Desmutagenicity of a Dibenzofuran-quinone Derivative toward the Mutagenicity of Trp-P-2

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2,6-Di-tert-butyl-8-hydroxy-dibenzofuran-1,4-quinone (BHDQ) is one of the oxidative derivatives of butylated hydroxyanisole. The suppressive effect of BHDQ on the mutagenicity of Trp-P-2 was investigated using Salmonella typhimurium TA 98. BHDQ (0.8 nmol) suppressed the mutagenicity of Trp-P-2 (0.2 nmol) by 30% in the presence of the S9 mix. The suppressive activity of BHDQ toward the mutagenicity of the activated metabolite of Trp-P-2, N-OH-Trp-P-2, in the absence of the S9 mix was 80%. The bio-antimutagenicity of BHDQ was also measured, but it was found to be negligible as compared to its desmutagenicity. The reaction of BHDQ with N-OH-Trp-P-2 re-produced Trp-P-2. It was considered that BHDQ should re-convert N-OH-Trp-P-2 to the original Trp-P-2, and therefore BHDQ should be a desmutagen.

Butylated hydroxyanisole (BHA) is one of the common antioxidants in foodstuffs. Recently, the carcinogenicity of BHA was reported by Ito et al.13 They showed that an oral dose of BHA induced squamous cell carcinomas of the forestomach in F344 rats. However, Wattenberg2 3) reported that BHA inhibited carcinogenesis when administered with other chemical carcinogens. These paradoxical bio-activities of BHA are confusing. It must be clarified as to whether BHA is a carcinogen, or an anti- or des-carcinogen. We have assumed that the bio-activities of orally administered BHA reflect not only the effect of BHA itself but also those of BHA derivatives, since BHA easily changes to its derivatives under the gastric acidic conditions through interaction with food components, such as nitrite, in the animal digestive tract.4) We have performed studies on the reaction of BHA with nitrite under acidic conditions,5) and found that two derivatives, 2-tert-butyl-p-quinone (t-BQ) and 3,3’-di-tert-butyl-biphenyldiquinone-(2,5,2’,5’) (BBDQ),6) are mutagens and that another derivative, 2,6-di-tert-butyl-8-hydroxy-dibenzofuran-1,4-quinone (BHDQ) (Fig. 1), exhibits highly suppressive activity toward chemical mutagens.7)

3-Amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), which is formed through the pyrolysis of tryptophan,8) is a potent mutagen9) and carcinogen.10,11) As to the mutagenicity of Trp-P-2 toward Salmonella typhimurium TA 98, it is believed that Trp-P-2 is converted to its activated form, 3-hydroxyamino-1-methyl-5H-pyrido[4,3-b]indole (N-OH-Trp-P-2),12-14) by a hepatic S9 system, which then modifies DNA.15) Another pyrolytic product of tryptophan, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), is also a mutagen, but its

Fig. 1. Structure of 2,6-di-tert-butyl-8-hydroxy-dibenzofuran-1,4-quinone (BHDQ).
mutagenicity is slightly lower than that of Trp-P-2.

In the present study, the suppressive effect of BHDQ on the mutagenic activities of Trp-Ps was clarified.

Materials and Methods

Chemicals. BHDQ was prepared and purified as shown previously.\(^7\) BHA was reacted with nitrite at pH 2 and one of the products, BBDQ, was purified. BBDQ was submitted to photolysis and the photolytic product, BHDQ, was purified by recrystallization.

Trp-P-2 and Trp-P-1 (acetic acid salts) were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan.

Bacterial strain. Salmonella typhimurium TA 98, which requires histidine for growth (His\(^-\)),\(^16\) was used. TA 98 carries a well-characterized frameshift-type mutation in the histidine operon and reverts to prototrophy in the presence of a wide variety of mutagens.\(^17\) The R-factor plasmid, pKM 101, has been incorporated in the TA 98.

