Title

Characterization of novel diphenylamine compounds as ferroptosis inhibitors

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Antiferroptotic diphenylamine compounds

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Nonstandard abbreviations

2-DG, 2-desoxyglucose; AA, antimycin A; AIF, apoptosis-inducing factor; Annexin V FITC, annexin V conjugated with fluorescein isothiocyanate, AMR plus, ATP monitoring reagent plus; BBB, Blood brain barrier; BCL-2, B-cell lymphoma-2; BID, BH3 interacting-domain death agonist; DCF, 2',7'-dichlorodihydrofluorescein diacetate; DMSO, dimethylsulfoxide; DMEM, Dulbecco’s Modified Eagle Medium; DPA, diphenylamine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ECAR, extracellular
acidification rate; Era, erastin FACS, fluorescence-activated cell sorting; FCCP, carbonylcyanide-4-(trifluormethoxy)-phenylhydrazone; GPX4, glutathione peroxidase 4; H₂O₂, hydrogen peroxide; hERG, human ether-a-go-go related gene; HT22, immortalized mouse hippocampal neurons; KO, knock out; LOX12/15, Lipoxygenase 12/15; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OCR, oxygen consumption rate; Oligo, oligomycin; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PI, propidium iodide; pIRES-tBid, tBid expressing plasmid; ROS, reactive oxygen species; Rot, rotenone; RSL-3: Ras-selective lethal small molecule 3; STS, staurosporine, tBid, truncated Bid; TMRE, tetramethylrhodaminethylester; xCT-System, cystine/glutamate antiporter

**Recommended sections**

Cellular and Molecular, Drug Discovery and Translational Medicine, Neuropharmacology
Ferroptosis is a form of oxidative cell death which is increasingly recognized as a key mechanism in neurodegeneration but also in regulated cell death causing disease in other tissues. In neurons, major hallmarks of ferroptosis involve the accumulation of lipid reactive oxygen species (ROS) and impairment of mitochondrial morphology and function. Compounds that interfere with ferroptosis could provide novel treatment options for neurodegenerative disorders and other diseases involving ferroptosis.

In the present study, we developed new compounds by refining structural elements of the BID inhibitor BI-6c9, that was previously demonstrated to block ferroptosis signaling at the level of mitochondria. Here, we inserted an antioxidative diphenylamine (DPA) structure to the BI-6c9 structure. These DPA compounds were then tested in models of erastin, and RSL-3 induced ferroptosis in neuronal HT22 cells. The DPA compounds showed an increased protective potency against ferroptotic cell death compared to the scaffold molecule BI-6c9. Moreover, hallmarks of ferroptosis like lipid, cytosolic and mitochondrial ROS formation were abrogated in a concentration and time-dependent manner. Additionally, mitochondrial parameters such as mitochondrial morphology, mitochondrial membrane potential as well as mitochondrial respiration were preserved by the DPA compounds, supporting the conclusion that lipid ROS toxicity and mitochondrial impairment are closely related in ferroptosis. Our findings confirm that the DPA compounds are very effective agents in preventing ferroptotic cell death by blocking ROS production and, in particular, via mitochondrial protection.
Significance Statement

Preventing neuronal cells from different forms of oxidative cell death was previously described as a promising strategy for treatment against several neurodegenerative diseases. In this study, we report novel compounds based on a diphenylamine structure that strongly protect neuronal HT22 cells from ferroptotic cell death upon erastin and RSL-3 induction by preventing the development of different ROS species and by protecting mitochondria from ferroptotic impairments.
Introduction

The term ferroptosis was coined by Stockwell and colleagues in 2012, describing an iron dependent and non-apoptotic form of oxidative cell death (Dixon et al., 2012). In recent years, studies on oxidative cell death and, in particular, on ferroptosis in the central nervous system are on the increase (Ratan, 2020), since there is compelling evidence for the involvement of these mechanisms and corresponding treatment strategies in model systems of neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, Huntington’s disease (Ren et al., 2020), and after acute brain damage, e.g. hemorrhagic stroke (Speer et al., 2013; Karuppagounder et al., 2018). Experimental induction of ferroptosis can be initiated by erastin or RSL-3 treatment in different cell lines, as for example the neuronal HT22 cell line, which has been widely established as a common model system for glutamate-induced oxidative cell death research (Tan et al., 2001). Notably, ferroptosis shares many features with the glutamate induced caspase-independent form of oxidative cell death, called oxytosis, and a close link between both pathways has been demonstrated in neuronal cells (Neitemeier et al., 2017). Initializing ferroptosis or oxytosis by the treatment of erastin and glutamate results in a disturbed redox balance by depletion of cysteine and corresponding glutathione (Tan et al., 2001) levels via inhibition of the cystine/glutamate antiporter (xCT-System) (Landshamer et al., 2008; Dixon et al., 2012; Tang et al., 2019). Furthermore, cells’ antioxidative machinery can also be targeted in a more direct way using the ferroptosis inducer RSL-3, which causes an inhibition and depletion of GPX4 protein (Jelinek et al., 2018; Tang et al., 2019), the main enzyme to remove ROS and lipid ROS in the cell (Ursini and Bindoli, 1987). All of these approaches result in the same downstream cascade including an accumulation of reactive oxygen species (ROS), especially lipid ROS, which are...
formed through autoxidation of polyunsaturated phospholipids via lipoxygenases, e.g. LOX 12/15.

Thereafter, the pro-apoptotic BCL-2 family member BH3 interacting-domain death agonist (BID) translocates to mitochondria (Landshamer et al., 2008; Neitemeier et al., 2017). This leads to an altered mitochondrial morphology possessing condensed mitochondrial membrane densities and vanished mitochondrial cristae (Angeli et al., 2017). Recent evidence supports the conclusion that mitochondria are not only involved in ferroptosis but in fact play a key role in the paradigms of caspase-independent forms of oxidative cell death. For example, loss of mitochondrial membrane potential, impaired mitochondrial respiration as well as release of mitochondrial apoptosis inducing factor (AIF) were regularly observed in different cell types when the cells died upon the aforementioned cell death paradigms (Seiler et al., 2008; Grohm et al., 2010; Friedmann Angeli et al., 2014). Thus, mitochondrial damage is widely considered as the point of no return in cells committed to die in regulated oxidative cell death (Galluzzi et al., 2009; Neitemeier et al., 2017; Jelinek et al., 2018).

Today, there are several strategies available for inhibiting ferroptosis involving radical trapping antioxidants, like ferrostatin-1 and liproxstatin-1 as well as lipoxygenase inhibitors, such as PD146176 (Angeli et al. 2017; Tobaben et al. 2011). Additionally, strategies by targeting the BID protein were successfully tested to counteract oxytosis and ferroptosis (Grohm et al., 2010; Yang et al., 2013; Oppermann et al., 2014). Inhibition or downregulation of BID using the BID inhibitor BI-6c9 (Fig.1 A), Bid siRNA or Bid CRISPR/Cas9 KO results in mitochondrial preservation and consequently protection against ferroptotic cell death (Becattini et al., 2006; Landshamer et al., 2008; Tobaben et al., 2011; Neitemeier et al., 2017). However,
novel compounds need to be developed to target ferroptosis, since until now there is a lack of effective treatment options for targeting neurodegenerative diseases (Angeli et al., 2017). In order to rectify this situation, we aimed to further refine the already promising BI-6c9 molecule. Starting this approach in 2014, BI-6c9 refinement led to the development of BID derivatives based on the N-phenyl-substituted thiazolidinediones structure (Barho et al., 2014; Oppermann et al., 2014). These inhibitors were very effective in preventing tBid induced cell death, although in the oxytosis paradigm the compounds were less effective than BI-6c9 (Oppermann et al., 2014). That is why we further refined this approach by introducing the antioxidative diphenylamine (DPA) structure into the novel compounds to increase the anti-ferroptotic potency, but also retaining the mitochondrial protection properties of BI-6c9. These novel DPA compounds were than tested in regard to their ferroptosis inhibiting capabilities and their potencies to prevent mitochondrial demise in the neuronal HT22 cells.
Materials and Methods

Compounds

The novel diphenylamine compounds were developed in cooperation with the Schlitzer group at our university’s department of pharmaceutical chemistry. These derivates were derived from the BI-6c9 backbone structure. The synthesis was similar to strategies previously established for the N-phenyl-substituted thiazolidinediones (Barho et al., 2014; Oppermann et al., 2014). Analytical data for the novel compounds is provided in the supplement (Supp. Analytical data). The compounds were dissolved in DMSO (100 mM) and then further dissolved in DMEM medium (Dulbecco’s Modified Eagle Medium, Capricorn Scientific GmbH, Ebsdorfergrund, Germany) to in-use concentrations of 10 and 1 mM, respectively. BI-6c9 (10 mM) (Sigma Aldrich, Taufkirchen, Germany), Trolox (50mM) (6-Hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid) (Sigma Aldrich, Taufkirchen, Germany), QVD-OPh (QVD) (Sigma Aldrich, Taufkirchen, Germany), staurosporine (STS) (2 mM) (Sigma Aldrich, Taufkirchen, Germany) and erastin (Era) (1 mM) (Merck KGaA, Darmstadt, Germany) were dissolved in DMSO. 1S, 3R-RSL-3 (1 mM) (later referred to as RSL-3) was synthesized as described before (Jelinek et al., 2018) and also dissolved in DMSO. H2O2 was further diluted from a 30% (9,79 M) solution to a 1 M stock solution in DMEM. Structural formulas in Figure 1 were drawn using ChemDraw® Professional (PerkinElmer Informatics, Inc).

