Substituent Effects of Pterin Derivatives on Singlet Oxygen Scavenging Activity

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The relationship of chemical structures of 6-formylpterin (6FP) and its derivatives with scavenging activity of singlet oxygen (1O2) was examined. First, effects of pterin derivatives on 1O2 released from activated human neutrophils were examined. The neutrophils, stimulated with opsonized zymosan, released 1O2 that was detected by chemiluminescence using a 1O2 specific probe, trans-1-(2′-methoxymethyl)pyrene. 6FP and its derivatives suppressed the 1O2 release. 6FP and other commercially available pterin derivatives, such as biopterin and neopterin, which have different substitutions at the 6-position, suppressed the 1O2 release with similar extent. On the other hand, newly synthesized pterin derivatives, which have different substitutions at the 2- and/or 3-position, such as 2-amino-6-formyl-3-methylpteridin-4-one, suppressed the 1O2 release in a dose-dependent manner and more potently than 6FP. Then, the 1O2 scavenging activity of pterin derivatives was examined photochemically by direct analysis of near-infrared luminescence at 1270 nm, the most sensitive method for the detection of 1O2. When rose Bengal, a photosensitizer, in D2O solution, was irradiated by 514 nm laser beam, the emission spectrum of 1O2 was observed. 6FP suppressed this emission spectrum of 1O2, and the newly synthesized pterin derivatives with different substituent at the 2- and/or 3-position suppressed the spectrum more potently than 6FP. The order of potency was similar to that obtained from biological assays. These findings indicate that the substitutions at the 2- and/or 3-position play an important role in 1O2 scavenging activity of pterin derivatives.

Key words pterin derivative; singlet oxygen; neutrophil; chemiluminescence; photosensitizer; near-infrared luminescence

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Materials and Methods

Reagents 6FP was obtained from Sankyo Kasei Kogyo (Hiratsuka, Japan). Pn, P6COOH, NP, and BP were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). 6FP and other pterin derivatives were initially dissolved in saline with 0.1 N NaOH to make a 30 mM solution, then diluted with phosphate-buffered saline (PBS) to a final concentration of 4 mM (pH=8.0). Novel pterin derivatives (Fig. 1), 2-amino-6-formyl-3-methylpteridin-4-one (FM), 2-(N,N-dimethylaminomethyl)eneamino)-6-formyl-3-methylpteridin-4-one (DFM) and 2-(N,N-dimethylaminomethyleneamino)-6-formyl-3-pivaloylpteridin-4-one (DFP), were synthesized in our laboratories as previously described. Novel pterin deriv-
Photochemical Generation of $^{1}$O$_{2}$ in a Cell-Free System

$^{1}$O$_{2}$ was photochemically generated by irradiation of RB solutions. Because of the short lifetime of $^{1}$O$_{2}$ in H$_{2}$O (3.8 $\mu$s), D$_{2}$O was used as the solvent in all experiments, where the lifetime is much longer (62 $\mu$s). $^{1}$O$_{2}$ was measured using a Raman spectroscopy (LabRAM HR-800; HORIBA, Kyoto, Japan). Quantum yields of $^{1}$O$_{2}$ production were determined by direct analysis of the weak $^{1}$O$_{2}$ near-infrared luminescence at 1270 nm, which was produced during continuous irradiation of RB solutions by a laser beam (wave length 514.5 nm, power 3 mW). The luminescence of $^{1}$O$_{2}$ was measured with a liquid nitrogen-cooled InGaAs photodiode in conjunction with a 1270 nm interference filter.

Statistical Analysis  Values are shown as means±S.D. Statistical comparisons were made using a one-way ANOVA followed by the Student’s paired t-test with a Bonferroni correction. $p$ values <0.05 were regarded as being statistically significant.

RESULTS AND DISCUSSION

When the neutrophils were activated with OZ, $^{1}$O$_{2}$ was released from neutrophils. 6FP, Pn, P6COOH, NP and BP suppressed the OZ-induced $^{1}$O$_{2}$ release, and their extent of suppression was almost equal at the concentration of 400 $\mu$m (Fig. 2). This result agreed with the finding that Thomas et al. showed in cell-free system. On the other hand, novel pterin derivatives, FM, DFM and DFP, suppressed the OZ-induced $^{1}$O$_{2}$ release in a dose-dependent manner, and their extent of suppression was more potent than that of 6FP. The inhibitory effects of novel pterin derivatives on $^{1}$O$_{2}$ release from activated neutrophils were enhanced according to the following order; 6FP, FM, DFM and DFP (Fig. 3).

The inhibition of $^{1}$O$_{2}$ release from activated neutrophils does not necessarily imply the scavenging of $^{1}$O$_{2}$. Activated neutrophils first generate O$_{2}^{-}$ by reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activity, and generated O$_{2}^{-}$ is dismuted to H$_{2}$O$_{2}$. H$_{2}$O$_{2}$ reacts with hypochlorite (HOCI) produced by myeloperoxidase (MPO) activity to form $^{1}$O$_{2}$. Therefore, the inhibitory effect of novel pterin derivatives on $^{1}$O$_{2}$ release might be attributed to that on O$_{2}^{-}$ production by NADPH-oxidase activity. Otherwise, they might inhibit the $^{1}$O$_{2}$ formation by MPO activity. Further evidence is required to determine if novel pterin derivatives directly scaveng $^{1}$O$_{2}$.