Preparation of the S9 mix. The S9 fraction was prepared from male Sprague-Dawley rats according to Ames et al.\(^16\) and then stored at \(-80^\circ\text{C}\) until use.

Preparation of a mixture containing activated metabolites of Trp-Ps. Around 0.2 nmol of Trp-P-2 or Trp-P-1 was dissolved in 0.1 ml water and then mixed with 0.5 ml of the S9 mix (containing 2.4 mg of S9 as protein), followed by incubation at 37°C for 20 min with shaking. The reaction was stopped by the addition of 0.6 ml of cold acetone and the resultant mixture was allowed to stand on ice for 15 min. After centrifugation of the mixture at 3500 rpm for 10 min, the supernatant was retained and evaporated in vacuo. When the resultant residue was analyzed by high performance liquid chromatography (HPLC),\(^15\) it was found to consist of an activated metabolite of Trp-Ps (N-OH-Trp-P-2 or N-OH-Trp-P-1) and its intact form, as shown in Fig. 2. N-OH-Trp-Ps are very unstable, their half-lives being only ca. 30 min.\(^18\) Here, the mixture prepared were dissolved again in water and immediately used in the following experiments.

Desmutagenic assay of BHDQ. The desmutagenicity of BHDQ was determined as the decreases in the number of revertants of TA 98 due to the mutagens, Trp-Ps and N-OH-Trp-Ps. The reversion (from His\(^-\) to His\(^+\)) of Salmonella typhimurium TA 98 was detected by the Ames test, as modified by Yahagi et al.\(^19\) Trp-Ps (0.2 nmol) in 0.1 ml of water were mixed, in order, with various concentrations of BHDQ in 0.1 ml of dimethyl sulfoxide, 0.5 ml of the S9 mix (when N-OH-Trp-Ps were used as mutagens, the same volume of 0.1 M phosphate buffer, pH 7.0, was added instead of the S9 mix) and 0.1 ml of a TA 98 medium-suspension. After incubation at 37°C for 20 min, 2 ml of soft agar was added and then the mixture was poured onto plates of a minimal-glucose agar-medium. The plates were cultured for 2 days at 37°C in the dark and then the numbers of colonies that developed on the surface of a lawn of unmutated bacteria were determined as revertants under a microscope. The desmutagenicity % of BHDQ was calculated using the equation, \((A - B) \times 100/A\), where \(A\) is the mean revertant number on the plates treated only with the mutagen and \(B\) the mean revertant number on the plates treated with both BHDQ and the mutagen.

Assaying of bio-antimutagenicity. A bio-antimutagen suppresses the process of mutagenesis (mutation fixation) after DNA has been damaged by a mutagen. The fixation of a His\(^+\) revertant, which has been induced by N-OH-Trp-P-2, can be evaluated by counting the number of His\(^+\) revertant colonies. It was examined, using the following method, as to whether or not BHDQ inhibits the process of mutation fixation. TA 98 cells were grown at 37°C overnight in liquid broth medium, washed with 1/15 M phosphate buffer 3 times and then resuspended in the same buffer. In order to mutate TA 98 cells, 5 ml of the bacterial suspension was incubated with 5 ml of a solution of each N-OH-Trp-P at 37°C for 30 min. The mixture was centrifuged at 3500 rpm for 10 min, washed with the buffer 3 times to remove the mutagen and then suspended in 5 ml...
of the phosphate buffer again. Aliquots of the bacterial
solution (0.1 ml) were mixed with various concentrations
of BHDQ (0.1 ml solution). Each mixture was mixed with
3 ml of soft agar with shaking and then poured onto plates
of the semi-enriched agar medium (MBB agar). The
mutagen-free bacterial solution was also subjected to
survival colony number determination. The solution was
diluted to 10^6 and then a 0.1 ml aliquot of it was poured
onto an MBB plate together with 3 ml of soft agar. After
incubation at 37°C for 4 days, the numbers of revertant
(His^+) and survival (His^-) colonies were determined. The
bio-antimutagenicity was determined by the induced mu-
tation frequency-method, as described in detail previ-
ously.

