In Silico Prediction of Excess Reactive Oxygen Species Generation by Metabolic Redox Cycling of Hydroxyphenols.

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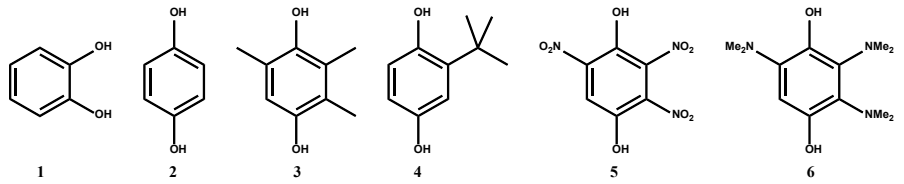
Abstract

The generation of excess reactive oxygen species (ROS) by hydroxyphenols that become 'trapped' in a redox cycle with their corresponding quinones, resulting in oxidative stress, is associated with toxicity[1] and can be detected in *in vitro* tests.[2] Briefly, the pathway involves reversible chemically- and enzymatically-mediated steps which include the formation of deprotonated hydroxyphenol ion and semiquinone radical ion intermediates. The positive control from *in vitro* tests for this phenomenon (1,1'-ethylene-2,2'-bipyridyldiylium dibromide, the herbicide Diquat dibromide) and four hydroxyphenols known to cause this cycling (2-hydroxyphenol (**1**), 4-hydroxyphenol (**2**), 2,3,5-trimethyl-4-hydroxyphenol (**3**) and 2-tert-butyl-4-hydroxyphenol (**4**))[3] were investigated in order to test the hypothesis that intermediates along the pathway are of comparable energy and can thus exist in equilibrium, thus there is little driving force to escape the cycle. In the absence of hydroxyphenols known not to undergo this process - other than 3-hydroxyphenols (resorcinols) which are unable to form quinones - the electronic scope of the reaction was investigated by considering a series of electron-poor hydroxyphenols substituted with nitro groups (e.g. **5**) and electron-rich ones substituted with dimethylamino groups (e.g. **6**). A ligand-based method was developed, using methanol-methanoate as a comparative acid-base pair for deprotonations and molecular oxygen-superoxide as an electron sink, and Density Functional Theory (DFT) calculations were performed at the B3LYP/6-311G** level of theory.[4,5]



Objectives

- Predict redox-cycling liability of confirmed positive hydroquinones
- Develop a method which can predict category membership for novel hydroquinones

Oxidative Stress and Redox Cycling

- Cells can handle some level of Reactive Oxygen Species (ROS)
  - Inevitable byproduct of using oxygen for energy/respiration
  - Also generated by oxidative enzymes such as peroxidases
  - Coping mechanisms such as the glutathione GSH/GSSG couple maintain homeostasis
- An increase in ROS levels beyond normal leads to Oxidative Stress:
  - DNA/RNA damage => mutagenicity, chromosome damage, carcinogenicity
  - Protein and lipid (per)oxidation => altered/destroyed functionality
  - Enzyme function disruption via cofactor oxidation, especially glutathione
- Reversible processes may generate dangerous levels of ROS by shuttling back and forth

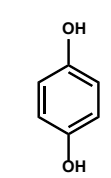
❖ Formation of ROS

$O_2 + e^- \rightarrow O_2^{\cdot -}$ (superoxide radical anion)
$O_2^{\cdot -} + e^- + 2H^+ \rightarrow H_2O_2$ (hydrogen peroxide)
$H_2O_2 + e^- \rightarrow HO^- + HO^{\cdot}$ (hydroxyl radical)
$HO^- + HO^{\cdot} + e^- + 2H^+ \rightarrow 2H_2O$

❖ Detection of ROS & Redox Cycling

- Rate of oxidation of NAD(P)H (UV spectroscopy)
- Oxygen consumption (Clark platinum electrode)
- Direct detection of ROS using, *inter alia*:
  - Spin trapping reagents
  - Electron Paramagnetic Resonance (EPR)