Cell culture and treatments

Mouse hippocampal HT22 cells and the mouse hypothalamus mHypo-CLU190 cells (Cedarlane®, Cellutions Biosystems Inc., Burlington, Canada) were grown in DMEM
medium, which was additionally enriched with 10 % heat-inactivated fetal calf serum (Merck KGaA, Darmstadt, Germany), 100 U/mL penicillin (Merck KGaA, Darmstadt, Germany), 100 mg/mL streptomycin (Merck KGaA, Darmstadt, Germany) and 2 mM glutamine (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and regularly checked for mycoplasma contamination (every 3-4 months). For experiments, cells were seeded and incubated (5 % CO₂/ 37 °C) 24 - 36 h in advance at a density of 6,000 - 8,000 cells/ well in 96-well plates, 96-well xCELLigence E-plates (Roche, Applied Science, Penzberg, Germany) or XF96-well microplates (Agilent Technologies, Waldbronn, Germany); 40,000 - 60,000 cells/ well in 24-well plates, 15,000 - 20,000 cells/ well in 8-well-ibidi plates (Ibidi, Munich, Germany) and 200,000 – 300,000 cells/ well in 6-well plates, respectively. For ferroptosis induction, 0.15 – 1 µM erastin or 0.1 - 1 µM RSL-3 were applied for the indicated times depending on well size and cell density. For apoptosis induction, 1 µM staurosporine was applied and for unspecific generation of reactive oxygen species, H₂O₂ solution was added to a final concentration of 0.7 mM. The different DPA compounds were added either together with cell death inducers (co-treatment), pre-incubated 1 h in advance to cell death induction (pre-treatment) or at the indicated time points after initial cell death induction (post-treatment).

**Cell viability and cell death determination**

These experiments were performed in 96-well plates. After 24 - 36 h of cell incubation in standard growth medium, the medium was exchanged for the respective treatment solution. Afterwards, cell viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described earlier (Diemert et al., 2012). Briefly, MTT was added after the indicated time points at a final concentration of 2.5 mg/ mL for 1 h at 37 °C. After that, the supernatant was
removed and the plate was frozen for at least 1 h at -80°C. The resulting product, a purple-colored formazan, was dissolved in 70 µL DMSO and absorbance (570 nm/630 nm) was measured with FluoStar OPTIMA photometer (BMG Labtech, Offenbach, Germany). The results were normalized to untreated control to ensure comparability between different experiments.

On the basis of 3 - 5 independent MTT experiments for each individual concentration, EC50 values were calculated exploiting the GraphPad Prism Software 8.2.1 (GraphPad Software Inc., La Jolla, USA) (non-linear fit: log (inhibitor) vs. response, variable slope) in accordance to earlier EC50 calculations (Jelinek et al., 2018).

Real time cell viability measurements were conducted utilizing the xCELLigence system as described previously (Diemert et al., 2012). Briefly, cells were seeded in 96-well E-plates and incubated for up to 24 h until the cell index reached approximately a value of 1. Thereafter, cells were treated as indicated and impedance was measured over 24 h.

**Bright field images**

For representative bright field images cells were seeded in 8-well ibidi plates and treated with erastin or RSL-3 +/- compounds for 15 - 16 h, respectively. Afterwards, bright field images were captured using a Leica (Wetzlar, Germany) DM6000 epi-fluorescence microscope. When images are additionally enlarged using digital zoom, this is mentioned in the legend.

**FACS measurements**

Lipid peroxidation (BODIPY 581/591C-11 (BODIPY), Invitrogen, Karlsruhe, Germany), cellular ROS formation (2',7'-dichlorodihydrofluorescein diacetate (DCF), Invitrogen, Karlsruhe, Germany), mitochondrial ROS formation (MitoSOX, Invitrogen,
Karlsruhe, Germany), mitochondrial membrane potential (TMRE, Immunochemistry Technologies, Germany) and cell death (Annexin V FITC/propidium iodide (Annexin V/PI), Invitrogen, Karlsruhe, Germany) were measured utilizing the FACS system. For endpoint measurements, 40,000 HT22 cells were seeded in 24-well plates. On the following day, cells were treated with the indicated treatment solutions and incubated for another 15-24 h. For experiments measured until 10 h of total treatment time, 60,000 HT22 cells were seeded in 24-well plates 24 h in advance of the respective treatment. After treatment, cells were stained and measured with the FACS analyzer Guava Easy Cyte 6-2 L system (Luminex Munich GmbH, Munich, Germany). The assays are described in further detail in the respective sections. For data analysis GuavaSoft Software package was used. For every FACS experiment at least three wells per condition were measured analyzing 5,000 cells per well.

**DCF assay**

At the indicated time points after treatments, treatment solutions were exchanged by serum-free medium containing DCF dye to a final concentration of 2.5 µM for 30 min at 37 °C. Afterwards, cells were incubated with medium containing serum for additional 30 min at 37 °C, harvested, washed once and resuspended in 150 µL PBS. Fluorescence was excited at 488 nm wavelength and emission was recorded at 525/30 nm.

**BODIPY assay**

After treatment, cells were stained with BODIPY for 0.5-1 h at 37 °C in DMEM medium at a final concentration of 2 µM in order to analyze lipid peroxide levels. Afterwards, the staining solution was removed, cells were harvested, washed and
resuspended in 150 µL PBS. The BODIPY signal was analyzed at 488 nm excitation and 525/30 nm emission wavelengths.

**MitoSOX assay**

To assess mitochondrial ROS formation, cells were incubated with MitoSOX red (1.25 µM) for 30 min at 37 °C after respective treatments. Next, the staining medium was removed from the cells, harvested, washed once and resuspended in 150 µL PBS and subjected to immediate FACS analysis (excitation: 488 nm, emission: 690/50 nm).

**TMRE assay**

In order to determine the mitochondrial membrane potential, cells were stained with TMRE dye (0.4 µM) for 30 min at 37 °C following treatment. After harvesting, and washing the cells once with PBS, HT22 cells were resuspended in 150 µL PBS. FACS measurements were exerted utilizing excitation at 488 nm and emission at 690/50 nm.

**Annexin V FITC/PI assay**

Cell death measurements were conducted using annexin V/PI co-staining. In this assay, annexin V (green) stains early apoptotic cells, whereas propidium iodide (PI) (red) could solely penetrate the cells in late necrotic state to bind DNA. After treatments, cells were washed once with PBS, trypsinized, centrifuged and resuspended in 150 µL annexin V/PI binding buffer containing 1 µL annexin V and PI solution for every well and incubated for another 5-10 min at RT in the dark. The staining was detected via FACS (excitation: 488 nm, emission: red filter: 690/50 nm, green filter: 525/30 nm).
Glutathione Assay

To determine glutathione levels, 200,000 – 300,000 HT22 cells were seeded in 6-well plates 24 h before treatment. For each condition, three wells were treated similarly. At indicated time points, the cells were harvested in DMEM, and similarly treated conditions were pooled. Thereafter, cells were centrifuged and washed once with PBS and centrifuged again. The PBS was removed, cell pellets were immediately frozen in N₂ and stored at -80 °C until all samples were collected. For conducting the GSH measurements, the Glutathione Assay Kit (Cayman Chemical Company, Ann Arbor, USA) was used according to the manufacturer's protocol. Briefly, cell pellets were re-suspended in MES-buffer (0.4 M 2-(N-morpholino) ethanesulphonic acid, 0.1 M phosphate, 2 mM EDTA, pH 6.0) and sonicated for several seconds. Cell debris was removed by centrifugation at 10,000 × g for 15 min. Next, metaphosphoric acid (1.25 M) was applied to the supernatant for protein precipitation. After incubation for 5 min, the sample was centrifuged at 17,000 × g for 10 min. Thereafter, the pH of the supernatant was increased using 4 M triethanolamine solution, before the samples were transferred into a 96-well plate. Subsequently, the freshly prepared assay cocktail was added to the supernatants in the 96-well plate and incubated in the dark for 10 min. Absorbance was measured at 405 nm in a photometer (SPARK 20M, TECAN, Germany). GSH amounts were normalized to the respective protein contents.