The most sensitive method for the detection of $^{1}$O$_{2}$ is a direct analysis of near-infrared luminescence at 1270 nm. When 10 $\mu$m RB solution in D$_{2}$O was irradiated by 514 nm laser beam, $^{1}$O$_{2}$ was generated and the emission spectrum of $^{1}$O$_{2}$ was clearly observed using Raman spectroscopy (Fig. 4, Control). 6FP suppressed the emission spectrum of $^{1}$O$_{2}$, and novel pterin derivatives suppressed the spectrum more potently than 6FP, according to the following order; 6FP, FM, DFM and DFP (Fig. 4). This result showed that novel pterin derivatives certainly had direct scavenging activity of $^{1}$O$_{2}$.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R$^2$</th>
<th>R$^3$</th>
<th>R$^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Formylpterin (6FP)</td>
<td>-NH$_2$</td>
<td>-H</td>
<td>-CHO</td>
</tr>
<tr>
<td>Pterin (Pn)</td>
<td>-NH$_2$</td>
<td>-H</td>
<td>-H</td>
</tr>
<tr>
<td>Pterin-6-carboxylic acid (P6COOH)</td>
<td>-NH$_2$</td>
<td>-H</td>
<td>-COOH</td>
</tr>
<tr>
<td>Bioppterin (BP)</td>
<td>-NH$_2$</td>
<td>-H</td>
<td>-(CHOH)$_2$-CH$_3$</td>
</tr>
<tr>
<td>Neopterin (NP)</td>
<td>-NH$_2$</td>
<td>-H</td>
<td>-(CHOH)$_2$-CHOH</td>
</tr>
<tr>
<td>2-Amino-6-formyl-3-methylpteridin-4-one (FM)</td>
<td>-NH$_2$</td>
<td>-CH$_3$</td>
<td>-CHO</td>
</tr>
<tr>
<td>2-[(2-[(2-Dimethylaminomethyl)eneamino]-6-formyl-3-methylpteridin-4-one (DFM)</td>
<td>-NH$_2$</td>
<td>-CH$_3$</td>
<td>-CHO</td>
</tr>
<tr>
<td>2-[(2-[(2-Dimethylaminomethyl)eneamino]-6-formyl-3-pivaloylpteridin-4-one (DFP)</td>
<td>-NH$_2$</td>
<td>-CH$_3$</td>
<td>-CHO</td>
</tr>
</tbody>
</table>

Fig. 1. Chemical Structures of Pterin Derivatives

Conventional numbering system for a pteridine ring and substituents on the 2-, 3- and 6-positions are shown in columns of R$^2$, R$^3$ and R$^6$, respectively.
with the same order of potency which was shown in biological assay.

The total rate constant of $^1\text{O}_2$ quenching ($k_t$) by pterin derivatives (P) consisted of rate constant of the chemical reaction of $^1\text{O}_2$ with pterin derivatives ($k_r$) and that of the $^1\text{O}_2$ physical quenching by pterin derivatives ($k_q$) ($k_t=k_r+k_q$).

$$P + ^1\text{O}_2 \xrightarrow{k_r} \text{PO}_2$$
$$P + ^1\text{O}_2 \xrightarrow{k_q} P + ^1\text{O}_2$$

In our result, there was no difference in $^1\text{O}_2$ scavenging activity among 6FP, Pn, P6COOH, NP and BP, despite different substitutions at the 6-position (Fig. 1, the column of R$^6$). Since $^1\text{O}_2$ reacts with C6–C7 double bond of the pyrazinic ring of the pterin moiety, the rate constant of chemical reaction ($k_r$) should be affected by the chemical nature of the 6-
substituent of pterin derivatives. It was reported, however, that the $k_q$ value for Pn (2.5 ± 0.3 × 10^5 M⁻¹ s⁻¹) is very low in comparison with the corresponding $k_q$ value, indicating that the deactivation of O₂ by Pn is mainly a physical quenching process ($k_q$ = 2.6 ± 0.3 × 10^5 M⁻¹ s⁻¹ is an order of magnitude larger than $k_e$). This indicated that the chemical reaction did not play a major role in 6FP, P6COOH, NP and BP as well as Pn, which is possibly the reason why there was no difference in ¹O₂ scavenging activity among 6FP, Pn, P6COOH, NP and BP.

In our result, there were distinct differences in ¹O₂ scavenging activity among 6FP, FM, DFM and DFP. Since the structure of the 6-position is the same in 6FP, FM, DFM, and DFP (Fig. 1, the column of R³), the difference of the substitutions at the 2- and/or 3-position affected the potency of ¹O₂ scavenging activity. It was reported that ¹O₂ is deactivated by the reaction with the pyrazinic or dihydropyrazinic ring of 6-position substituted pterin derivatives. ¹O₂ quenching efficiency of tertiary amines are larger than primary amines because of its low ionization potentials. Further, a hydro- gen atom on the 3-position of 6FP is replaced by the pivaloyl group in DFP, and the ¹O₂ scavenging activity of DFP was more potent than that of 6FP which in which the 3-position is free. Further, a hydrogen atom on the 3-position of 6FP is replaced by the pivaloyl group in DFP, and the ¹O₂ scavenging activity of DFP was more potent than that of DFM in which the 3-position is replaced by methyl group. These results indicated that modification of the 3-position also contributes to ¹O₂ physical quenching.

In conclusion, the substitutions at the 2- and/or 3-position play an important role in ¹O₂ scavenging activity of pterin derivatives. For several years, growing interest has been given to the role of ¹O₂ in pathological situations and particularly in the oxidative stress induced by postischemic reperfusion. The neuroprotective effects of 6FP shown in experimental brain ischemia in our previous study might be also attributed to its ¹O₂ scavenging activity. On the basis of the findings of this study, more potent ¹O₂ scavengers should be developed, which may serve as more potent neuroprotective agents.

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REFERENCES