Supporting Data and Information (SI)

Redox modulators determine luminol luminescence generated by porphyrin-coordinated iron and may repress 'suicide inactivation'.

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SI 1: Luminol protects heme compounds against suicide inactivation

(Supporting Data)

Table S 1.1  The half-inhibitory peroxide concentrations IC\textsubscript{50} of the HRP-catalysed luminol reaction and its dependence on the luminol concentration [LH]. Data were extracted from Fig 1 of the main manuscript by sigmoidal four-parameter logistic curve fitting to the data on the right hand side of the peak.

| [LH] (μM) | IC\textsubscript{50} (μM H\textsubscript{2}O\textsubscript{2}) |
|-----------|-----------------|
| 2.5       | 1120            |
| 25        | 2080            |
| 250       | 3800            |
| 2500      | 7100            |

![Figure S 1](image_url) Dependence of luminescence on [H\textsubscript{2}O\textsubscript{2}] and luminol concentration [LH] with Cyt\textsubscript{c} as catalyst. 

**A**: Assay conditions: 100 mM Tris/HCl pH = 9; [Cyt\textsubscript{c}] = 15 μg/ml; [LH] is indicated in the inset; 

**B**: Data were normalized by the area under the curve for better comparison. Luminescence data represent light yield (I\textsubscript{LY}) integrated over the first 15 min of the reaction. Data are averages of n = 4 technical replicates. Error bars represent StDv. StDv is below symbol size, where no error bar can be seen.
Table S 1.2  The half-inhibitory peroxide concentrations (IC$_{50}$) of the HRP-catalysed luminol reaction and its dependence on the GSH concentration. Data were extracted from Fig 2 of the main manuscript by sigmoidal four-parameter logistic curve fitting to the data on the right hand side of the peak.

| [GSH] (µM) | IC$_{50}$ (µM H$_2$O$_2$) |
|------------|--------------------------|
| 0          | 3900                     |
| 20         | 3630                     |
| 80         | 3980                     |
| 320        | 4660                     |

SI 2: Luminescence recovery in dependence on [GSH] (Supporting Data)

Figure S 2 Effect of GSH on the kinetics of the luminol reaction catalysed by HRP. Antioxidants inhibit the luminol reaction and render it futile. However, light production of the luminol reaction starts when all AOs in the reaction are consumed. A: Various [GSH], as indicated in the inset, were used to demonstrate the effect. These are the data shown in Fig 3A of the main manuscript, but plotted here on a linear scale B: The delay time the catalyst needs to consume all GSH correlates with the [GSH] over several orders of magnitude. Assay conditions: Tris/HCl 100 mM pH = 9; [HRP] = 0.75 µg/ml; [LH] = 750 µM; [H$_2$O$_2$] = 1.1 mM.
SI 3: Luminescence quenching in dependence on reducing agents

(Supporting Data)

Figure S 3.1 Comparison of the initial quenching effects of reducing agents on the luminol reaction in the presence of diverse catalysts. Several concentrations of various reducing agents were tested for their ability to reduce the light emission of the luminol reaction: A glutathione (data presented in Fig 4A) B di-thiothreitol; C ascorbate; D uric acid; E trolox; F sulfite; Relative light yield integrated over the first minute of each reaction is plotted against the respective concentration of the reducing agent. Data are normalized by the luminescence obtained without reducing agent. Assay conditions: Tris/HCl 100 mM pH = 9; [LH] = 750 µM; [H₂O₂] = 1.1 mM; Catalysts are indicated in the respective insets: [HRP] = 0.5 µg/ml; [Cytc] = 20 µg/ml; [Hb] = 20 µg/ml; [FeEDDHA] = 200 µM; [FeSO₄] = 200 µM; Data are means of n = 3 technical replicates. Error bars represent StDv. StDv is below symbol size where no error bar can be seen.
Figure S 3.2 The quenching effect of GSH on the luminol reaction catalysed by Cyt c and HRP. Four-parameter logistic functions were fitted to the data shown in Fig 4A of the main manuscript. This revealed IC\textsubscript{50} values of the quenching effect produced by GSH and demonstrates that there is a single IC\textsubscript{50} when Cyt c-catalysed luminescence is quenched (A) whereas GSH exerts two different effects on HRP which reveal themselves by two EC\textsubscript{50} values (B) Assay conditions: Tris/HCl 100 mM pH = 9; [L] = 750 µM; [H\textsubscript{2}O\textsubscript{2}] = 1.1 mM; [HRP] = 0.75µg/ml; [Cyt\textsubscript{c}] = 15 µg/ml; Data are means of n = 3 technical replicates. Error bars are given in Fig 4A of the main manuscript.
SI 4: The effects of enhancers (Supporting Data)

