Abstract. Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is now under active investigation as a neuroprotective and anticancer agent. In the present study, the induction period method was used to investigate the antioxidant activity of ebselen in the radical polymerization of methyl methacrylate (MMA) at 70°C. The reaction of ebselen with growing MMA radicals (lipid radicals) showed a $k_{inh}$ of $4 \times 10^4$ M$^{-1}$s$^{-1}$. This value was similar to that for mercaptomethylimidazole (MMI, a thiol) and 10-fold greater than that for butylated hydroxyanisole (BHA). The ratio of the rate of chain inhibition to that of chain propagation (CI/CP) for ebselen, MMI and BHA was 0.1, 0.01 and 0.001, respectively, whereas the stoichiometric factor (n, the number of free radicals trapped by one mole of antioxidant moiety) for the corresponding compounds was 0.02, 0.2 and 2, respectively. Ebselen preferentially affected CP rather than CI, indicating that it was an effective scavenger (supressor) of growing MMA radicals. These results suggest that ebselen is a potent suppressor of polyunsaturated fatty acid (PUFA) radicals, which are harmful radicals in biological systems.

Ebselen (Figure 1) is a non-toxic seleno-organic drug that has been extensively studied during the last decade. Selenium-containing enzymes exert their enzymatic activity through oxidation of their seleno moiety to selenic acids. Ebselen displays glutathione (GSH) peroxidase (Px) (GSH-Px)-like activity. It is now under active investigation in clinical trials as a neuroprotective agent (1). Ebselen suppresses inflammation in a variety of experimental animal models. Ebselen inhibits 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced down-regulation of gap-junctional intercellular communication (GJIC) (2). Reduction of GJIC may be involved in the development of a number of pathological conditions, particularly carcinogenesis. Indeed, ebselen has been reported to inhibit aflatoxin B1-induced hepatocarcinogenesis in Fischer 344 rats (3). The pharmacological profile of ebselen appears to be due to its action as an antioxidant, and recent studies have led to a broader appreciation of the role of selenium in cellular antioxidant defense mechanisms. Ebselen efficiently scavenges peroxynitrite (ONOO$^-$/ONOOH), yielding the selenoxide at 1:1 stoichiometry (4). Additionally, ebselen exhibits potent inhibition of $O_2$ generation (5, 6). In contrast, ebselen does not suppress the oxidation of low density lipoprotein initiated by the thermal decomposition of azo-iniators (7). The kinetics of the reaction of ebselen with lipid radicals remain to be fully elucidated.

We have previously proposed a quantitative model rationalizing the radical-scavenging activity of butylated hydroxytoluene (BHT)-related compounds in the polymerization of methyl methacrylate (MMA) initiated by the thermal decomposition of 2,2'-azobisisobutylnitrile (AIBN) or benzoyl peroxide (BPO) under nearly anaerobic conditions (8). The model was well able to explain the mechanism of radical-scavenging activity and to predict the chain-breaking activity of polyamines (9) and beta-carotenes (10). The advantage of this model system is that measurements using differential scanning calorimetry (DSC) are highly sensitive; furthermore, use of anaerobic conditions makes this system relatively biomimetic, since oxygen is sparse in living cells (11, 12). In the present study, the investigation was extended to ebselen. Here, kinetic studies of the radical-scavenging activity of ebselen are reported.

Materials and Methods

The following chemicals and reagents were obtained from the indicated companies: ebselen (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 4-hydroxy-3-tert-butylanisole (BHA) and 2-mercapto-1-methylimidazole (MMI). MMA (Tokyo Kasei Chem. Co., Tokyo, Japan) was purified by distillation. AIBN and BPO (Tokyo Kasei Chem. Co.) were recrystallized from methanol and...
methanol/chloroform (1:1 v/v), respectively. The chemical structures of ebselen, BHA and MMI are shown in Figure 1.