HPLC analysis of Trp-P-2 and N-OH-Trp-P-2. Trp-P-2
(194 nmol) was incubated with the S9 fraction and
1.5 mg/ml NADPH as described above. Trp-P-2 and N-
OH-Trp-P-2 were extracted from the incubation mixture
with an equal volume of cold acetonitrile, centrifuged and
then subjected to HPLC analysis with a Hitachi 655 liquid
chromatograph. The HPLC conditions were as follows:
column, ZORBAX ODS (4.6 mm x 15 cm); eluting solvent
system, 30% acetonitrile in 20 mM potassium dihydrogen-
phosphate; flow rate, 0.7 ml/min; detection, at 254 nm,
which corresponds to the \( \lambda_{max} \) of N-OH-Trp-P-2. The
amount of Trp-P-2 on the chromatogram was calculated
using a standard calibration curve. The amount of N-OH-
Trp-P-2 was estimated using a modified calibration curve
for Trp-P-2, i.e., as to the ratio of their molecular extinc-
tion coefficients, Trp-P-2 (4.3 x 10^4) versus N-OH-Trp-P-
2 (4.7 x 10^4).

Results

Desmutagenicity of BHDQ toward Trp-Ps and
N-OH-Trp-Ps

BHDQ markedly reduced the mutation
caused by Trp-Ps and a mixture containing
N-OH-Trp-Ps (Fig. 3). The number of His^+
revertant colonies due to Trp-P-2 (0.2 nmol)
proportionally decreased with the addition of
less than 3 nmol of BHDQ (Fig. 3A). Trp-P-2
was converted to its N-OH form by incubation
with the S9 mix and then the effect of BHDQ
on the mutagenicity of N-OH-Trp-P-2 was
evaluated. BHDQ suppressed the mutagenicity
of N-OH-Trp-P-2, depending proportionally
on the concentration of BHDQ up to 1 nmol.
The addition of 0.8 nmol of BHDQ decreased
the mutagenicities of Trp-P-2 and N-OH-Trp-
P-2 by 30% and 80%, respectively. Thus, the
suppressive effect of BHDQ on the mutagen-
icity of N-OH-Trp-P-2 was greater than that
in the case of Trp-P-2. BHDQ also exhibited
centrifugation-dependent activity toward Trp-
P-1 and N-OH-Trp-P-1, and the extent of its
desmutagenicity toward N-OH-Trp-P-1 was
greater than that toward Trp-P-1 (Fig. 3B). On
the other hand, the desmutagenicity of 1.5
nmol BHDQ toward Trp-P-2 and Trp-P-1
was 60% and 30%, respectively. Thus, the
effect of BHDQ was observed greater in the
case of Trp-P-2. The following experiments
were carried out with Trp-P-2 and N-OH-Trp-
P-2.

Bio-antimutagenicity of BHDQ

It is still not clear whether this suppressive
effect of BHDQ on mutagenicity is due to its
bio-antimutagenicity or its desmutagenicity.
Table I. Bio-antimutagenicity of BHDQ on Bacteria Mutated by the Activated Metabolite of Trp-P-2