Western blot

After respective treatments, cells were washed once with PBS and subsequently lysed in the 6-well plates using lysis buffer containing 0.25 M Mannitol, 0.05 M Tris, 1 M EDTA, 1 M EGTA, 1 mM DTT, 1% Triton-X enriched with Complete Mini Protease
Inhibitor Cocktail and PhosSTOP (Roche Diagnostics, Penzberg, Germany). Following cell lysis, cell suspensions were centrifuged for 15 min (10,000 g) at 4°C. Thereafter, protein content of the supernatant was determined using Pierce BCA Protein Assay Kit (Perbio Science, Bonn, Germany). To separate proteins by size, 50 – 70 µg of protein were subjected to SDS-PAGE gel electrophoresis (10 % SDS gels) and afterwards blotted on a PVDF-membrane (325 mA, 1.5 h). Primary antibodies for GPX4 (1:500 in milk, Abcam, Cambridge, UK) and vinculin (1:20,000 in milk, Sigma Aldrich, Taufkirchen, Germany) as loading control were incubated over night at 4°C. Corresponding HRP-labelled secondary antibodies were incubated for 1 – 2 h on the following day at RT before western blot bands were detected using the Chemidoc system (Bio-Rad, Munich, Germany). Quantification was performed using the Image Lab 4.0.1 Software (Bio-Rad, Munich, Germany). Here, background-corrected intensities of the western blot bands were determined, followed by intrinsic normalization to the respective loading control. To assure comparability between different experiments, band intensities were compared to respective control set as 100 %.

**Radical scavenging activity**

Radical scavenging activity of the compounds was measured using DPPH assay (2,2-diphenyl-1-picrylhydrazyl) (Cayman Chemical Company, Ann Arbor, USA). DPA compounds and Trolox were prepared in 75 % ethanol in respective concentrations. Afterwards, 90 µL DPPH solution (150 µM) and 10 µL sample solution were incubated in 96-well plates in the dark for 30 min and absorbance was assessed at 517 nm using a plate reader (SPARK 20M, TECAN, Germany). Respective DPPH scavenging effect was calculated in accordance to the formula:
\((A_0 - A_x) / (A_0) \times 100\); where \(A_0\) reflects the intrinsic absorbance of ethanol and \(A_x\) reflects the absorbance of the individual samples.

**Mitochondrial morphology**

In order to analyze mitochondrial morphology, HT22 cells were seeded in 8-well ibidi dishes. Twenty-four hours later, cells were incubated for another 10 h with 0.4 µM erastin with or without compound co-treatment, before they were stained with MitoTracker DeepRed (0.2 µM) (Invitrogen, Karlsruhe, Germany) for 30 min at 37 °C and subsequently fixed for 20 min with PFA 4 % (paraformaldehyde) solution and washed with PBS. MitoTracker DeepRed fluorescence was excited using a 620/60 nm laser and emission was detected via a red filter (700/75 nm). Thereafter, mitochondrial appearance was classified as previously described (Grohm et al., 2010) in three different categories. Category I: mitochondria were elongated and equally distributed in the cell. Category II: mitochondria appeared largely dotted (not coherent) but were still widely distributed in the whole cell. Category III: mitochondria were completely fragmented and located around the nucleus. At least 500 cells per condition were analyzed based on this classification system and calculated as percentage of all cells for each category. For quantification, the results of three independent experiments were taken into consideration.

Representative images were acquired via 63 x oil objective of the Leica DM6000 epi-fluorescence microscope (Wetzlar, Germany). Microphotograph magnification were performed using digital zoom.
Seahorse measurements

The seahorse system XF96-Analyzer (Agilent Technologies, Waldbronn, Germany) was utilized to investigate alterations in energy metabolism of the cell via analysis of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). For this measurement, 7,000 cells were seeded in XF96-well microplates. Different treatments were performed 36 h later. After ferroptosis initiation and compound co-treatments overnight (16 h), treatment solutions were replaced by seahorse assay medium (DMEM with 4.5 g/L (25 mM) glucose, 2 mM glutamine, 1 mM sodium pyruvate, pH 7.35) and incubated at 37 °C for 60 min without CO₂. In the meantime, the injection solutions were prepared as follows: injection A (OLIGO): oligomycin (3 µM); injection B (FCCP): FCCP (0.5 µM); injection C (AA/ROT): antimycin A (0.1 µM)/ rotenone (1 µM); injection D (2-DG): 2-desoxyglucose (50 mM). Three measurements were conducted before the first injection, representing the commencement of OCR followed by three measurements after each injection. Immediately after each injection, a three-minute mixing step was performed to ensure proper compound distribution in the well before measurements started for an additional three minutes. For evaluation, the respiration rates were normalized to respective cell density, determined as protein content of each well. Basal respiration was determined by subtracting the OCR generated by non-mitochondrial respiration (after AA/ROT application) from the commencement of OCR (before oligomycin injection). Maximal respiration was determined by subtracting the OCR, generated by non-mitochondrial respiration (after AA/ROT application), from the maximal OCR (after FCCP injection). To ensure comparability between the single experiments when three independent experiments were pooled, untreated controls of basal
respiration and maximal respiration were set to 1 for each experiment. All other values were calculated according to the respective untreated controls.

**ATP measurement**

To assess ATP levels, measurements were performed in accordance with the ViaLight™plus Bioassay Kit (Lonza, Verviers, Belgium) protocol. Briefly, cells were plated in a normal 96-well cell culture plate. After the indicated treatments, cells were lysed with respective lysis buffer. Afterwards, cell lysates were transferred to a white-walled luminescence plate (Greiner, Frickenhausen, Germany), AMR plus reagent was added and incubated for 2 min in the dark, followed by luminescence measurement via FluoStar OPTIMA photometer (BMG Labtech, Offenbach, Germany).

**Plasmid transfection**

PcDNA3.1+ vector was purchased from Invitrogen (Invitrogen, Karlsruhe, Germany). pIRES-tBid vector was used as described earlier (Landshamer et al., 2008). Plasmid transfection was performed according to the manufacture’s protocol. In short, Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) (1 µL/ well) was diluted in OptiMEM I reduced serum medium (OptiMEM) (Invitrogen, Karlsruhe, Germany) and incubated for 5 min. In the meantime, the different plasmids (0.5 µg) were diluted in OptiMEM. Thereafter, plasmid solution and Lipofectamine 2000 solution were combined and incubated for another 20 min at RT. After incubation, the transfection solution was applied drop-wise to the cells previously seeded in 24-well plates and incubated overnight. Cell viability was measured via MTT assay 16 h later.
In silico ADME and toxicity analysis

*In silico* ADME and toxicity studies were performed using the freely available *PreADMET online tool* (Bioinformatics and Molecular Design Research Bioinformatics Center, 2004) and *admetSAR online server* (Cheng *et al.*, 2012).

**Statistical analysis**

The statistical analysis was performed using Winstat standard statistical software (R. Fitch Software, Germany). For multiple comparisons, analysis of variance (ANOVA) was conducted combined with Scheffé’s post-hoc test. Data are presented as mean and standard deviation.
Results

Development of novel diphenylamine compounds based on BI-6c9

For the development of the novel compounds against ferroptotic cell death, BI-6c9 (Fig.1 A) served as a scaffold molecule. However, the diphenylthioether was replaced by a diphenylamine (DPA) structure to additionally insert an antioxidant moiety. The antioxidative effect of the diphenylamine is due to the relatively labile N-H bond, which is prone to transfer its H-atom in a radical reaction (Pratt et al., 2002; Zilka et al., 2017). Moreover, the central γ-aminobutyric acid-linker sequence is replaced by a more rigid system to ensure less flexibility of the compound. The piperidyl-N-substituent was individually designed, whereby the esterified aspartic acid (Fig.1 B), the serin derivative (Fig.1 C) and the tert-butyl residue (Fig.1 D) provided the best inhibition properties in a first preliminary MTT screening. Therefore, these compounds were further investigated for their activity to block different detrimental hallmarks of ferroptosis.

DPA compounds provide protection against ferroptosis

To analyze the protective effects of the novel DPA compounds against ferroptotic cell death, the neuronal HT22 cell line was used. In this model, cell death occurred after 10 – 12 h following initial ferroptosis induction. We initially evaluated the protective potency of the compounds upon erastin treatment by increasing concentrations of the novel compounds between 0.025 - 1 µM (Fig.2 A, C, E) in co-treatment with erastin for 16 h. After treatment time, metabolic activity was analyzed via MTT assay. Results from at least three independent experiments were pooled and fitted for EC50 determination. The calculation revealed EC50 values at a low micromolar range.
between 0.30 µM (Fig. 2 A) and 0.32 µM (Fig. 2 C) for the amino acid derivatives and 0.23 µM for the tert-butyl derivative (Fig. 2 E). Notably, compound concentrations of 1 µM provided full protection against ferroptotic cell death for all three compounds. In addition, real-time impedance measurements using the xCELLigence system confirmed the results of the MTT assays (Fig. 2 B, D, F). After 8 - 12 h of erastin treatment a drop in impedance was detected, reflecting the detachment of the dying cells from the well bottom. At EC50 the compounds were only able to attenuate the decline in cell index following ferroptosis induction, whereas erastin induced decline of the cell index was fully prevented at EC100, without intrinsically altering cell index in absence of erastin treatment (Fig. 2 B, D, F). Moreover, the protective effects were confirmed across different classes of ferroptosis inducers. While 16 h exposure to erastin or RSL-3 resulted in massive cell death (Fig. 3 A, Erastin; RSL-3), co-treatment with 1 µM of each DPA compound effectively counteracted these deleterious effects (Fig. 3 A, DPA 1-3 + Era, DPA 1-3 + RSL-3). Correspondingly, cell death measurements using Annexin V/PI staining unveiled a clear dose-dependent protection against erastin (Fig. 3 B) as well as RSL-3 (Fig. 3 C) induced cell death. Moreover, the DPA compounds alone did not show any toxic effects, even when treated at concentrations 10-100-fold higher (10-100 µM) than needed for the maximal protective effect (1µM) (Supp. Fig. 1 A). Furthermore, to exclude a cell line-specific effect of the DPA compounds, the compounds were additionally tested in the mouse hypothalamus (mHypo) cell line. Corresponding to the results obtained in the HT22 cell line, morphological alterations upon erastin treatment were attenuated by EC50 concentrations of the compounds and completely blocked by the respective EC100 concentrations (Supp. Fig. 2 A). To validate this impression, MTT assays as well as Annexin V/PI cell death measurements were conducted and revealed
protection against ferroptotic cell death comparable to previous observations in the HT22 cells (Supp. Fig. 2 B, C). Intriguingly, apoptotic cell death, induced by 1 µM staurosporine (STS) treatment in HT22 cells, could only be blocked by the broad spectrum caspase inhibitor QVD but not by any of the novel DPA compounds (Supp. Fig. 3).

