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:** $C_{23}H_{30}Cl_2N_4O_4$

**MW:** 497.42 g/mol

**Solubility**

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

**Mp.:** 181 °C (decomposition).

**$^1$H-NMR:** 400 MHz, DMSO-$d_6$; $\delta$ = 9.90 (s, 1H, NH), 8.43+8.24 (s, 3H, NH$_3$), 7.47 – 7.43 (m, 2H, H$_{arom}$), 7.16 – 7.10 (m, 2H, H$_{arom}$), 6.99 – 6.94 (m, 4H, H$_{arom}$), 6.75 – 6.68 (m, 1H, H$_{arom}$), 4.40 – 4.20 (m, 2H, CH, H$_{Pip}$), 3.86 – 3.78 (m, 1H, H$_{Pip}$), 3.68 (s, 3H, CH$_3$), 3.08 – 2.99 (m, 3H, CH$_2$, H$_{Pip}$), 2.66 – 2.56 (m, 2H, H$_{Pip}$), 1.81 – 1.75 (m, 2H, H$_{Pip}$), 1.64 – 1.52 (m, 1H, H$_{Pip}$), 1.49 – 1.38 (m, 1H, H$_{Pip}$) ppm.

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

**$^{13}$C-NMR:** 101 MHz, DMSO-$d_6$; $\delta$ = 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 $C_{23}H_{29}N_4O_4^+$; 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$^{-1}$. 
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**DPA-2**

<table>
<thead>
<tr>
<th><strong>MF:</strong></th>
<th>$\text{C}<em>{21}\text{H}</em>{28}\text{CL}<em>{2}\text{N}</em>{4}\text{O}_{3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MW:</strong></td>
<td>455.38 g/mol</td>
</tr>
<tr>
<td><strong>Solubility</strong></td>
<td>DMSO &gt; 100 mM</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_{2}\text{O} &gt; 100 \mu\text{M} (10 % \text{DMSO})$</td>
</tr>
<tr>
<td><strong>Mp.:</strong></td>
<td>181 °C.</td>
</tr>
</tbody>
</table>
| **$^1\text{H-NMR:}$** | 400 MHz, DMSO-$\delta_6$; $\delta = 9.88$ (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-$\delta_6$; $\delta = 172.7, 144.6, 139.1, 132.7, 129.7$ (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$^{-1}$. |
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DPA-3

MF: \( \text{C}_{23}\text{H}_{29}\text{N}_{3}\text{O}_{2} \)
MW: 379.50 g/mol
Solubility: DMSO > 100 mM, H\(_2\)O > 100 µM (10 % DMSO)
Mp.: 206 °C.

\(^1\text{H}-\text{NMR:} \) 400 MHz, DMSO-\( \text{d}_6 \); \( \delta = 9.69 \) (s, 1H, NH), 7.94 (s, 1H, NH), 7.50 – 7.40 (m, 2H, H\(_{\text{arom}}\)), 7.20 – 7.11 (m, 2H, H\(_{\text{arom}}\)), 7.01 – 6.90 (m, 4H, H\(_{\text{arom}}\)), 6.76 – 6.66 (m, 1H, H\(_{\text{arom}}\)), 4.36 – 4.22 (m, 2H, H\(_{\text{Pip}}\)), 2.86 – 2.75 (m, 2H, H\(_{\text{Pip}}\)), 2.59 – 2.51 (m, 1H, H\(_{\text{Pip}}\)), 1.81 – 1.73 (m, 2H, H\(_{\text{Pip}}\)), 1.54 – 1.40 (m, 2H, H\(_{\text{Pip}}\)), 1.17 (s, 9H, CH\(_3\)) ppm.

\(^{13}\text{C}-\text{NMR:} \) 101 MHz, DMSO-\( \text{d}_6 \), \( \delta = 175.3, 172.9, 144.6, 139.1, 132.8, 129.7 \) (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 \( \text{C}_{23}\text{H}_{29}\text{N}_{3}\text{O}_{2}\text{Na}^+ \), 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\(^{-1}\).
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).
Supp. Fig. 2: Protective effects of the DPA compounds against ferroptosis in mouse hypothalamus (mHypo) cells. 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. 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 < 0.001 compared to untreated control, #p < 0.05, ##p < 0.01, ###p < 0.001 compared to erastin treated control (ANOVA, Scheffé’s test).
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).
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.
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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>
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<tr>
<td>2</td>
<td>Caco2 (nm/sec)</td>
<td>17.2368</td>
<td>15.1995</td>
<td>24.7099</td>
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<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
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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>
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<tr>
<td>6.</td>
<td>Cyp-2C19 Inhibition</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<td>7.</td>
<td>Cyp-1A2 Inhibition</td>
<td>No</td>
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<td>No</td>
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<td>8.</td>
<td>Cyp inhibitory promiscuity</td>
<td>No</td>
<td>No</td>
<td>No</td>
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