed which is a highly reactive electrophile compound. NAPQI rapidly forms adducts with glutathione and is eliminated as APAP-GSH conjugate (**Larson**, **2007**; **Ghanem *et al.***, **2016**). However, NAPQI rapidly depletes cytosolic and mitochondrial glutathione causing depletion and oxidative stress. Following the depletion of glutathione (lower than 30% of physiologic values) NAPQI forms adducts with biomolecules (nucleophile macromolecules, proteins, DNA, unsaturated lipids) resulting in hepatotoxicity (**Ghanem *et al.***, **2016**). Cytotoxicity which leads to cell death is assumed to occur in a consequence of damage to specific target proteins and not general adduct formation. This may result in mitochondrial dysfunction, increased ROS production, the inhibition of mitochondrial respiration and depletion of cellular ATP (**Meyers *et al.***, **1988**; **Ramsay, Rashed and Nelson**, **1989**; **Jaeschke**, **1990**; **Tirmenstein and Nelson**, **1990**; **Burcham and Harman**, **1991**; **Ghanem *et al.***, **2016**).

Pharmacologic ascorbate-induced tumorspecific cytotoxicity

Although ascorbate is a crucial cofactor and water soluble antioxidant in human cells, seemingly in an ambivalent manner it is able to act as a pro-oxidant as well. This capability and the cytotoxic effect of ascorbate is seen in the presence of iron and/or copper ions and oxygen. These are the requirements for the so-called Haber-Weiss reaction:

(1.) $\mathrm{Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -}}$

(2.) $\mathrm{O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+}}$

(3.) $\mathrm{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-}}$

while in the presence of ascorbate ($\mathrm{Asc^-}$):

(4.) $\mathrm{Fe^{3+} + Asc^- \rightarrow Fe^{2+} + Asc^{\bullet -}}$

According to equation (4.) ascorbate is capable of recycling of ferric iron by reduction, which in accordance with (1.) is responsible of superoxide ($O_2^{\bullet-}$) production. In equation (3.) which is the Fenton reaction, $O_2^{\bullet-}$ can form the toxic hydroxyl radical ($HO^{\bullet}$) by hydrogen peroxide (H_2O_2) intermediate. In addition $O_2^{\bullet-}$ can form H_2O_2 in a disproportion reaction as well. H_2O_2 has increased membrane permeability, its' elimination is a burden for the cellular antioxidant capacity and its' further decomposition (which can be accelerated by the elevation of the labile iron pool) results in the toxic $HO^{\bullet}$ production. $HO^{\bullet}$ is potentially cytotoxic as it can damage numerous bioactive molecules. The resulting oxidative stress and DNA damage can lead to PARP activation which then can lead to NAD^+ and ATP depletion. As the regeneration of the cellular antioxidant capacity is dependent on NADPH which in terms is dependent on glucose by the means of the pentose-phosphate shunt, it intensifies ATP depletion (**Schraufstatter *et al.***, **1986**).