**Induction period and initial rate of polymerization.** The induction period (IP) and initial rate of polymerization in the presence (Rp\text{inh}) or absence (Rp\text{con}) of an antioxidant were determined by the method previously reported (9). In brief, the experimental resin consisted of MMA and AIBN (or BPO), with or without additives. AIBN or BPO were added at 1.0 mol\%, and the additives were used at 0, 0.1, 0.2, 0.5, 0.7 and 1.0 mol\%. Approximately 10 µl of the experimental resin (MMA: 9.12-9.96 mg) was loaded into an aluminum sample container and sealed by applying pressure. The container was placed in a differential scanning calorimeter (model DSC 3100; MAC Science Co., Tokyo, Japan) kept at 70°C, and the thermal changes induced by polymerization were recorded for the appropriate periods. The heat due to polymerization of MMA was 13.0 kcal/mole in this experiment. The conversion of all samples, as calculated from the DSC thermograms, was 92-95%.

Polymerization curves were derived from the DSC thermograms using the integrated heat evoked by the polymerization of MMA. Polymerization curves break when an inhibitor is consumed (Figure 2). These breaks are sharp and provide a reliable measure of the IP of the inhibitor. The presence of oxygen retards polymerization because oxygen reacts with MMA radicals activated by the initiator and then, subsequently, produces a non-radical product. Thus, polymerization of the control was slightly inhibited, even though the reaction was carried out in a sealed DSC pan, because the pan contained a small amount of oxygen, having been sealed in air. Tangents were drawn to the polymerization curves at an early stage in the run. The IP of the test compounds was determined from the length of time between the zero point on the abscissa and the point of intersection of the tangents drawn to the early stage of polymerization. The IP was calculated from the difference between the induction period of specimens and that of controls. The initial rates of polymerization in the absence (Rp\text{con}) and presence (Rp\text{inh}) of ebselen, BHA and MMI were calculated from the slope of the plots of the first linear line of the conversion rate of MMA polymerization (tangent drawn at the early polymerization stage).

**Measurement of stoichiometric factor (n).** The relative \( n \) value in Eq. (1) can be calculated from the induction period in the presence of an inhibitor:

\[
n = R_p \frac{[\text{IP}]}{[\text{IH}]} \tag{1}
\]

where \([\text{IP}]\) is the induction period in the presence of an inhibitor. The number of moles of peroxo radicals trapped by the antioxidant was calculated with respect to 1 mole of inhibitor moiety unit. The \( R_p \) values of AIBN and BPO were 5.66x10^{-6} M\text{s}^{-1} and 2.28x10^{-6} M\text{s}^{-1}, respectively (9).

**Measurement of inhibition rate constant (\( k_{inh} \)).** When \( R_p \) is constant, i.e. when new chains are started at a constant rate, a steady-state treatment can be applied and the initial rate of polymerization of MMA is given by Eq. (2) (9, 13):

\[
R_p\text{con} = \frac{(k_p [\text{MMA}] R_i^{1/2})}{(2k_t)^{1/2}} \tag{2}
\]

where MMA represents methyl methacrylate and \( k_p \) and \( k_t \) are the rate constants for chain propagation and termination, respectively.

The \( k_p(2k_t)^{1/2} \) rate of polymerization of MMA (9.4 M) initiated by AIBN (1 mol\%) or BPO (1 mol\%) at 70°C was a constant value, 9.86x10^{-2} M^{-1/2} s^{-1/2} (9). The \( R_p\text{inh} \) rates are determined by Eq. (3):

\[
R_p\text{inh} = \frac{(k_p [\text{MMA}] R_i)}{(n k_{inh} [\text{IH}])} \tag{3}
\]

in which \( R_p\text{inh} \) is the initial rate of inhibited polymerization, \([\text{MMA}], n, [\text{IH}] \) and \( k_p \) are defined above, and \( k_{inh} \) is the rate constant for scavenging (inhibiting) of MMA radicals by an antioxidant. From Eq. (2) and Eq. (3), \( k_{inh}/k_p \) can be calculated (Eq. (4)):

\[
k_{inh}/k_p = \frac{[\text{MMA}]}{([\text{IP}][R_p\text{inh}])} \tag{4}
\]

The ratio of the rate of chain inhibition to that of chain propagation was determined by Eq. (5):

\[
\text{chain inhibition/chain propagation (CI/CP)} = \frac{(k_{inh} [\text{ROO.}][\text{IH}])/(k_p [\text{ROO.}][\text{MMA}]])}{(k_{inh} [\text{IH}])(k_p [\text{MMA}]])} = \frac{([\text{IH}])([\text{IP}][R_p\text{inh}])}{([\text{IP}] R_p\text{inh})} \tag{5}
\]