Table S 4.1  Dependence of half-inhibitory H$_2$O$_2$-concentrations (IC$_{50}$) on the iodophenol concentration [IP]. Data from Fig 6 of the main manuscript were plotted on log-log scale and the IC$_{50}$ was determined by sigmoidal four-parameter logistic curve fitting to the data on the right hand side of the peak.

| [IP] (µM) | IC$_{50}$ (µM H$_2$O$_2$) |
|-----------|--------------------------|
| 0         | 2000                     |
| 0.5       | 3200                     |
| 2.5       | 6700                     |
| 12.5      | 15300                    |

Figure S 4.1 Effect of benzidine and coumarine on the luminol reaction in the presence of diverse catalysts. Various concentrations of enhancers (A: benzidine; B: coumarine) were tested for their ability to boost the light yielding reaction. The relative light yield of each reaction is plotted against the respective enhancer concentration. Data are normalized to the luminescence obtained without enhancer. Assay conditions: Tris/HCl 100 mM pH = 9; [L] = 750 µM; [H$_2$O$_2$] = 1.1 mM; Catalysts are indicated in the inset: [HRP] = 0.375 µg/ml; [Cyt c] = 15 µg/ml; [Hemin] = 0.75 µM; [FeEDDHA] = 150 µM; [Hb] = 3.75 µg/ml. Data are means of n = 3 technical replicates. Error bars represent StDv. StDv is below symbol size where no error bar can be seen.
Figure S 4.2  Dependence of luminescence on \([H_2O_2]\) in presence of benzidine [Benzidine] is indicated in the inset and luminescence is plotted against \([H_2O_2]\) in order to demonstrate that the peroxide-induced inhibition of the reaction shifts towards higher \([H_2O_2]\) with increasing [Benzidine]. Assay conditions: Tris/HCl 100 mM pH = 9; \([L]\) = 750 µM; \([HRP]\) = 0.125 µg/ml; Data are averages of n = 3 technical replicates. Error bars represent StDv. StDv is below symbol size, where no error bar can be seen.

SI 4: The effects of enhancers (Supporting Information)

Table S 4.2  Reaction equations representing the mechanism of the enhanced catalytic peroxidative cycle and the subsequent luminol reaction. The following reactions from Tao et al. (2013) and Bhandari et al. (2010)\(^1,2\) are crucial for understanding the enhanced peroxidase luminol cycle and refer to Fig 8 of the main paper.

\[
\begin{align*}
\text{POX} + H_2O_2 & \rightarrow \text{CMP I} & \text{Eq S 1} \\
\text{CMP I} + LH^- & \rightarrow \text{CMP II} + L^- & \text{Eq S 2} \\
\text{CMP II} + LH^- & \rightarrow \text{POX} + L^- & \text{Eq S 3} \\
\text{CMP I} + AH & \rightarrow \text{CMP II} + A^- & \text{Eq S 4} \\
\text{CMP II} + AH & \rightarrow \text{POX} + A^- & \text{Eq S 5} \\
A^- + LH^- & \rightarrow AH + L^- & \text{Eq S 6} \\
L^- + L^- & \rightarrow LH^- + AQ & \text{Eq S 7}
\end{align*}
\]
SI 5: Luminol-based quantitative assays (Supporting Information)

Quantitative light detection against a dark background (i.e. photon counting luminometry) is highly sensitive as well as easy to perform \(^3\)\(^-\)\(^6\). The sensitivity range of modern light detectors exceeds eight orders of magnitude when calibrated neutral density filters are used \(^7\).

Consequently, chemiluminescence assays are among the most sensitive quantification methods allowing the detection of analytes in concentrations as low as pico- or even attomolar \(^8\)\(^-\)\(^{12}\).

Assays based on luminol are used for a broad variety of analytes (e.g. \(\text{H}_2\text{O}_2\) \(^{13}\)\(^-\)\(^{17}\); vitamin \(\text{B}_{12}\) \(^18\); antioxidants \(^{19}\)\(^-\)\(^{27}\); chlorine \(^{28}\); phenols in wastewater \(^29\); nitrate and nitrite in freshwater \(^30\); Dissolved iron \(^31\); \(\text{NO}_2\) and \(\text{NO}_3\) \(^{32}\)\(^,\)\(^{33}\); transition metals and metal-proteins \(^{34}\)\(^-\)\(^{36}\), tagged proteins in western blots \(^37\); proteases \(^38\); peroxidases \(^19\)\(^,\)\(^{39}\)). Also forensic investigators take advantage of luminol-based detection of blood (reviewed in \(^{40}\)\(^,\)\(^{41}\)) and even luminol luminescence imaging of tumors in mice has been successfully demonstrated \(^42\).
SI 6: The complexity of the luminol reaction (Supporting Information)