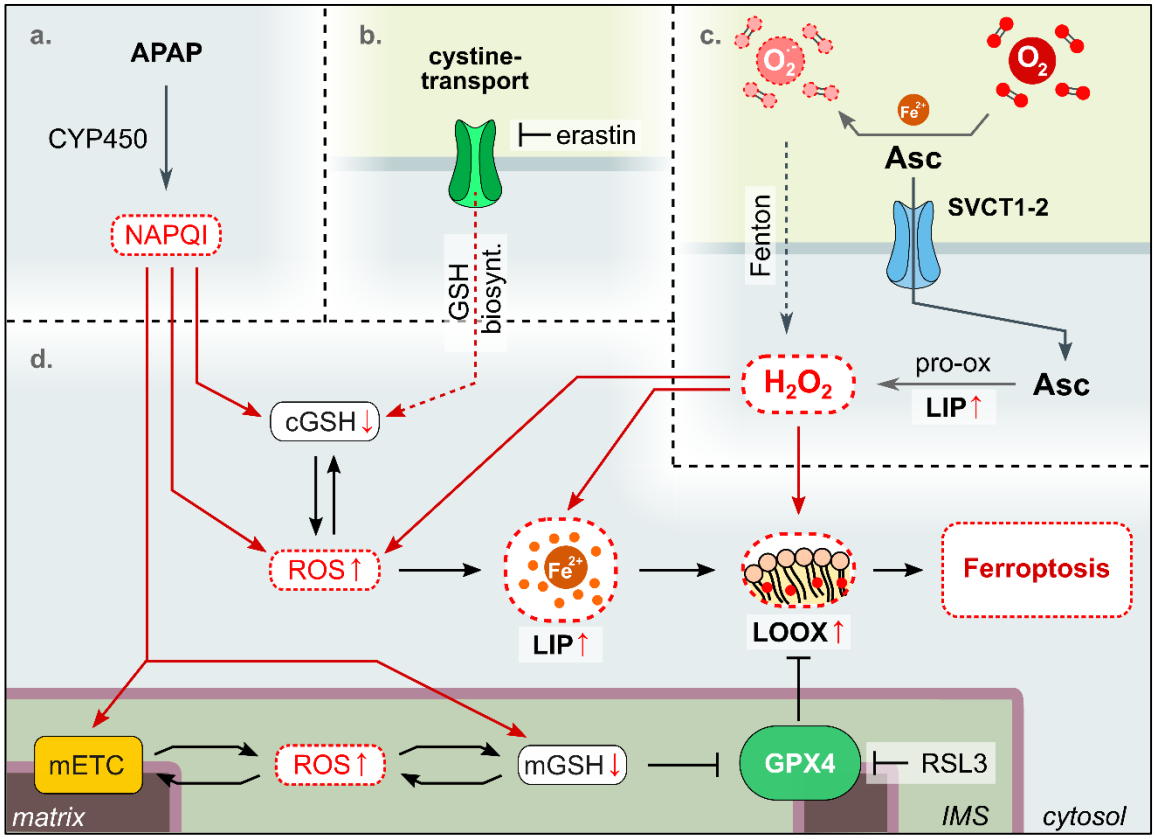
Tumor cells as opposed to somatic cells are characterised by increased ROS ($O_2^{\bullet-}$ and H_2O_2) production, increased sensitivity towards glucose-deprivation induced cytotoxicity and oxidative stress (**Ahmad *et al.***, **2005**; **Aykin-Burns *et al.***, **2009**). Furthermore it was shown that tumor cells have an elevated LIP which by the means of the Fenton reaction increase the cytotoxic effect of oxidative agents (**Torti and Torti**, **2013**).

There is extensive research on the tumor-specific cytotoxic effect of ascorbate which is called pharmacologic ascorbate (**Chen *et al.***, **2005**; **Deubzer *et al.***, **2010**; **Klingelhoeffer *et al.***, **2012**; **Parrow, Leshin and Levine**, **2013**). To achieve cytotoxicity ascorbate is administered in the mM range which *in vivo* can only be achieved parenterally. Administration of pharmacologic ascorbate *in vivo* was capable of slowing the growth of mouse hepatoma (**Verrax and Calderon**, **2009**), cervival and pancreatic-derived tumors and as well as glioblastoma (**Chen *et al.***, **2008**).

Parenteral administration of ascorbate resulted in a twelve-fold increase of ascorbate radical of the blood and intercellular fluid, however H_2O_2 levels responsible for downstream toxicity was only measurable from the intercellular fluid (**Chen *et al.***, **2007**). This might be the result of the high peroxidase capacity of cellular elements in the blood.

Pharmacologic ascorbate induced cytotoxicity as a result of Fenton chemistry is iron dependent, the inhibitory effect of iron chelators is well documented (**Verrax and Calderon**, **2009**; **Ullah *et al.***, **2011**). It was also showed that the process of labile iron pool elevation (**Baader *et al.***, **1996**; **Caltagirone, Weiss and Pantopoulos**, **2001**; **Drisko, Chapman and Hunter**, **2003**) and iron exocytosis also occurs and results in a self-reinforcement effect (**Deubzer *et al.***, **2010**).

Theoretical figure based on the investigated processes



a. Acetaminophen (APAP) biotransformation; **b.** Ferroptosis induction by erastin; **c.** The initiation of ascorbate induced cytotoxicity; **d.** Common points of the investigated process in the cytosol and mitochondrial compartment (IMS: mitochondrial intermembrane space): cytosolic/mitochondrial GSH depletion (c/mGSH ↓), increased ROS production (ROS↑), increase in labile iron pool (LIP↑), lipid-peroxidation (LOOX↑), mitochondrial electron transport chain (mETC) dysfunction.