All in all, these results showed that the novel DPA compounds provide concentration-dependent, cell line independent protection against ferroptotic cell death in neurons induced by different ferroptosis inducing agents, i.e. erastin and RSL-3.

**Major ROS dependent hallmarks of ferroptosis are effectively blocked by the novel compounds**

We extended the foregoing experiments to determine whether the novel compounds affect different pivotal hallmarks of ferroptosis. Initially, lipid ROS formation was examined as it is considered to be the most conclusive hallmark of ferroptosis. In HT22 cells, elevated lipid peroxide levels occur 4 - 8 h after ferroptosis induction (Jelinek et al., 2018). Coinciding with this event, soluble, cytosolic ROS levels, measured as DCF fluorescence, also rose. Increased lipid peroxidation as well as soluble ROS formation were inhibited in a concentration-dependent manner by all novel DPA compounds (Fig. 4 A, B). Using the MitoSOX assay, we investigated superoxide formation specifically in mitochondria as the next step in the ferroptosis cascade. In line with our previous results, DPA compounds demonstrated a dose-dependent suppression of mitochondrial ROS formation (Fig. 4 C). To exclude adverse effects on these ferroptosis hallmarks due to the DPA compound treatment per se, the novel compounds were also tested without erastin, where no alterations to untreated controls could be detected. (Supp. Fig. 1 B-D). To further investigate the
DPA compound’s potency to directly abrogate radicals in this paradigm, we performed the established DPPH assay for evaluating radical scavenging effects. Notably, DPA compounds did not exhibit direct radical scavenging capacity when tested at full protective concentrations (EC100) in this assay (Fig. 4 D).

This prompted us to examine if the protective effects seen in the aforementioned assays upon erastin treatment were also transferrable to ferroptosis induction by the GPX4 targeting agent RSL-3. Indeed, the novel compounds clearly demonstrated a concentration-dependent protection against cytosolic and lipid, as well as mitochondrial ROS formation in the paradigm of RSL-3 induced ferroptosis (Supp. Fig. 4 A-C).

Since the compounds prevented ROS-dependent hallmarks of ferroptosis, we next tested whether the compounds also affected the upstream cascade of ferroptosis including glutathione levels and GPX4 regulation. Former studies demonstrated that glutathione levels were depleted after erastin treatment (Tan et al., 2001). In contrast, GSH levels were not altered by RSL-3 since RSL-3 induces ferroptosis via direct GPX4 inhibition and subsequent downregulation of the protein (Jelinek et al., 2018).

Therefore, we evaluated glutathione levels 6 h after respective treatments with the DPA compounds in absence or presence of erastin or RSL-3. Our results clearly demonstrate that the DPA compounds affected neither glutathione levels under basal control conditions nor after RSL-3 treatment, nor did they prevent the decrease of GSH levels during erastin-mediated ferroptosis (Supp. Fig. 5 A). Moreover, using Western blot analysis of GPX4 levels, we observed that the DPA compounds influenced neither basal GPX4 levels after 16 h of DPA treatment alone (Supp. Fig. 5 B, D), nor did they prevent the decrease of GPX4 expression upon RSL-3 treatment.
(Supp. Fig. 5 C, E). These results indicate that the novel compounds inhibit ferroptosis without affecting GSH depletion or GPX4 downregulation after erastin or RSL-3 treatment, respectively.

In conclusion, these experiments demonstrate, that the novel compounds counteract ferroptotic cell death downstream of GSH and GPX4 depletion by preventing the development of different ROS species in the cytosol as well as in mitochondria without possessing intrinsic radical scavenging effects.

**DPA compounds preserve mitochondrial membrane potential and morphology**

As previously reported, upstream ferroptotic effects such as lipid and cytosolic ROS formation lead to mitochondrial impairments in the proceeding cascade, resulting in fragmented mitochondrial morphology in combination with impaired mitochondrial function and a loss of mitochondrial membrane potential. We expanded our studies to investigate the mitochondrial morphology by microscopic analysis after 10 h of erastin treatment. Ferroptosis induction caused a significant change from elongated mitochondria, which were distributed throughout the whole cell (referred to as category I) (Fig. 5 A Control) to fragmented mitochondria, which were located around the nucleus (referred to as category III) (Fig. 5 A Erastin). All three compounds led to a totally preserved mitochondrial morphology when treated together with erastin for 10 h (Fig. 5 A DPA 1-3 + Era). To additionally quantify this impression, mitochondrial morphology of three independent experiments of at least 500 cells per condition were rated in accordance to the aforementioned rating system and afterwards pooled for statistical analysis. As expected, the erastin conditions with DPA compound co-treatment showed a significant reduction in mitochondria classified as category III compared to erastin (Fig. 5 B). This preserved mitochondrial morphology by the novel
compounds in co-treatment with erastin was associated with a maintained mitochondrial membrane potential, which was analyzed using TMRE assay after 15 h (Fig. 5 C). The preservation of mitochondrial membrane potential through the DPA compounds was provided in a dose-dependent manner (Fig. 5 C). Moreover, the dose-dependent protection of mitochondrial membrane potential was also applicable to the RSL-3 induced ferroptotic paradigm (Supp. Fig. 4 D), whereas a direct interference of the novel compounds with the TMRE fluorescence dye could be excluded (Supp. Fig. 1 E).

DPA compounds maintain mitochondrial respiration and function

To investigate whether the compounds affect mitochondrial bioenergetics, we utilized the seahorse system to analyze important respiration parameters such as the oxygen consumption rate (OCR), which is an indicator for energy supply through oxidative phosphorylation in mitochondria, as well as the extracellular acidification rate (ECAR), as an indicator for energy supply based on glycolysis. When HT22 cells were treated with 0.15 µM erastin over 15 - 16 h, a striking drop in basal OCR and ECAR was observed (Fig. 6 B, D). Furthermore, the capacity for maximal respiration, which was evoked by the application of the protonophore FCCP, was remarkably reduced by 0.15 µM erastin application (Fig. 6 B). When DPA compounds were applied without additional erastin treatment, basal respiration seemed to be slightly increased above control levels (Fig. 6 A), although the statistical analysis of three independent experiments revealed, that this effect was not significant for any of the compounds (Fig. 6 E). Co-treatment of the different DPA compounds with erastin provided a complete maintenance of basal respiration (Fig. 6 B), which was substantiated in the pooled results (Fig. 6 E). Moreover, statistical analysis further demonstrated no intrinsic effects on FCCP stimulated maximal respiration, when the
compounds were applied in absence of erastin, but a totally preserved maximal respiration when applied together with erastin (Fig.6 F). Furthermore, mitochondrial independent energy metabolism via glycolysis was explored. Basal glycolytic activity was not affected, when DPA compounds were applied at concentrations of 1 µM without additional erastin treatment (Fig.6 C). However, the detrimental effects of erastin on glycolysis were completely blocked by DPA compound co-treatment (Fig.6 D). Furthermore, we checked the ATP status of the cells to investigate an additional parameter for mitochondrial function. Sixteen hours of erastin treatment caused an intense decline in ATP levels due to marked mitochondrial damage, which could be prevented by the compounds in a dose-dependent manner (Fig.6 G).

The results obtained imply that the novel DPA compounds are very effective in maintaining mitochondrial integrity and function against ferroptotic demise, thereby, providing a significant contribution to cell survival.

**Post-treatment time windows after ferroptosis induction**

As a last step in the characterization process of the compounds, we investigated in which post-treatment time window the compounds could be applied to effectively inhibit cell death and prevent the development of the aforementioned hallmarks of ferroptosis. For these experiments, ferroptosis was initiated by erastin treatment in advance to DPA compound application. To the indicated post-treatment time points, erastin solution was exchanged with an erastin solution containing the novel compounds. Afterwards, the cells were further incubated to a total incubation time of 24 h.