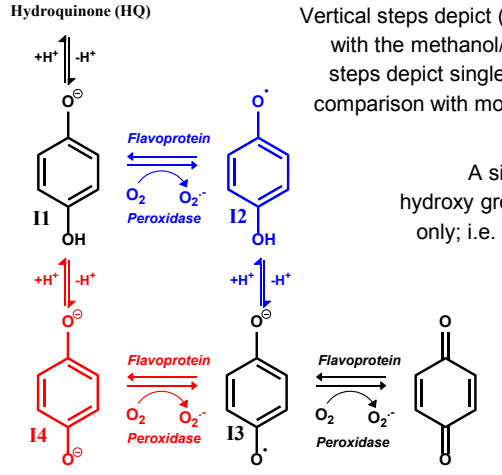
Methods



The ground-state structures of the steps depicted were minimised (MMFF94) for each of the hydroquinones under investigation, then submitted to NWChem as open-shell DFT calculations *in vacuo* at the B3LYP/6-311G** level of theory.[4,5] The free energies of the structures were then compared (see equations) to the energies of the methoxide/methanol and oxygen/superoxide pairings to generate relative $\Delta_r G$ values for each step of the reaction.

Vertical steps depict (de)protonation, modelled by comparison with the methanol/methoxide deprotonation, and horizontal steps depict single-electron transfer reactions, modelled by comparison with molecular oxygen/superoxide redox couple. Example is compound **2**.

A similar mechanism starting at the opposite hydroxy group exists for asymmetric hydroquinones only; i.e. initial deprotonation at the other hydroxyl group. This is referred to as 'route b'.



HQ + MeO⁻ → I1 + MeOH

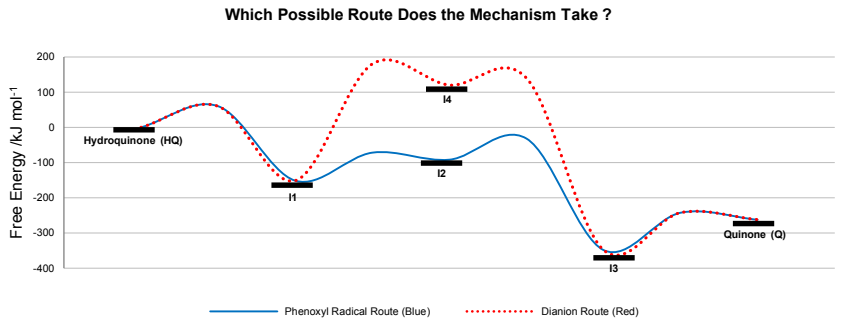
I1 + O_2 → I2 + $O_2^{\cdot -}$
I2 + MeO⁻ → I4 + MeOH

I1 + MeO⁻ → I3 + MeOH
I3 + O_2 → I4 + $O_2^{\cdot -}$

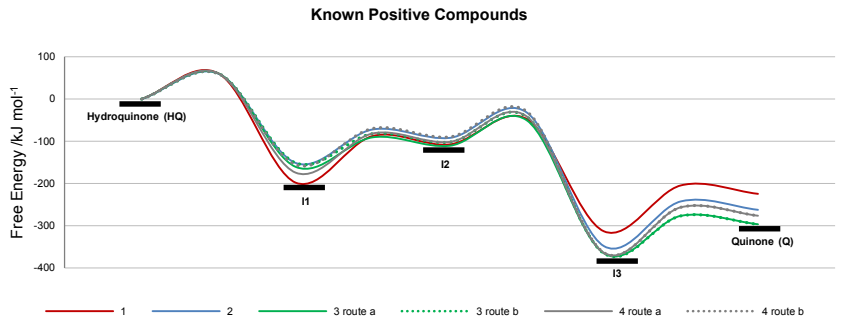
I4 + O_2 → Q + $O_2^{\cdot -}$

Results

Comparing the two possible routes (red and blue), it becomes clear that the red route, via the dianion, is disfavoured, presumably due to the high energy of that dianion intermediate. Arbitrary barrier heights are included for clarity. This is compound **2**, others are similar.