<table>
<thead>
<tr>
<th>Added amount of BHDQ (nmol/plate)</th>
<th>Mean number of survival (His\textsuperscript{+}) colonies (\times 10^6/plate)</th>
<th>Mean number of induced (His\textsuperscript{-}) colonies (Mean number/plate)\textsuperscript{a}</th>
<th>Frequency of induced His\textsuperscript{+} revertants/10\textsuperscript{8} survivors\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>140</td>
<td>7384</td>
<td>5300</td>
</tr>
<tr>
<td>1.5</td>
<td>123</td>
<td>6542</td>
<td>5300</td>
</tr>
<tr>
<td>3.0</td>
<td>141</td>
<td>6753</td>
<td>4800</td>
</tr>
<tr>
<td>7.7</td>
<td>153</td>
<td>6853</td>
<td>4500</td>
</tr>
<tr>
<td>15.0</td>
<td>145</td>
<td>6706</td>
<td>4600</td>
</tr>
<tr>
<td>30.7</td>
<td>138</td>
<td>5599</td>
<td>4000</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The treated bacterial suspensions (0.1 ml) were plated for observation of mutation induction. From the mean number of induced (His\textsuperscript{+}) colonies appearing per plate, the mean number of spontaneous mutant colonies per plate\textsuperscript{20} was subtracted.

\textsuperscript{b} The induced mutation frequency was taken as the number in the third column divided by that in the second column multiplied 100.\textsuperscript{20}

Therefore, the bio-antimutagenicity of BHDQ was evaluated (Table I). The number of survival colonies (His\textsuperscript{+}) remained constant and the number of revertant colonies slightly decreased with an increase of the amount of BHDQ. In the present case, the induced mutation frequency is expressed as the ratio of the number of His\textsuperscript{+} colonies \textit{versus} the number of His\textsuperscript{-} colonies, a decrease in the value reflecting the bio-antimutagenic effect of BHDQ. The frequency on plates without BHDQ was 5300 \times 10^{-8}, \textit{i.e.}, the same as the original induced frequency. On the addition of 1.5 nmol BHDQ the frequency remained unchanged, but the addition of 30 nmol BHDQ reduced it to 4000 \times 10^{-8}. Thus, the high concentration of BHDQ exhibited 25\% of the bio-antimutagenicity, but the low level of BHDQ (1.5 nmol), as shown in Fig. 3A, none at all. It was considered that the suppressive effect of BHDQ on the mutation caused by Trp-Ps should be ascribed to its desmutagenicity.

The reaction of BHDQ with N-OH-Trp-P-2

Trp-P-2 is metabolically changed to its activated form by the microsomal S9 system in animal liver. Here, the effect of BHDQ on the production of N-OH-Trp-P-2 from Trp-P-2 by the S9 system was measured \textit{in vitro} (Table II). The remaining Trp-P-2 and the N-OH-Trp-P-2 produced were determined by HPLC. A typical chromatography is presented in Fig. 2. With an increase in added BHDQ, the remaining amount of Trp-P-2 increased and the production of N-OH-Trp-P-2 decreased. The mutagenicity of the reaction mixture decreased, too. Thus, BHDQ depressed the production of N-OH-Trp-P-2 by the S9 system.

There remained the question of whether or
Table III. The Re-production of Trp-P-2 from N-OH-Trp-P-2 by BHDQ

<table>
<thead>
<tr>
<th>Added amount of BHDQ (nmol)</th>
<th>Amounts of Trp-P-2 (nmol)</th>
<th>Amounts of N-OH-Trp-P-2 (nmol)</th>
<th>Sum of them (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>94.6</td>
<td>24.5</td>
<td>119</td>
</tr>
<tr>
<td>1</td>
<td>98.2</td>
<td>24.4</td>
<td>123</td>
</tr>
<tr>
<td>3</td>
<td>103.7</td>
<td>13.8</td>
<td>118</td>
</tr>
<tr>
<td>6</td>
<td>105.5</td>
<td>8.6</td>
<td>114</td>
</tr>
<tr>
<td>12</td>
<td>107.3</td>
<td>6.5</td>
<td>114</td>
</tr>
<tr>
<td>30</td>
<td>110.5</td>
<td>2.3</td>
<td>113</td>
</tr>
<tr>
<td>60</td>
<td>117.0</td>
<td>ND</td>
<td>117</td>
</tr>
</tbody>
</table>

a BHDQ was incubated with a mixture of Trp-P-2 and N-OH-Trp-P-2 in a 50% acetonitrile solution at 37°C for 20 min, and then the amounts of Trp-P-2 and N-OH-Trp-P-2 in the incubation mixture were estimated by HPLC analysis.