Objectives

Our research focused on the investigation of glutathione-dependent cell deaths. The processes examined were acetaminophen-induced hepatotoxicity, high dose ascorbate induced tumorspecific cytotoxicity and ferroptosis. These processes share the common feature of increased reactive oxygen species (ROS) production, which in the case of insufficent antioxidant elimination consequently leads to lipid peroxidation (LOOX). Oxidative damage may lead to mitochondrial dysfunction or the increase of the labile iron pool (LIP). The latter increases ROS cytotoxicity through the Fenton-reaction. As numerous features of acetaminophen- and high dose ascorbate-induced cytotoxicity are shared with ferroptosis, we examined its' possible activation. As glutathione plays a crucial role in the aforementioned processes we examined the available bioanalytical methods in their performance of glutathione determination and the need for possible optimisation. During our investigation of the mitochondrial elements of ferroptosis we aimed to examine the mitochondrial localisation of the key protein of ferroptosis, glutathione peroxidase 4 (GPX4), using *in silico* techniques.

Based on the above, our objectives were:

1. the comparision and optimisation of the avaliable glutathione determination methods in their ability to determine subcellular glutathione levels and redox state even during glutathione depletion,

2. the investigation of the *in vitro* relationship of acetaminophen induced cytotoxicity and ferroptosis in hepatocarcinoma and primary mouse hepatocytes,

3. the investigation of the *in vitro* relationship of high dose ascorbate induced cytotoxicity and ferroptosis in N-RAS mutant fibrosarcoma,

4. the investigation of the subcellular localisation of glutathione peroxidase 4 using *in silico* techniques.

Methods

- **Cell culture**
- **Preparation of primary mouse hepatocytes**
- **In vitro citotoxicity assay**
- **Assesment of cell viability by flow cytometry**
- **Assesment of ROS production by the Muse® cell analyzer**
- **Assesment of ROS production (superoxide and hydroxyl-radical) and lipid peroxidation by FACSCalibur**
- **Preparation of cell organells from lat river**
- **Measurement of GSH by mClB derivatisation and HPLC-fluorescent method**
- **Measurement of GSH by NEM derivatisation and HPLC-UV-VIS method**
- **Measurement of GSH by DTNB enzyme recycling method**
- **Analysis of proteins by Western Blot**
- **Analysis of protein localisation by in silico methods**

Results and discussion

Analysis of glutathione measurement methods

Our results highlight the importance of choosing the most suitable method. *N*-ethyl maleimide is an excellent thiol-protecting group and the quantification of GS-NEM adducts

does not need high qualifications nor is instrument demanding besides an HPLC-UV, however the methods' high limit of detection we found the method inappropriate for the quantification of subcellular GSH levels nor for the DTT-dependent redox-state measurement.

The pharmacologically and toxicologically relevant GSH levels of subcellular organelles were readily quantifiable by mClB derivatisation followed by HPLC-fluorescent detection. As the isolation of subcellular organelles is a time-demanding process, the GSH content of the samples is prone to auto-oxidation. In order to counter this undesired process, we investigated the effect of mClB addition before tissue homogenisation. Our results demonstrated that pre-conjugation with mClB resulted in significantly higher GSH levels in more than one subcellular organelles.

Glutathione S-transferase (GST) can catalyse the formation of the GS-mClB adduct. By measuring the kinetics of adduct formation we concluded that it is essential to take into account the endogenous GST activity of biological samples and during calibrator preparation. According to our results at least 100 mU/ml GST activity is needed for complete GSH conjugation.

The method involving DTNB and enzyme recycling is simple and high throughput, however, our investigation showed increased matrix effect which without sufficient dilution can lead to inaccurate results. Reagents used for the determination of GSH redox-status (e.g. NEM, 2VP) influence the recycling reaction, hence their extraction with organic solvents is needed which prolong sample preparation time and reduces the methods sample throughput.