Remarkably, the DPA compounds were able to rescue HT22 cells from ferroptotic cell death when the compounds were added no later than 6 h after the initial erastin treatment (Fig.7 A). In line with this, lipid peroxidation as well as cytosolic and
mitochondrial ROS formation could also be prevented up to 6 h after ferroptosis induction (Fig. 7 B - D), whereas mitochondrial membrane potential was still largely maintained when DPA compounds were applied 8 h after erastin challenge (Fig. 7 E).
Discussion

In the present study we introduced new DPA compounds which were very effective in inhibiting ferroptotic cell death in neuronal cells. These novel compounds were developed to enhance the antiferroptotic potency of the BID inhibitor BI-6c9 by inserting an antioxidative diphenylamine structure. The obtained compounds were able to block major hallmarks of ferroptosis such as lipid peroxide, soluble ROS and mitochondrial ROS formation. Our results indicate that mitochondrial protection is crucial for cell survival after ferroptosis induction.

Initially, the novel DPA compounds were tested in a MTT assay to determine their potencies to inhibit ferroptotic cell death. The EC50 values ranged from 0.23 µM for the tert-butyl compound DPA-3 to 0.30 and 0.32 µM for the amino acid derivatives DPA-1 and DPA-2, respectively. Full protection was observed at concentrations of 1 µM for all DPA compounds. Remarkably, the novel DPA compounds were 10-fold more potent than the established scaffold molecule BI-6c9, which provides full protection at concentrations of 10 µM (Culmsee et al., 2005; Oppermann et al., 2014; Jelinek et al., 2018). Furthermore, the DPA compounds were about 20-fold more effective in inhibiting ferroptosis than the former BI-6c9 derivatives based on the N-phenyl thiazolidinedione structure which possess EC50 values between 6.78 – 9.87 µM (Oppermann et al., 2014).

Notably, no intrinsic cytotoxic effects were found for concentrations up to 100 µM. The DPA compounds were unable to inhibit apoptotic cell death through staurosporine treatment, indicating a specificity of these compounds towards caspase-independent cell death mechanisms like ferroptosis and oxytosis. This disability to block staurosporine induced cell death was also shown for the scaffold
molecule BI-6c9 (Landshamer et al., 2008), so that similar mechanisms of action can be surmised.

Moreover, we investigated the compounds with regard to several important hallmarks of ferroptosis. Remarkably, only the ROS dependent parameters downstream of glutathione and GPX4 depletion were affected. Accordingly, the compounds prevented the development of lipid peroxidation and cytosolic as well as mitochondrial ROS formation upon erastin and RSL-3 treatment in a concentration-dependent manner. Protection against these ROS hallmarks was also demonstrated for other ferroptosis inhibitors such as liproxstatin-1 or ferrostatin-1 upon RSL-3 induced ferroptosis (Jelinek et al., 2018), which are established as ferroptosis inhibiting radical-trapping antioxidants. This could indicate an antioxidant capacity of the novel DPA compounds that is presumably directly related to the diphenylamine moiety (Sugihara et al., 1993; Conrad and Pratt, 2019). However, unlike with ferrostatin-1 (Dixon et al., 2012), no direct radical scavenging effect could be demonstrated for the DPA compounds in the DPPH assay. For this reason, we additionally tested the compounds in a H$_2$O$_2$ model. H$_2$O$_2$, as one of the ROS species occurring in HT22 cells during ferroptosis, can enter cell membranes and thereby directly alter ROS levels inside the cell (Kang et al., 2012). The DPA compounds were able to prevent H$_2$O$_2$ mediated cell death as expected, although these effects were not as pronounced as the effects obtained by the radical scavenger Trolox (Supp. Fig. 6). This fact suggests that the ability of the novel compounds to provide protection against ferroptosis is at least to some extent attributed to antioxidant effects probably through the newly introduced DPA structure.

Next, we tested the ability of the novel compounds to abrogate mitochondrial demise. Mitochondrial impairment is widely believed to play a key role in the mechanisms of
oxidative stress induced neurodegeneration (Knott et al., 2008; Jelinek et al., 2018; Battaglia et al., 2020). As also studied here, this mitochondrial demise upon ferroptosis induction comprises mitochondrial fragmentation, a loss of mitochondrial membrane potential and impaired mitochondrial energy metabolism. Hence, pharmacological treatments, focusing on the rescue of mitochondria for instance via MitoQ, a specific mitochondrial ROS scavenger, were recently described as a way to inhibit cell death in oxidative stress models (Friedmann Angeli et al., 2014; Jelinek et al., 2018).

As a consequence, maintenance of mitochondrial integrity and functional cellular energy metabolism seems to be crucial for cell survival in the ferroptotic paradigm and therefore protecting mitochondria serves as a promising way to prevent neuronal cells from ferroptotic cell death. In fact, all novel DPA compounds prevented the loss of mitochondrial membrane potential in a concentration-dependent manner in co-treatment with ferroptosis inducers, confirming the close link between upstream ROS dependent effects, mitochondrial demise and cell death.

Accordingly, mitochondrial morphology and mitochondrial oxidative phosphorylation processes, measured as the oxygen consumption rate in the Seahorse system, were preserved when cells were treated with erastin in the presence of the novel DPA compounds. Moreover, ATP levels of the cells, as the end product of the oxidative phosphorylation and a well-accepted measure for mitochondrial respiratory function, were preserved in a concentration-dependent manner by the DPA compounds against erastin treatment, further confirming the maintained mitochondrial integrity.

In examining the mode of action of the DPA compounds, an effective time window was determined in which the compounds could be added following ferroptosis initiation to prevent the development of the ferroptotic hallmarks as well as ferroptotic
cell death. Ferroptotic cell death could be largely blocked when compounds were applied up to 6 h after initial erastin treatment. This is in line with post-treatment results from experiments conducted with BI-6c9 in HT22 cells upon RSL-3 induced ferroptosis (Jelinek et al., 2018). Moreover, lipid peroxidation as well as cytosolic and mitochondrial ROS formation could also be prevented until 6 h after ferroptosis induction. However, at treatment timepoints beyond 6 h, formation of these ROS species could no longer be prevented via the DPA compounds. These results indicated that the DPA compounds did not act as radical scavengers but interfered with the ROS formation process. Intriguingly, mitochondrial membrane potential is still largely maintained at control levels up to 8 h after first ferroptosis induction even when cell death has already been determined. This could indicate a direct protective interaction of the novel compounds on the level of mitochondria suggesting that mitochondria once harmed by ferroptotic upstream effects, a cascade including another boost of all ROS parameters including lipid peroxide, cytosolic and mitochondrial ROS formation occurs. Although, the DPA compounds could stop a further decrease of mitochondrial membrane potential at this stage, the cell death response could not be halted implying that these effects were driven independently from mitochondria, for instance, via apoptosis inducing factor (AIF) released from mitochondria upon ferroptotic signals to execute cell death. (Landshamer et al., 2008; Tobaben et al., 2011; Jelinek et al., 2018).

However, the precise target of the novel DPA compounds leading to the strong ferroptosis inhibition is yet to be identified. It seems obvious that this mechanism of action may involve the inhibition of the pro-apoptotic protein BID, since BI-6c9 was the scaffold molecule for these compounds. This supposition is supported by other studies demonstrating that the aforementioned hallmarks of ferroptosis like lipid
peroxide and mitochondrial ROS formation as well as mitochondrial demise were also abrogated by BID inhibition or genetic BID depletion (Neitemeier et al., 2017). However, unlike BI-6c9 (Landshamer et al., 2008; Oppermann et al., 2014), the DPA compounds failed to protect HT22 cells in truncated Bid (t-Bid) induced cell death (Supp. Fig. 7). However, it is still unclear whether Bid cleavage in its “activated” t-Bid form is mandatory in the paradigm of caspase-independent cell death pathways in neuronal cells. So far, BID cleavage could not be demonstrated in HT22 cells in models of glutamate induced oxytosis (Tobaben et al., 2011). Additionally, other studies indicate that in neurons BID migrates to mitochondria in its full-length form (Ward et al., 2006; König et al., 2007). It appears that tBid overexpression is unsuitable in finally clarifying the potential role of full length BID in ferroptotic cell death and, therefore, the question whether BID inhibition is involved in the mechanism of the DPA compounds needs further investigation.

Nevertheless, the protective effect of the novel DPA compounds against ferroptotic neuronal cell death at low micromolar concentrations together with the low intrinsic neurotoxicity, as demonstrated in this study, encourages for further in vivo application in the future. For example, the DPA compounds may be applied in model systems of intracerebral hemorrhage (ICH), where hallmarks of neuronal ferroptosis like GSH depletion, lipid peroxidation as well as mitochondrial demise have recently been detected (Li et al., 2017; Wu et al., 2018) (Li et al., 2017; Wu et al., 2018).