5-amino-2,3-dihydro-1,4-phthalazinedione also known as luminol (LH₂) emits blue light when oxidized in an alkaline environment in the presence of a strong oxidising agent such as hydrogen peroxide (H₂O₂). The oxidising peroxide donates the oxygen needed for the reaction and converts luminol into aminophthalate (AP*), which is the light emitting product. Thereby molecular nitrogen (N₂) is formed (Fig S 6.1)

\[
\text{LH}_2 + 2[\text{O}] \xrightarrow{\text{catalyst}} \text{AP} + \text{N}_2 + h \cdot \nu
\]

\[
2\text{H}_2\text{O}_2 + 2\text{OH} \xrightarrow{\text{Catalyst}} + \text{N}_2 + 4\text{H}_2\text{O} + h \cdot \nu
\]

Figure S 6.1 Summary of the luminol reaction. A: Luminol (LH₂) is oxidised to aminophthalate (AP) with the emission of light (h·ν) and nitrogen (N₂). The reaction only occurs under strong oxidising conditions (e.g. H₂O₂), at high pH (e.g. NaOH), and is boosted in presence of a catalyst (e.g. transition metals). B: The oxygen incorporated into the molecule by this reaction is derived from the peroxide.

The luminol reaction is more complex than Fig S 6.1 suggests, because a catalyst is indispensable for detectable light emission (h·ν). During catalysis, several intermediates (Table S 6), reactive radicals, transient oxidation states and reactive oxygen species (ROS) are formed before an excited aminophthalate (AP*) can release a photon.

In addition, different routes of electron transfer and hydrogen abstractions may occur. This specifically depends on the molecular environment and the type of catalyst. Despite this complexity, the luminol reaction is one of the most closely studied chemiluminescence reactions 43.
Table S 6 Structures of luminol, intermediates and product of the reaction

|   | Structures                                                                 |
|---|---------------------------------------------------------------------------|
| 1 | Luminol (LH₂)                                                             |
| 1a&1b | Luminol-monoanion (LH⁻) & Luminol-dianion (L²⁻)                          |
| 2 | Luminol-radical (L•⁻)                                                     |
| 3 | Luminol-di-azaquinone (AQ)                                                 |
| 4 | Luminol-(di-oxetane)endo-peroxide (LO₂²⁻)                                 |
| 5 | Aminophthalate (AP²⁻)                                                     |
In aqueous solutions at pH = 9, about 98% of luminol is in its monoanion form (LH\textsuperscript{-}; 1a in Table S 6). This is because the dissociation constants are pK\textsubscript{a1} = 6.3 and pK\textsubscript{a2} > 12 .

\[
\begin{align*}
LH_2 &\rightleftharpoons pK_{a1}=6.3 & LH^{-} &\rightleftharpoons pK_{a2}>12 & \rightarrow L^{2-} & \\
& & & & & \text{Eq S 6.1}
\end{align*}
\]

Generally, the first step of the luminol reaction is an electron abstraction from the luminol monoanion (LH\textsuperscript{-}) in presence of a molecular species C\textsubscript{ox} with high oxidation power (Eq S 6.3). LH\textsuperscript{-} is oxidised to its radical form (L\textsuperscript{-}; 2 in Table S 6) . C\textsubscript{ox} is reduced to C\textsubscript{red} and then somehow recycled by the peroxide (e.g. H\textsubscript{2}O\textsubscript{2}) to C\textsubscript{ox} . Two L\textsuperscript{-} recombine to L\textsuperscript{2-} and Azaquinone (AQ; 3 in Table S 6) (Eq S 6.4; ) . At a sufficiently high H\textsubscript{2}O\textsubscript{2} concentration and high pH, AQ is then converted to the intermediate cyclic peroxide adduct (LO\textsubscript{2}\textsuperscript{2-}; 4 in Table S 6; Eq S 6.5a). Thereby oxygen which originates from the peroxide is introduced. The acid dissociation constant of the peroxide intermediate of luminol (LO\textsubscript{2}\textsuperscript{-}; 4 in Table S 6) is 11.1-12.1 (Eq S 6.2a) and only the basic form of this intermediate is decomposed to excited aminophtalate (AP\textsuperscript{2-}; 5 in Table S 6) .

\[
\begin{align*}
HLO_2 &\rightleftharpoons 11.2 < pK_{a} < 12.1 & \rightarrow LO_2^{2-} + H^{+} & \\
& & & & \text{Eq S 6.2}
\end{align*}
\]

This is one reason why light emission increases with increasing pH. In addition, the acid dissociation constant of H\textsubscript{2}O\textsubscript{2} is around pK\textsubscript{a} = 11.7 and the peroxide anion HO\textsubscript{2}\textsuperscript{-} is a stronger oxidising agent than H\textsubscript{2}O\textsubscript{2}. So, when AQ is preferably oxidised by the peroxide anion (Eq S 6.5b) then the dissociation of H\textsubscript{2}O\textsubscript{2} could be responsible for the increase of light yield with increasing pH .