Analysis of acetaminophen-induced cell cytotoxicity

Our investigation of acetaminophen-induced cytotoxicity showed that the viability of low CYP activity bearing HepG2 hepatocarcinoma cells was affected only by very high doses of acetaminophen while the viability of isolated primary mouse hepatocytes showed high sensitivity to lower doses (20 mM) of acetaminophen as well. By using primary mouse hepatocytes we showed that the ferroptosis inhibitor ferrostatin-1, the necroptosis inhibitor necrostatin-1 and the antioxidants α-tocopherol and dehydroascorbic acid had a significant inhibitory effect on the cytotoxicity initiated by acetaminophen.

The loss in cell viability induced by ferroptosis inducers (e.g. erastin, RSL3) is almost entirely countered by the presence of specific ferroptosis inhibitors (e.g. ferrostatin-1, liproxstatin-1). As in the case of our findings with acetaminophen, the moderate inhibitory effect suggests that the cytotoxic mechanism is not entirely ferroptotic although as the inhibition by ferrostatin-1 suggests ferroptosis is also involved in the process as well as necroptosis. Acetaminophen-induced cytotoxicity is suggested to involve more than one cell death pathways which are not separable.

Analysis of the relationship between ascorbate and ferroptosis

Both pharmacologic ascorbate and ferroptosis inducer compounds show tumour-specific cytotoxicity which is an oxidative process involving increased ROS production and lipid peroxidation. According to our results albeit the two processes show a high degree of similarity, we did not find an overlap in their molecular program as the specific ferroptosis inhibitors ferrostatin-1 and liproxstatin-1 were unable to counter ascorbate-induced cytotoxicity. The oxidative stress induced by the two distinct pathways did not show a cumulative effect moreover in these circumstances ascorbate at even low concentrations (~0.1 mM) showed a remarkable inhibitory effect. Ascorbate diminished both RSL3 induced ROS production and lipid peroxidation assumably by its' antioxidant effect. We found that the inhibitory effect of ascorbate can be increased even further by the neutralisation of its' pro-oxidant effect (e.g. by pyruvate).

Our results with sublethal ascorbate doses (0.5-0.6 mM) showed that autophagy is involved in the process as autophagy induction was observed on protein level and accordingly the examined autophagy inhibitors wortmannin and bafilomycin A1 showed a moderate but significant inhibitory effect on cytotoxicity. We found no activation of the apoptotic pathway based on the examination of cleaved PARP. Our results indicate that necroptosis is also involved at sublethal ascorbate as RIPK1 protein levels increased even at 0.1 mM ascorbate.

In silico analysis of GPX4 localisation

According to our recent knowledge, the only key protein in ferroptosis is the lipid peroxide eliminating glutathione-dependent enzyme GPX4. Either direct chemical inhibition, the knockout of its gene or the deprivation of its substrate (GSH) leads to ferroptosis. Hence with the aid of *in silico* techniques, we investigated the protein localisation of the two physiologically relevant isoforms of GPX4 (sGPX4 and lGPX4) as well as of the SLC25A11 transporter with a special focus on mitochondrial involvement. Our results showed that numerous rule- and neural-based algorithms calculated specific pathway localisation for both lGPX4 and sGPX4 hence their targeting to multiple organelles is assumed. In the case of sGPX4, the probability of non-canonical import pathways arose hence we evaluated the efficiency of the algorithms to recognising either the chimeric signal of CYP2B1 with known multi-organellar targeting or the canonical mitochondrial signal in CYP27A1. Our investigation showed that in this scenario the efficiency of *in silico* methods is limited. Only CELLO algorithm was able to predict mitochondrial import based on neighbouring sequences. In the case of the presumed GSH transporter SLC25A11 our results showed that two out of seven algorithms calculated high, while other three calculated moderate mitochondrial localisation.

These results confirm the assumption that SLC25A11 is the mitochondrial transporter of GSH which can be utilised as a substrate by GPX4 and that the two proteins work together in eliminating mitochondrial lipid peroxides. We raise the possibility that direct cooperation between the two proteins in the mitochondrion is possible which could influence the regulation of the ferroptotic pathway.

Possible application of the results

Ferroptosis was described less than a decade ago as a programmed cell death capable of specific elimination of tumour cells. The molecular mechanism of ferroptosis is currently a hot research topic. The investigation of pathological activation of the process could lead to the discovery of new inhibitor compounds and pathways which could be able to eliminate unwanted cell death.

