PROGRESS REPORT FOR ALNGRA10082

**PROJECT TITLE**
How does the structure of nitroxides modulate their efficacy as antioxidants against protein-derived free radicals?

**INVESTIGATOR(S)**

<table>
<thead>
<tr>
<th>Chief Investigator</th>
<th>Institution and Department</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prof Michael Davies</td>
<td>University of Sydney, Heart Research Institute, EPR Group</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other Investigators</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Assoc Prof Bob Anderson, University of Auckland, NZ</td>
<td></td>
</tr>
<tr>
<td>Dr. David Pattison, The Heart Research Institute, University of Sydney</td>
<td></td>
</tr>
</tbody>
</table>

**Students**
None

**ANSTO Investigators**
Assoc Prof Bob Anderson

**Specialist Committee**
BSB

**SCIENTIFIC OBJECTIVES**

Radical mediated protein oxidation occurs during aging and some human diseases (e.g. cataracts, heart disease). Tyrosine (Tyr) and tryptophan (Trp) residues are particularly susceptible to oxidation, resulting in the formation of protein-bound Tyr and Trp radicals which can readily propagate damage to other cellular components. As protein oxidation is detrimental in biological systems, it is desirable to minimise such damage. One approach is to prevent the damage by scavenging the highly reactive attacking radicals, although this is difficult to achieve due to the abundance of targets for radical-induced damage in vivo. Alternatively, it may be more beneficial to introduce exogenous compounds that can effectively repair the protein radicals once they are formed. For this approach to be successful the compounds must react rapidly with protein radicals, as the concentration of exogenously added compounds rarely exceeds ca. 100 µM in vivo.

We postulated that stable nitroxide radicals (RR'NO•) may be suitable candidates, as they are tolerated at relatively high concentrations (up to 0.5 mM) in vivo and have been shown to react with low-molecular-mass radicals with second-order rate constants from 10^6 - 10^9 M⁻¹ s⁻¹. Furthermore, the steric and electronic properties of nitroxides can be readily tailored to achieve maximal antioxidant efficacy by introduction of substituents and modification of the ring structure (Scheme 1). Consistent with our hypothesis, our recent studies have shown that nitroxides can successfully scavenge protein radicals and modulate the formation of biomarkers of protein oxidation in isolated systems. In order to gain further insight into their likely efficacy as antioxidants against protein damage in more complex systems, we aimed to:

1) Demonstrate that stable nitroxide radicals are effective antioxidants against a range of peptide- and protein-derived radicals by pulse radiolysis.

2) Determine absolute rate constants for the reactions of these nitroxides with model peptide and protein radicals.

3) Investigate whether the electronic and steric properties of nitroxides affect their reactivity with protein-derived radicals.

![Scheme 1](image)

**Scheme 1:** Structures of nitroxides used in the pulse radiolysis studies. (a) X = H, TEMPO; X = OH, 4-hydroxy-TEMPO; X = COO⁻, 4-carboxy-TEMPO. (b) Y = COO⁻, 3-carboxy-PROXYL.
PROGRESS REPORT and RESEARCH OUTCOMES

We have made considerable progress with the studies outlined in the research proposal. We have already undertaken comprehensive studies with TEMPO demonstrating that it reduces the yields of Trp and Tyr radicals in a range of peptides and proteins and is therefore an effective antioxidant against these species. These studies have been extended to determine the absolute second-order rate constants for TEMPO with a variety of Trp and Tyr radicals, allowing the relative antioxidant efficacy against radicals in various environments to be established. The final aim of the proposal has been initiated with preliminary studies using the other nitroxides shown in Scheme 1; these studies are ongoing with further funds provided by AINSE Award ALNGRA11047.

DATA

The studies undertaken by pulse radiolysis at the Auckland Free Radical Facility in 2010 initially focused on the reactions of model Tyr and Trp radicals generated by selective attack of azide radicals ($N_3^\cdot$) on N-acetyl-tyrosine-amide (NAc-Tyr-NH$_2$) and N-acetyl-tryptophan-amide (NAc-Trp-NH$_2$). The reactions of these species were all investigated in buffered solutions at pH 7.4 and 22 °C, and the kinetic decays were monitored at 405 nm (Tyr radicals) and 510 nm (Trp radicals). The second order rate constants for the reaction of the TEMPO with these species were determined as $k \approx 10^8$ and $5 \times 10^6$ M$^{-1}$ s$^{-1}$, respectively (Figure 1, Table 1). Preliminary studies have derived similar rate constants for a range of other nitroxide derivatives. The second-order rate constants for the direct consumption of $N_3^\cdot$ by TEMPO and related nitroxides were also determined with $k$ typically ca. $3 \times 10^9$ M$^{-1}$ s$^{-1}$ (Table 1). Determination of these rate constants facilitated experimental design to ensure that the yield of Trp and Tyr residues was maximal. The radical yields calculated for the acquired data indicated that the yield of Tyr and Trp radicals in these model peptides was typically 60 – 100 % of the maximal theoretical dose under all conditions, with direct scavenging of $N_3^\cdot$ becoming increasingly important at higher nitroxide concentrations. Once the principles of the approach had been validated with the model peptides, second-order rate constants for the reactions of TEMPO with a range of protein-bound Trp and Tyr radicals were determined. In the case of lysozyme (Figure 2), TEMPO reacted with lysozyme-bound Trp radicals with $k = 1.5 \times 10^7$ M$^{-1}$ s$^{-1}$.