not BHDQ directly attacks N-OH-Trp-P-2 and thus decomposes it. Therefore, Trp-P-2 and N-OH-Trp-P-2 were extracted from the reaction mixture of Trp-P-2 with the S9 system. BHDQ was added to the extracts, followed by incubation without the S9 system (Table III). The amount of N-OH-Trp-P-2 inversely decreased and the amount of Trp-P-2 proportionally increased with the concentration of BHDQ. When 60 nmol of BHDQ was added to the mixture, N-OH-Trp-P-2 completely disappeared. The sum of N-OH-Trp-P-2 and Trp-P-2 remained almost constant in all the incubation mixtures. This indicates that N-OH-Trp-P-2 was non-enzymatically re-converted to Trp-P-2 by BHDQ.

Discussion

We reported an interesting result in our previous paper, i.e., a product of the reaction of BHA and nitrite, BHDQ, exhibited a suppressive effect on the mutagenicities of Trp-P-2 and Trp-P-1. The present study proved that this suppressive effect of BHDQ on the mutagenicity is due to its desmutagenicity.

The bio-antimutagenic activity of BHDQ was negligible as compared to its desmutagenicity (Table I). BHDQ was considered not to be a bio-antimutagen suppressing the process of mutation fixation. On the other hand, BHDQ markedly reduced the production of N-OH-Trp-P-2 from Trp-P-2 by the S9 system (Table II). The suppressive effect of BHDQ on the mutation was greater when the mutation was induced by N-OH-Trp-P-2 than when induced during the metabolism of Trp-P-2 by the S9 system (Fig. 3). BHDQ re-converted N-OH-Trp-P-2 to Trp-P-2 (Table III). Thus, BHDQ acted directly on the N-OH-forms of Trp-Ps, but not on the metabolic system of S9. It was concluded that BHDQ should be a desmutagen. The desmutagenicity of BHDQ was ascribed to its non-enzymatic effect, i.e., the conversion of N-OH-Trp-P-2 to its inactive form.

BHDQ also exhibited suppressive effects on the mutagenicities of benzo[a]pyrene and 1-nitropyrene (data not shown). A hundred nanomoles of benzo[a]pyrene and 2 nmol of 1-nitropyrene gave 148 and 822 revertant colonies, respectively. The addition of 30 nmol BHDQ decreased the numbers to 60 and 439, respectively.

Thus, we showed that the reaction products of BHA with nitrite under gastric conditions can act as both a mutagen (t-BQ and BBDQ) and a desmutagen (BHDQ). We consider that the paradoxical bio-activities of BHA on oral administration are due to both its mutagenic and desmutagenic abilities.

BHDQ is photolytically produced from BBDQ (quinone dimer) via its semiquinone radical in vitro. It is generally believed that quinones are reduced in vivo through one electron-reduction to their semiquinone radicals by microsomal NADPH-cytochrome P-450 or mitochondrial NADH-ubiquinone oxidoreductase. Since the specificities of these enzymes for quinones are low, it may be possible that BBDQ is also changed to BHDQ in vivo. On the other hand, BBDQ is produced in vitro through the oxidation of nitrite via a stable intermediate, which is a dimer of BHA (2,2',2'-dihydroxy-3,3'-di-tert-butyl-5,5'-dimethoxybiphenyl). Recently, Armstrong and Wattenberg reported that the
dimer was formed from administered BHA in rat liver microsomes. It has also been reported that t-BQ was produced as one of the microsomal metabolites of BHA in cultured hepatocytes.26) On the basis of this information, it is predicted that some mutagenic and desmutagenic derivatives of BHA are also formed in the hepatic microsomes of animals.

References