During our investigations, we focused on the optimisation of a bioanalytical method for the determination of one of the key molecules of ferroptosis, glutathione. Our results showed that GSH conjugation with mClB prior to homogenisation makes possible to quantitate subcellular GSH levels and redox-state more accurately.
Our investigation uncovered that the ferroptosis is activated during acetaminophen-induced cytotoxicity. These results contributed to the better understanding of the mechanism of hepatotoxicity.

By investigating the interaction between ascorbate and ferroptosis we found an interfering effect by which ascorbate could remarkably inhibit ferroptosis induction. These results contributed to the better understanding of the mechanism of ferroptosis and also highlighted the importance of monitoring and controlling tissue ascorbate levels in the case of clinical application of ferroptosis.

Theses

1. We showed that the most suitable method for monitoring subcellular glutathione levels is the method involving monochlorobimane (mClB) conjugation and HPLC-fluorescent detection. The advantages of this method were its low limit of detection (<0.1 μM), the possibility of redox state differentiation, and thiol protection. According to our results in order to avoid under-measurement of glutathione at least 100 mU/ml glutathione S-transferase activity is needed for the complete conjugation of glutathione by mClB during the 15 minutes of incubation of biological samples [4].

2. Our investigation on the mechanism of acetaminophen-induced cell death showed that in primary mouse hepatocytes the ferroptosis inhibitor ferrostatin-1 can significantly decrease cytotoxicity without the alteration of either cellular glutathione levels or the mRNA level of the CYP2E1 isoenzyme which is responsible for acetaminophen conversion, thus ferroptosis is involved in acetaminophen-induced cell death. Using primary mouse hepatocytes we showed that the necroptosis inhibitor necrostatin-1, as well as the antioxidant compounds α-tocopherol and dehydroascorbic acid are able to decrease acetaminophen-induced cytotoxicity [3].

3. By investigating high-dose ascorbate induced cell death we measured the ascorbate sensitivity of the HT-1080 RAS-mutant fibrosarcoma cell line and identified two compounds which can inhibit ascorbate cytotoxicity: the iron-chelator deferoxamine and the antioxidant glutathione [7].

4. With our investigation, we excluded that high-dose ascorbate induces ferroptosis in HT-1080 fibrosarcoma cell line as ferrostatin-1 and liproxstatin-1 specific ferroptosis inhibitors were unable to counter cytotoxicity. We showed that in a cell-free medium the hydrogen peroxide produced by ascorbate in the Fenton reaction is undetectable in the presence of either glutathione or pyruvate while its level is increased in the presence of deferoxamine. By examining protein activation after ascorbate cytotoxicity we showed that the activation of autophagic and necroptotic pathways only occur at low ascorbate concentrations and specific inhibitors can only inhibit cytotoxicity in this concentration range [7].

5. Our results showed that ascorbate is able to inhibit RSL3 and erastin induced ferroptosis. Our investigation uncovered that low and moderate dose ascorbate (200-500 µM) is able to counter the effect of ferroptosis inducers on cell viability decrease, reactive oxygen production and lipid peroxidation [7].

6. Based on our *in silico* investigations, we concluded that the mitochondrial localisation of the short (sGPX4) and long (sGPX4) isoforms of the lipid peroxide eliminating enzyme GPX4 as well as the presumed glutathione transporter SLC25A11 is probable. Our results suggest that the two proteins as well as mitochondrial play a crucial role in the lipid peroxidation process during ferroptosis [6]

Publication list

Publications in relation with dissertation:

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Posters in relation with dissertation:

Tamás Lőrincz, András Szarka: *Ascorbate and ferroptosis.* **Paris Redox Congress 2018**, Párizs (2018. június 25-26)

Tamás Lőrincz, András Szarka: *Ascorbate and ferroptosis.* **10th World Congress on Medicinal Chemistry and Drug Design 2018**, Barcelona (2018. június 14-15)

Tamás Lőrincz, András Szarka: *Pharmacologic ascorbate and ferroptosis.* **FEBS Advanced Course 2018**, Budapest (2018. április 4-6)

Tamás Lőrincz, András Szarka: *Glutation meghatározása szöveti és szubcelluláris szinten.* **47. Membrán-transzport konferencia 2017**, Sümeg (2017. május 16-19)

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