Figure 1: (a) Kinetic data (pH 7.4, 22 °C, 405 nm) for the reaction of the Tyr radical generated on NAc-Tyr-NH$_2$ with increasing concentrations of TEMPO. (b) Quenching plot demonstrating the linear nature of the TEMPO concentration dependence on the observed rate of decay of the Tyr radical on NAc-Tyr-NH$_2$. The gradient of the line gives the second-order rate constant quoted in Table 1.

with TEMPO (≤ 100 µM) effectively inhibiting the long range electron transfer (LRET) reactions that result in intramolecular conversion of Trp radicals to Tyr radicals (Scheme 2, Figure 2b). The ability of TEMPO to scavenge protein-bound Trp radicals was also demonstrated in chymotrypsin, with $k = 1.6 \times 10^7$ M$^{-1}$ s$^{-1}$ and almost complete inhibition of LRET with 100 µM TEMPO. In these experiments, Tyr radicals are generated directly through reaction of the lysozyme and chymotrypsin with $N_3^\cdot$ as well as by LRET. The reaction of TEMPO...
with these Tyr radicals was slow on the pulse radiolysis timescale for both of these proteins, preventing accurate determination of these rate constants.

A third protein, pepsin, was also investigated to ascertain whether the scenario identified for both lysozyme and chymotrypsin was a general phenomenon for proteins. In pepsin, TEMPO was found to react more rapidly with Trp radicals ($k = 1.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) than in the other proteins (Figure 3). However, the LRET process resulting in conversion of Trp to Tyr radicals is faster in pepsin, thus TEMPO was unable to completely prevent this process at the concentrations investigated (up to 100 µM; Figure 3b). In contrast with the other proteins, TEMPO reacted with pepsin-derived Tyr radicals on the pulse radiolysis timescale, yielding $k = 4.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. Preliminary experiments undertaken with 4-carboxy-TEMPO and 3-carboxy-PROXYL instead of TEMPO yielded very similar second-order rate constants for the scavenging of these radicals in pepsin.

**Scheme 2:** Schematic diagram showing the processes investigated by these pulse radiolysis studies.

**Figure 2:** Kinetic data (pH 7.4, 22 °C) for the reaction of (a) lysozyme-bound Trp radicals (at 510 nm) with increasing concentrations of TEMPO, (b) lysozyme-bound Tyr radicals (at 405 nm) with increasing concentrations of TEMPO.
The pulse radiolysis data described above strongly support our hypothesis that stable nitroxide radicals can act as potent antioxidants against radical-mediated protein oxidation. These data indicate that TEMPO can scavenge a variety of peptide- and protein-bound Trp and Tyr radicals with relatively high second-order rate constants that are comparable to those for endogenous antioxidants such as ascorbate. Thus, we would expect that at physiologically achievable concentrations, TEMPO would supplement the natural antioxidant defense mechanisms and contribute to attenuating the propagation of damage by these radicals. These data are of particular interest and potential importance, as previous studies have shown that nitroxides can be fed to animals over very long periods (many months) without noticeable toxicity, and that nitroxides prolong lifespan and decrease tumour incidence. These highly positive effects may arise from the antioxidant action of nitroxides as proposed here. Furthermore, our preliminary studies suggest that the steric and electronic properties of nitroxides do not have a major impact on their ability to scavenge Trp and Tyr radicals, therefore development of novel nitroxides with enhanced biological stability and solubility are likely to remain effective antioxidants against radical-induced protein oxidation. These studies are ongoing through AINSE Award ALNGRA11047.

\[
\begin{array}{c|c}
\text{Radical} & k_2 / \text{M}^{-1} \text{s}^{-1} \\
N_3^\cdot & (2.7 \pm 0.3) \times 10^9 \\
\text{TyrO}^\cdot (\text{NAC-Tyr-NH}_2) & (1.6 \pm 0.1) \times 10^8 \\
\text{Trp}^\cdot (\text{NAC-Trp-NH}_2) & (7.5 \pm 0.5) \times 10^6 \\
\text{Trp}^\cdot (\text{Lysozyme}) & (1.5 \pm 0.5) \times 10^7 \\
\text{TyrO}^\cdot (\text{Lysozyme}) & \text{Too slow to determine} \\
\text{Trp}^\cdot (\text{Pepsin}) & (1.1 \pm 0.4) \times 10^8 \\
\text{TyrO}^\cdot (\text{Pepsin}) & (4.3 \pm 0.2) \times 10^7 \\
\text{Trp}^\cdot (\text{Chymotrypsin}) & (1.6 \pm 0.2) \times 10^7 \\
\text{TyrO}^\cdot (\text{Chymotrypsin}) & \text{Too slow to determine} \\
\end{array}
\]

Table 1: Rate constants derived for reactions of various radicals with TEMPO in these studies. Errors represent 95% confidence intervals.

Signature of Investigator preparing the report for
After signing this report please fax this page with your signature for our files

Proj: ALNGRA10082
Date:

PUBLICATIONS / REPORTS arising as a result of your work.


Invited lecture: Michael J. Davies “Nitroxide radicals and nitric oxide as inhibitors of oxidative damage” at Pacifichem 2010 in Honolulu, Hawaii, 15-20th December 2010.

**PhD STUDENTS**

No students are involved in this project.