For preliminary predictions on metabolism in vivo, DPA-1 to DPA-3 have been evaluated in silico for their ADME and Tox Parameters using the PreADMET (Bioinformatics and Molecular Design Research Bioinformatics Center, 2004) and
admetSAR online server (Cheng et al., 2012). The *in silico* analysis (Supp. Table 1) revealed no major issues which would preclude further development of these compounds. The only exemption is the predicted activity of DPA-2 and 3 on the hERG channel indicating a potential risk for cardiac arrhythmia. These predictions have to be experimentally proved or disproved in course of future development. Parameters indicating oral bioavailability were in acceptable ranges, however low BBB permeability was predicted for DPA-1 and -2, possibly due to their charged nature. In contrast, the predications suggest a good penetration in the brain for the non-charged DPA-3. Moreover, compounds were predicted to be non-mutagenic and non-carcinogenic. P-glycoprotein inhibition was predicted for DPA-1 and 2, whereas DPA-1 and 3 could be possible substrates for the Cyp3A4 enzyme, however this is also the case for most drugs in use. Furthermore, none of the compounds show inhibition of the other Cyp-enzymes used in the in-silico prediction (Supp. Table 2).

To sum up, our research highlights novel compounds that were 10 to 20-fold more effective in protecting neuronal cells against ferroptotic cell death than the scaffold molecule BI-6c9 and the analogue N-phenyl thiazolidinediones. This protection was associated with efficiently preventing the development of several ROS dependent hallmarks of ferroptosis and with a preserved mitochondrial integrity. Post-treatment experiments further confirmed that mitochondria are pivotal in the ferroptotic paradigm, indicating that strategies for mitochondrial protection should be taken into account for further development or improvement of antiferroptotic compounds, since targeting ferroptosis could be a valuable tool for treatment of neurodegenerative diseases in the future.
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Authorship contribution

Participated in research design: Hinder, Michels, Culmsee

Conducted experiments: Hinder, Emmerich

Contributed new reagents: Pfaff, Schlitzer

Performed data analysis: Hinder, Emmerich, Michels, Schlitzer, Culmsee

Wrote or contributed to the writing of the manuscript: Hinder, Michels, Schlitzer, Culmsee
References


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Tobaben S, Grohm J, Seiler A, Conrad M, Plesnila N, and Culmsee C (2011) Bid-mediated mitochondrial damage is a key mechanism in glutamate-induced


Footnotes

This work received no external funding, and no author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

Fig. 1: Structure of BI-6c9 and novel ferroptosis inhibiting compounds.

A) Structure of Bid inhibitor BI-6c9; B-D) Novel compounds are composed of a diphenylamine moiety linked to a piperidine-4 carboxamide via an amid bond and a third varying residue. B) DPA-1 compound (Schl-30172); C) DPA-2 compound (Schl-30174), D) DPA-3 compound (Schl-30736)

Fig. 2: EC50 determination and real-time impedance measurement reveal a marked antiferroptotic effect of novel diphenylamine compounds.

A, C, E) Diphenylamine compounds provided concentration-dependent protection upon erastin treatment (0.4 µM) in HT22 cells. For EC50 determination, MTT results after 16 h of erastin co-treatments with respective concentrations from 3 – 5 independent experiments were used. EC50 values were calculated using the prism software. Dotted lines mark the EC50 values: A) EC50 (DPA-1): 0.30 µM; C) EC50 (DPA-2): 0.32 µM; E) EC50 (DPA-3): 0.23 µM.

B, D, F) Real-time impedance measurements revealed a dose-dependent protection of the novel DPA compounds over 24 h. Compounds alone did not influence the cell index. Each curve contains different measurement point of n = 6 - 8 wells.

All data are shown as mean +/- S.D. ###p < 0.001 compared to erastin treated control (ANOVA, Scheffé’s test).
Fig. 3: DPA compounds abrogate cell death upon different ferroptosis inducers in HT22 cells.

A) Representative bright field image of HT22 cells (20x + 3x digital zoom) treated with either erastin (0.4 µM) or RSL-3 (0.2 µM) for 15 - 16 h in absence or presence of novel diphenylamine compounds (1 µM) revealed pronounced protection against the different ferroptosis inducers. Scale bar: 100 µm

B), C) Cell death measurements using Annexin V/ PI staining unveiled a dose-dependent protection of cell demise upon erastin (0.4 µM) B) and RSL-3 (0.1 µM) C). B), C) show representative results from n = 3; all experiments were repeated at least three times with similar outcomes; all data are shown as mean + S.D. ***p < 0.001 compared to untreated control; #p < 0.05, ##p < 0.01, ###p < 0.001 compared to erastin or RSL-3 treated control (ANOVA, Scheffé’s test).

Fig. 4: DPA compounds prevent cytosolic and mitochondrial ROS as well as lipid ROS formation without possessing intrinsic ROS scavenging capabilities.

A) HT22 cells were treated with erastin 0.4 µM for 8 h A) or 6 h B), respectively, and with or without co-treatment of the indicated concentrations of differing compounds. Following, cells were stained with BODIPY A) or DCF B) and subjected to FACS analysis. Lipid peroxidation A) and soluble ROS formation B) were prevented in a dose-dependent manner according to the EC50 and EC100 concentration.
C) Mitochondrial ROS formation, detected by MitoSOX staining, was measured after 15 h erastin treatment in presence or absence of the DPA compounds. Thereby, a dose-dependent abrogation of mitochondrial ROS formation was observed. D) DPPH assay revealed no intrinsic ROS scavenging abilities of the compounds. Trolox
100 µM served as a positive control and ethanol as a negative control. Ethanol effect was subtracted as background correction for all results. A-C) show representative results from n = 3 and D) shows representative results from n = 6; all experiments were repeated at least three times with similar outcomes; all data are shown as mean + S.D. ***p < 0.001 compared to untreated control; #p < 0.05, ##p < 0.01, ###p < 0.001 compared to erastin treated control (ANOVA, Scheffé’s test).

**Fig. 5: Compounds preserve mitochondrial morphology and mitochondrial membrane potential following erastin challenge.**

A) Representative images (63 x oil-objective) of mitochondria after 10 h of erastin treatment (erastin) (0.4 µM) or medium control (control). DPA compounds (1 µM) were applied as co-treatment. Cells were stained with MitoTracker DeepRed (0.2 µM) before being fixed using 4 % PFA solution. Scale bar: overview: 20 µm, zoom: 5 µm. B) Categorization of mitochondria was performed in accordance to the protocol detailed in methods. For quantification at least n = 500 cells per condition of three independent experiments were taken into consideration and thereby a strong preservation of mitochondrial morphology was revealed. ***p < 0.001 of cells classified as category III compared to untreated control; ##p < 0.01 of cells classified as category III compared to erastin treated control (ANOVA, Scheffé’s test). C) Representative results of mitochondrial membrane potential analysis using TMRE staining followed by FACS assessment 15 h after erastin treatment or combined erastin compound treatment (n = 3) demonstrate a concentration-dependent maintenance of mitochondrial membrane potential. All data are shown as mean + S.D. ***p < 0.001 compared to untreated control; #p < 0.05, ###p < 0.001 compared to erastin treated control (ANOVA, Scheffé’s test).
Fig. 6: Novel compounds maintain mitochondrial respiratory function upon erastin treatment.

A - D) Representative results from Seahorse measurements after 16 h of respective treatment (n = 6 - 8/ condition). Plots show the effects of 1 µM compound treatment on oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) after 16 h incubation without erastin treatment A), C) or in combined treatment with 0.15 µM erastin B), D). 16 h of erastin treatment resulted in strong decrease in OCR B) and ECAR D), which was completely prevented by the compounds. E), F) Quantification of basal respiration (before oligomycin injection) E) and maximal respiration (after FCCP injection) F) of three independent experiments. DPA compounds led to slight, but not significant rise of basal respiration in absence of erastin (white columns E)). In presence of erastin, both, basal and maximal respiration were fully restored to control levels (black columns E) and F)). G) ATP levels were investigated via ATP luminescence assay 16 h after erastin treatment. Novel diphenylamine compounds maintain ATP levels in accordance with their EC50 and EC100 concentration (n = 8). All data are shown as mean ± S.D. or +/- S.D. **p < 0.01, ***p < 0.001 compared to untreated control; #p < 0.05, ###p < 0.001 compared to respective erastin treated control (ANOVA, Scheffé’s test).

Fig. 7: Cell viability and formation of different ROS species could be blocked up to 6 h and loss of mitochondrial membrane potential could be prevented up to 8 h after ferroptosis induction.