\[
\begin{align*}
C_{ox} + LH^{-} &\rightarrow C_{red}^{-} + L^{2-} + H^{+} & \text{Eq S 6.3} \\
L^{2-} + L^{2-} &\rightarrow L^{2-} + AQ & \text{Eq S 6.4} \\
AQ + H\textsubscript{2}O\textsubscript{2} &\rightarrow LO_2^{2-} + 2H^{+} & \text{Eq S 6.5a} \\
AQ + HO\textsubscript{2} &\rightarrow LO_2^{2-} + H^{+} & \text{Eq S 6.5b} \\
LO_2^{2-} &\rightarrow AP^{2-} + N\textsubscript{2} & \text{Eq S 6.6} \\
AP^{2-} &\rightarrow AP^{2-} + h\cdot\nu & \text{Eq S 6.7}
\end{align*}
\]
LO$_2^-$ instantaneously decomposes to molecular nitrogen and aminophthalate (AP$^*$; 5 in Table S 6; Eq S 6.6). The latter is the light emitting molecular species (Eq S 6.7; Fig S 6.2).

Figure S 6.2 Light generation by the luminol reaction: The general principle. A molecule (C$_{ox}$) with high oxidation power abstracts electrons from luminol (LH$^-$) and converts it to its radical form (L$^-$•). Two L$^-$• can recombine to L$_2^-$ and azaquinone (AQ). AQ is oxidized by the peroxide anion HO$_2^-$ to an excited state of aminophthalate (AP$_2^*$) which emits blue light ($h\nu$; $\lambda = 420$ nm) to reach its ground state.

The typical color of the light released by the luminol reaction is blue with a spectral maximum around $\lambda_{\text{max}} = 424$ nm $^{16, 54-56}$. This is due to the confined $\pi$-electron cloud of AP$_2^-$ (5 in Table S 6). The emission peak shifts towards $\lambda_{\text{max}} = 485$ nm in the presence of DMSO $^{57-59}$. Naphthalene analogues of luminol, in contrast, have a more expanded conjugated system and consequently emit at longer wavelengths in the blue-green range $^{60}$.

Figure S 6.3 The catalytic peroxidative cycle and the luminol reaction catalysed by it. A: The peroxidative cycle of a transition metal porphyrin (TMP) protein converts H$_2$O$_2$ to H$_2$O and is converted to 'compound I' (CMP I). Here, a ferric iron centre of the porphyrin (Fe III) is oxidised and converted to ferryl iron (Fe IV). This complex is converted to a 'compound II' complex (CMP II) and thereby subducts electrons from an aromatic hydrogen donor cosubstrate (AH) leaving it as radical (A$^*$). Another AH is converted to A$^*$ when CMP II is recycled to TMP. B: If the aromatic hydrogen donor substrate is luminol (LH$^-$) then luminol radicals (L$^-$•) appear. In consequence, the same sequence of events occur as already shown in Fig S 6.2. Azaquinone (AQ) is formed by electron transfer between two L$^-$•. AQ is oxidised by H$_2$O$_2$ to an excited state of aminophthalate (AP$_2^*$) and nitrogen is emitted. Finally the AP$_2^*$ emits blue light ($\lambda = 420$ nm) to reach its ground state (AP$_2$). ($^{51, 61}$; Schemes adapted from $^{19, 20}$.)

Biogenic porphyrins carrying a central iron (heme compounds) are preferably used as catalysts of the luminol reaction. The oxidation state switching of their central iron is between III (ferric state) and IV (ferryl state) and traverses through a three stage cycle, the so-called
peroxidative cycle when a hydrogen donor (AH) and a peroxide (H$_2$O$_2$) are present (Fig S 6.3A).

Peroxidases represent an extensive and heterogeneous class of enzymes which catalyse the reduction of peroxides and the concurrent oxidation of a cosubstrate\textsuperscript{62, 63}. Aromatic hydrogen donors (AH) are often typical cosubstrates. A sub-class, the heme peroxidases, with an iron-porphyrin as a catalytically active prosthetic group, also accept luminol as electron-donor and are thus able to produce luminescence (Fig S 6.3B). The most prominent representative of this sub-class, the horseradish peroxidase (HRP), has been characterized in detail\textsuperscript{64} and is widely used for luminol-based applications. The pH optimum of HRP activity is in a range below 8 and the enzyme is stable up to pH = 9\textsuperscript{65}. This range (pH < 9) opposes the alkaline pH > 9 needed for a good light yield from the luminol reaction (Fig S 6.2; Eqs S 6.2, S 6.6). Consequently, a pH value of 9, used in this study, is a compromise to keep the enzyme stable and, at the same moment, obtain a maximum light yield.
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