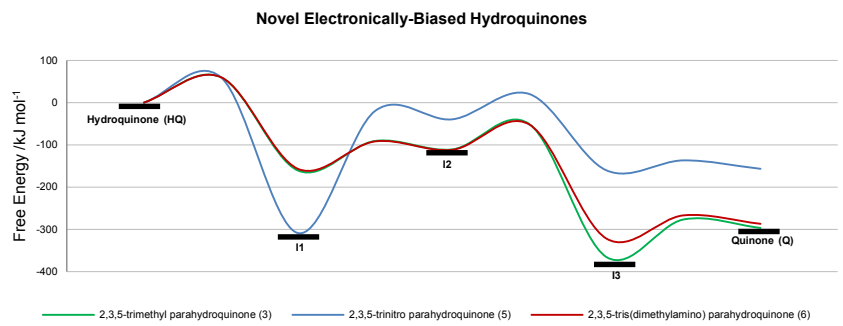


Taking the preferred hydroxyl radical route (via I2) in the above calculations for the known experimental positives **1-4**, we can create the following graph:



- Intermediate I1 is most stable for orthoquinone **1** (blue) and the quinone least stable, resulting in a gap of merely 20 kJ mol^{-1} and thus highest persistence in the redox cycle.
- In all cases, the semiquinone radical anion (I3) is the most stable, corresponding to its detectability under conditions where test systems become hypoxic[2].
- Little effect of regioselectivity (dotted vs solid lines) on asymmetric compounds **3** and **4**.

All of the positive compounds are reasonably electroneutral. In order to investigate substituent effects, the energy profiles for a series of electron poor and electron rich hydroquinones were calculated. Results for trinitro (**5**) and triamino (**6**) analogues are shown.



- The profiles for compound **3** and the triamino compound **6** are similar, though **6** gives a less stable radical anion as the dimethylamino groups are more electron donating.
- The trinitro compound **5** gives the most stable phenoxide anion and the least stable quinone and radical anion. The energy difference between dihydroquinone and quinone is the least, and the pathway may be fully reversible if the phenoxide anion to phenoxyl radical energy barrier can be overcome.

Conclusions

For all four hydroxyphenols with experimental data, the semiquinone radical anion is comparatively stable relative to other species in the pathway, potentially slowing formation of the quinone and allowing the ROS-forming cycle to occur. In addition, the deprotonated hydroxyphenol is of comparable energy to the quinone, meaning that the two can exist in equilibrium, prolonging the cycle. This energy gap is particularly small (<20 kJ mol^{-1}) in the case of the ortho-substituted species where the remaining hydroxyl group in the deprotonated species can form a strong intramolecular hydrogen bridge between the two oxygens, stabilising the negative charge, yet the two electron-rich oxygens in the ortho-quinone repel each other, destabilising it. In conclusion, a method has been developed which allows characterisation of the Gibbs free energy profiles of the hydroxyphenol-quinone equilibrium. In the absence of negative examples, this method will assist in confirming the suitability of category membership (i.e. that they are energetically similar to known compounds) for specific hydroxyphenols in respect of Read-Across analyses.

1) Chem. Res. Toxicol., (2000) **13**, 135-160. 2) Front. Biosci., (2000) **5**, D629-D638. 3) Studies in *RepDose* (http://fraunhofer-repdose.de) and *ToxRef* (https://cfpub.epa.gov/si) databases. 4) a) J. Chem. Phys., (1993), **98**, 5648-5652; b) J. Phys. Chem., (1994), **98**, 11623-11627. 5) J. Chem. Phys., (1980), **72**, 650-654.