A-E) Ferroptosis was induced 0 – 10 h in advance to novel compound application (1 µM). After the last DPA compound treatment, cells were incubated to a total erastin treatment time of 24 h. A) Compounds largely preserved cell viability
measured via MTT assay when applied no later than 6 h after initial erastin treatment. B-D) Cytosolic B), lipid C) and mitochondrial D) ROS formation were also widely abolished until 6 h of previous ferroptosis initiation. Interestingly, loss of mitochondrial membrane potential E) could largely be blocked until 8 h after first erastin exposure. All data are shown as representative examples with mean + S.D. of n = 3, all experiments were repeated in similar ways at least three times with similar outcomes. ***p < 0.001 compared to untreated control; #p < 0.05, ##p < 0.01, ###p < 0.001 compared to erastin treated control (ANOVA, Scheffé’s test).
Fig. 2

A

![Graph showing metabolic activity of Ctrl, DPA-1 1 μM, and DPA-1 0.3 μM treatments.](image)

EC$_{50}$(DPA-1): 0.30 μM

B

![Graph showing normalized cell index over time of treatment for Ctrl, DPA-1 1 μM, and DPA-1 0.3 μM treatments.](image)

Erastin 0.4 μM

C

![Graph showing metabolic activity of Ctrl, DPA-2 1 μM, and DPA-2 0.3 μM treatments.](image)

EC$_{50}$(DPA-2): 0.32 μM

D

![Graph showing normalized cell index over time of treatment for Ctrl, DPA-2 1 μM, and DPA-2 0.3 μM treatments.](image)

Erastin 0.4 μM

E

![Graph showing metabolic activity of Ctrl, DPA-3 1 μM, and DPA-3 0.2 μM treatments.](image)

EC$_{50}$(DPA-3): 0.23 μM

F

![Graph showing normalized cell index over time of treatment for Ctrl, DPA-3 1 μM, and DPA-3 0.2 μM treatments.](image)
**Fig. 3**

**A**

Control | Erastin | RSL-3
---|---|---
DPA-1 | DPA-1 + Era | DPA-1 + RSL-3
DPA-2 | DPA-2 + Era | DPA-2 + RSL-3
DPA-3 | DPA-3 + Era | DPA-3 + RSL-3

**B**

Erastin 0.4 μM, 15 h

Annexin V+ PI positive cells [% of gated cells]

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**C**

RSL-3 0.1 μM, 16 h
**A**

Control Erastin
DPA-1 + Era DPA-2 + Era DPA-3 + Era

**B**

![Graph showing Mitochondrial categories (%)]

- **Ctrl**
- **Era**
- **DPA-1 + Era**
- **DPA-2 + Era**
- **DPA-3 + Era**

**C**

Erastin 0.4 μM, 15 h

- **Ctrl**
- **Era**
- **DPA-1**
- **DPA-2**
- **DPA-3**

TMRE fluorescence [% of gated cells]
Fig. 7

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Erastin 0.4 μM, 24 h

A

Metabolic activity [% of control]

B

DCF fluorescence [% of gated cells]

C

BODIPY fluorescence [% of gated cells]

D

TMRE fluorescence [% of gated cells]

E

MitoSOX fluorescence [% of gated cells]
Supplemental Data

**Article Title:** Characterization of novel diphenylamine compounds as ferroptosis inhibitors  
**Authors:** Hinder L., Pfaff A.L., Michels S., Schlitzer M., Culmsee C.  
**Journal Title:** Journal of Pharmacology and Experimental Therapeutics  
**Manuscript Number:** JPET-AR-2021-000534

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**Supp. Analytical data**

**DPA-1**

**MF:** \( \text{C}_{23}\text{H}_{30}\text{Cl}_{2}\text{N}_{4}\text{O}_{4} \)

**MW:** 497.42 g/mol

**Solubility** DMSO > 100 mM  
H₂O > 100 µM (10 % DMSO)

**Mp.:** 181 °C (decomposition).

**\(^1\)H-NMR:** 400 MHz, DMSO-\(\text{d}_6\); \(\delta = 9.90\) (s, 1H, NH), 8.43+8.24 (s, 3H, NH₃), 7.47 – 7.43 (m, 2H, \(\text{H}_{\text{arom}}\)), 7.16 – 7.10 (m, 2H, \(\text{H}_{\text{arom}}\)), 6.99 – 6.94 (m, 4H, \(\text{H}_{\text{arom}}\)), 6.75 – 6.68 (m, 1H, \(\text{H}_{\text{arom}}\)), 4.40 – 4.20 (m, 2H, \(\text{CH, H}_{\text{Pip}}\)), 3.86 – 3.78 (m, 1H, \(\text{H}_{\text{Pip}}\)), 3.68 (s, 3H, CH₃), 3.08 – 2.99 (m, 3H, \(\text{CH, H}_{\text{Pip}}\)), 2.66 – 2.56 (m, 2H, \(\text{H}_{\text{Pip}}\)), 1.81 – 1.75 (m, 2H, \(\text{H}_{\text{Pip}}\)), 1.64 – 1.52 (m, 1H, \(\text{H}_{\text{Pip}}\)), 1.49 – 1.38 (m, 1H, \(\text{H}_{\text{Pip}}\)) ppm.

The signal of the diphenylammonium was not detected. Due to conformational restrictions some signals are doubled.

**\(^{13}\)C-NMR:** 101 MHz, DMSO-\(\text{d}_6\); \(\delta = 172.8, 170.8, 167.4+167.3, 144.7, 139.2, 132.8, 129.6 (2C), 121.0 (2C), 119.4, 118.4 (2C), 116.3 (2C), 53.3, 49.3+49.2, 45.0+44.9, 42.8+42.7, 41.4+41.3, 33.5+33.3, 29.2+29.1, 28.6 ppm.

**HRMS:** ESI(+) m/z for \(\text{C}_{23}\text{H}_{29}\text{N}_{4}\text{O}_{4}^+\); calculated: 425.2183, found: 425.2191.

**FT-IR:** ATR, \(\tilde{\nu} = 2924\) (w), 2857 (w), 1744 (w), 1597 (m), 1546 (m), 1509 (vs), 1493 (s), 1443 (s), 1320 (m), 1233 (m), 1175 (m), 1110 (w), 1073 (w), 1028 (w), 953 (m), 841 (m), 738 (m), 692 (m), 602 (s), 512 (s), 477 (s) cm⁻¹.
**Article Title:** Characterization of novel diphenylamine compounds as ferroptosis inhibitors  
**Authors:** Hinder L., Pfaff A.L., Michels S., Schlitzer M., Culmsee C.  
**Journal Title:** Journal of Pharmacology and Experimental Therapeutics  
**Manuscript Number:** JPET-AR-2021-000534

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**DPA-2**

**MF:** \( \text{C}_{21}\text{H}_{26}\text{Cl}_{2}\text{N}_{4}\text{O}_{3} \)

**MW:** 455.38 g/mol

**Solubility**  
DMSO > 100 mM  
H\(_2\)O > 100 µM (10 % DMSO)

**Mp.:** 181 °C.

**\(^1\text{H-NMR:}\)**  
400 MHz, DMSO-\text{d}_6; \( \delta = 9.88 \) (s, 1H, NH), 8.12 (s, 3H, NH\(_3\)), 7.49 – 7.41 (m, 2H, Harom), 7.17 – 7.12 (m, 2H, Harom), 7.01 – 6.94 (m, 4H, Harom), 6.75 – 6.68 (m, 1H, Harom), 4.39 – 4.29 (m, 2H, CH, HPip), 4.00 – 3.92 (m, 1H, HPip), 3.73 – 3.56 (m, 2H, CH\(_2\)), 3.13 – 3.05 (m, 1H, HPip), 2.73 – 2.58 (m, 2H, HPip), 1.84 – 1.76 (m, 2H, HPip), 1.65 – 1.56 (m, 1H, HPip), 1.52 – 1.43 (m, 1H, HPip) ppm.  
The signal of the diphenylammonium was not detected the OH-signal is masked by the HDO-signal.

**\(^{13}\text{C-NMR:}\)**  
101 MHz, DMSO-\text{d}_6; \( \delta = 172.7, 144.6, 139.1, 132.7, 129.7 \) (2C), 121.0, 120.9, 119.4 (2C), 118.3 (2C), 116.2 (2C), 60.2, 52.9, 42.7, 41.8, 30.7, 28.7 ppm.  
The signal of one aliphatic Carbon is masked by the DMSO-signal.

**HRMS:**  
ESI(+) for \( \text{C}_{21}\text{H}_{27}\text{N}_{4}\text{O}_{3} \); calculated: 383.2078, found: 383.2091.

**FT-IR:**  
ATR, \( \tilde{\nu} = 3237 \) (w), 2927 (w), 1644 (m), 1595 (m), 1510 (s), 1493 (s), 1445 (m), 1401 (w), 1316 (m), 1257 (m), 1229 (m), 1173 (m), 1029 (m), 955 (m), 880 (w), 829 (m), 744 (m), 693 (m), 602 (m), 512 (s) cm\(^{-1}\).
Supplemental Data

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**DPA-3**

MF: $C_{23}H_{29}N_3O_2$

MW: 379.50 g/mol

**Solubility**

- DMSO > 100 mM,
- H$_2$O > 100 µM (10 % DMSO)

**Mp.:** 206 ºC.

**$^1$H-NMR:** 400 MHz, DMSO-$d_6$; δ = 9.69 (s, 1H, NH), 7.94 (s, 1H, NH), 7.50 – 7.40 (m, 2H, H$_{arom}$), 7.20 – 7.11 (m, 2H, H$_{arom}$), 7.01 – 6.90 (m, 4H, H$_{arom}$), 6.76 – 6.66 (m, 1H, H$_{arom}$), 4.36 – 4.22 (m, 2H, H$_{Pip}$), 2.86 – 2.75 (m, 2H, H$_{Pip}$), 2.59 – 2.51 (m, 1H, H$_{Pip}$), 1.81 – 1.73 (m, 2H, H$_{Pip}$), 1.54 – 1.40 (m, 2H, H$_{Pip}$), 1.17 (s, 9H, CH$_3$) ppm.

**$^{13}$C-NMR:** 101 MHz, DMSO-$d_6$, δ = 175.3, 172.9, 144.6, 139.1, 132.8, 129.7 (2C), 121.0 (2C), 119.4, 118.4 (2C), 116.2 (2C), 44.7, 43.3, 38.7 (2C), 29.3 (2C), 28.6 (3C) ppm.

**HRMS:** ESI(+), m/z for $C_{23}H_{29}N_3O_2Na^+$, calculated: 402.2152, found: 402.2165.

**FT-IR:** $\tilde{\nu}$ = 3318 (m), 2954 (w), 2925 (w), 2860 (w), 1654 (m), 1615 (s), 1597 (s), 1540 (s), 1509 (s), 1494 (vs), 1446 (s), 1404 (m), 1363 (m), 1322 (s), 1258 (m), 1233 (w), 1165 (s), 1100 (w), 1030 (w), 1010 (w) 956 (m), 874 (w), 822 (m), 806 (m), 767 (w), 749 (m), 697 (m), 647 (w), 581 (m), 561 (m), 515 (s), 499 (m) cm$^{-1}$. 
Supplemental Data

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Supp. Fig. 1

Supp. Fig. 1: DPA compounds are well tolerated and show no neurotoxic effects or intrinsic alterations of ferroptosis hallmarks. A) Cell viability was not altered compared to control, when HT22 cells were treated with DPA compounds at concentrations of 1 – 100 µM for 16 h (n = 8). (B - D) Intrinsic effects of the DPA compounds (1 µM) on B) lipid peroxidation, C) cytosolic ROS formation, D) mitochondrial ROS formation, and E) mitochondrial membrane potential were not found. All data are shown as representative results with mean + S.D. (ANOVA, Scheffé’s test).
Supplemental Data

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**Supp. Fig. 2**

**A)** Representative bright field images of mHypo cells (10x magnification) treated with erastin (1 µM, 16h) in absence or presence of EC50 and EC100 concentrations of the novel compounds, respectively (Scale bars 100 µm). Cell viability **B)** and cell death **C)** measurements revealed a dose-dependent protection for all DPA compounds against ferroptotic cell demise in the neuronal mHypo cells. B), C) show representative results from n = 3; all data are shown as mean ± S.D. **p < 0.001** compared to untreated control, **##p < 0.001** compared to erastin treated control (ANOVA, Scheffé’s test).

**Supp. Fig. 2: Protective effects of the DPA compounds against ferroptosis in mouse hypothalamus (mHypo) cells.**
Supp. Fig. 3: DPA compounds do not prevent apoptotic cell death in HT22 cells upon staurosporine treatment. Cells were pre-incubated with diphenylamine compounds (1 µM) and QVD (10 µM) for 1 h. After that, cells were treated with staurosporine (STS) and staurosporine/compound co-treatment solution for 17 h. Only the caspase inhibitor QVD prevented cell death in this apoptosis cell death paradigm. Cell death rates were determined via annexin V/PI staining and subsequent FACS analysis. All data are shown as representative examples with mean ± S.D. ***p < 0.001 compared to untreated control; ###p < 0.001 compared to erastin treated control (ANOVA, Scheffé’s test).
Supp. Fig. 4: DPA compounds prevent ferroptotic hallmarks upon RSL-3 treatment. Concentration-dependent protection of the novel DPA compounds against RSL-3 (0.1 - 0.2 µM) induced lipid peroxidation A), cytosolic ROS formation B), mitochondrial ROS formation C) and loss of mitochondrial membrane potential D). All data are shown as representative examples with mean ± S.D. **p < 0.01 compared to untreated control, #p < 0.05, ##p < 0.01, ###p < 0.001 compared to erastin treated control (ANOVA, Scheffé’s test).
Supplemental Data

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Supp. Fig. 5

Supp. Fig. 5: Novel DPA compounds do not affect glutathione and GPX4 levels.  
A) DPA compounds (1 µM) were treated in absence or presence of erastin (1 µM) and RSL-3 (1 µM) for 6 h (n = 5). B, C) Representative western blots of GPX4 levels and vinculin as loading control after 16 h of DPA treatment alone B) or after a 4 h-co-treatment of the DPA compounds with RSL-3. D) Quantification of n = 4 western blot results from DPA treatment alone for 16 h or E) quantification of n = 5 western blot results from DPA RSL-3 co-treatment for 4 h. All data are shown as mean ± S.D. ***p < 0.001 compared to untreated control (ANOVA, Scheffé’s test).
Supplemental Data

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**Supp. Fig. 6**

**Supp. Fig. 6:** Novel compounds prevent H2O2 induced cell death, however, not as pronounced as Trolox. Diphenylamine compounds (1 µM) and Trolox (100 µM) as positive controls were co-treated with 0.7 mM H2O2 solution. After 16 h, cell viability was assessed using MTT assay. All diphenylamine compounds were protective against H2O2 treatment, although solely Trolox co-treatment brought metabolic activity back to control levels. All data are shown as representative examples with mean + S.D. ***p < 0.001 compared to untreated control, ###p < 0.001 compared to erastin treated control (ANOVA, Scheffé’s test).
Supp. Fig. 7: DPA compounds did not prevent pIRES-tBid induced cell death. HT22 cells were transfected with pIRES-tBid vector (0.5 μg) or pcDNA 3.1 + vector (0.5 μg) as control for 16 h. Co-treatment using diphenylamine compounds (1μM) did not prevent decrease of cell viability measured in MTT assay. All data are shown as representative examples with mean ± S.D. ***p < 0.001 compared to untreated control.
**Supplemental Data**

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**Supp. Table 1: In-silico ADME-parameters predicted with the preADMET-server:**

<table>
<thead>
<tr>
<th>No.</th>
<th>Properties</th>
<th>DPA-1</th>
<th>DPA-2</th>
<th>DPA-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>BBB-Penetration</td>
<td>0.0651745</td>
<td>0.0646825</td>
<td>2.64996</td>
</tr>
<tr>
<td>2.</td>
<td>Caco2 (nm/sec)</td>
<td>17.2368</td>
<td>15.1995</td>
<td>24.7099</td>
</tr>
<tr>
<td>3.</td>
<td>HIA (%)</td>
<td>87.538415</td>
<td>85.01442</td>
<td>94.491544</td>
</tr>
<tr>
<td>4.</td>
<td>MDCK (nm/sec)</td>
<td>6.81055</td>
<td>2.51159</td>
<td>0.0944907</td>
</tr>
<tr>
<td>5.</td>
<td>PPB (%)</td>
<td>73.398999</td>
<td>49.861928</td>
<td>90.898779</td>
</tr>
<tr>
<td>6.</td>
<td>hERG-Inhibition</td>
<td>ambiguous</td>
<td>high risk</td>
<td>high risk</td>
</tr>
<tr>
<td>7.</td>
<td>AMES Test</td>
<td>non-mutagen</td>
<td>non-mutagen</td>
<td>non-mutagen</td>
</tr>
<tr>
<td>8.</td>
<td>Carcinogenicity</td>
<td>non-carcinogen</td>
<td>non-carcinogen</td>
<td>non-carcinogen</td>
</tr>
</tbody>
</table>

(1) BBB: Blood-Brain Barrier; a value >1 indicates an CNS-active compound  
(2) Caco-2: Human intestinal cell line used for *in-silico* simulation for cell permeability; low permeability: <4, medium permeability 4~70; high permeability: more than 70.  
(3) HIA: Human Intestinal Permeability; value closer to 1 represents better absorption through the intestine.  
(4) MDCK: Madin-Darby Canine Kidney; Represents the renal clearance of the molecule. Lower values indicate higher clearance  
(5) PPB: Plasma Protein Binding  
(6) hERG: human Ether-a-go-go Related Gene  
(7) Ames Test for potential mutagenicity of the compounds  
(8) Carcinogenicity indicates the cancer inducing ability of the molecule.
## Supplemental Data

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**Supp. Table 2:** *in-silico* metabolism properties of DPA-1, DPA-2 and DPA-3 obtained from the *admetSAR*-server

<table>
<thead>
<tr>
<th>No.</th>
<th>Properties</th>
<th>DPA-1</th>
<th>DPA-2</th>
<th>DPA-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>P-gp Inhibition/Substrate</td>
<td>Yes/No</td>
<td>Yes/No</td>
<td>No/No</td>
</tr>
<tr>
<td>2.</td>
<td>Cyp-3A4 Inhibition/Substrate</td>
<td>No/Yes</td>
<td>No/No</td>
<td>No/Yes</td>
</tr>
<tr>
<td>3.</td>
<td>Cyp-2D6 Inhibition/Substrate</td>
<td>No/No</td>
<td>No/No</td>
<td>No/No</td>
</tr>
<tr>
<td>4.</td>
<td>Cyp-2C9 Inhibition/Substrate</td>
<td>No/No</td>
<td>No/No</td>
<td>No/No</td>
</tr>
<tr>
<td>5.</td>
<td>Cyp-2C19 Inhibition</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6.</td>
<td>Cyp-1A2 Inhibition</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>7.</td>
<td>Cyp inhibitory promiscuity</td>
<td>No</td>
<td>No</td>
<td>No</td>
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</tbody>
</table>