**Results and Discussion**

**Radical-scavenging activity.** Time-exothermic (top) and time-conversion (bottom) curves for the AIBN and BPO systems are shown in Figure 2. Typical time-conversion curves for the BPO system in the presence of ebselen, BHA or MMI are shown in Figure 3. First, the radical-scavenging activity of ebselen was evaluated kinetically. Plots of IP or \( R_p\text{inh} \) vs. concentration of ebselen in the AIBN-MMA and BPO-MMA systems are shown in Figures 4A and 4B, respectively. The IP and \( R_p\text{inh} \) for ebselen in both systems increased linearly in a dose-dependent manner. A linear relationship between IP and [IH]/\( R_i \) for ebselen was found in both systems, and the values could be plotted on the same
The $n$ value for ebselen was rather low, approaching zero ($n \approx 0.02$). A linear relationship between $([R_{\text{inh}}][IP])$ and $[IH]$ was also found for both systems, and the values could be plotted on the same line (Figure 5A). The CI/CP value calculated from Eq. (5) was approximately 0.1. When we compared the values of $n$ and CI/CP for the compounds tested, the $n$ value declined in the order BHA(2) > MMI(0.2) > ebselen(0.02), whereas the CI/CP value declined in the order ebselen(0.1) > MMI(0.01) > BHA(0.001).

BHA is a well-known chain-breaking antioxidant (14). In the present study, BHA showed the highest $n$ value and the lowest CI/CP value. Conversely, ebselen showed the lowest $n$ value and the highest CI/CP value. These findings clearly indicate that ebselen acts as a retarder. Ebselen was a poor radical scavenger (inhibitor) against $R'$ derived from AIBN or against PhCOO$^-$ derived from BPO, but was an effective retarder (suppressor) of growing MMA radicals. This strongly suggests that ebselen may suppress polyunsaturated fatty acid (PUFA) radicals induced by reactive oxygen species (ROS) in biological systems. MMI was used as a representative thiol, since GSH cannot be used in this system because of its limited solubility in MMA. MMI is an antioxidant and its activity was between those of ebselen and BHA. Ebselen was previously reported to be oxidized easily by hydrogen peroxide, tert-butyl-hydroperoxide and m-
Figure 4. Relationship between the induction period (IP) (A) or the initial rate of polymerization in the presence of inhibitor (Rp_{inh}) (B) and the concentration of ebselen in the AIBN and BPO systems.

Figure 5. Plots of induction period (IP) vs. ([IH]/R_i) (A) and of (Rp_{inh}IP) vs. concentration of ebselen (B). [IH], ebselen, an inhibitor; Rp_{inh}, the initial rate of polymerization in the presence of inhibitor; IP, induction period; R_i, the initiation rate of AIBN or BPO.
chlorobenzoic acid to yield seleneoxides (15). In the present study, the low but measurable \( k_{\text{inh}} \) value for ebselen suggests that this compound is oxidized by \( \text{R}^- \) or \( \text{PhCOO}^- \), although to a very limited extent. In contrast, ebselen does not react with diphenylpicrylhydrazyl (DPPH) radicals, nor does it suppress the oxidation of methyl linoleate in acetone solution or in aqueous dispersions induced by free radical initiators, suggesting that ebselen does not act as a potent radical-scavenging antioxidant (7). Similarly, ebselen was previously reported to exhibit little radical-scavenging activity against DPPH radicals (16). Ebselen shows peroxy nitrite-scavenging activity induced by pyrogallol red dye (16). The second-order rate constant for the reaction of ebselen with peroxynitrite, as determined by a stop-flow technique, was previously reported to be 2.0±0.1x10^6 M^−1 s^−1 at pH values of 8 or above and 25°C and to be three orders of magnitude greater than that for cysteine (17), possibly because of the high reactivity of the seleno moiety in ebselen with peroxynitrite. On the other hand, ebselen appears to be incapable of suppressing the oxidation of methyl linoleate (7), which may be a result of the fully oxygenated experimental system used. β-Carotene, quinones and secondary aromatic amines have been reported to be very poor antioxidants at high oxygen tensions in aqueous systems containing linoleic acid, since their inhibition rate constants were too small to be measured (13). In the present study, which used highly sensitive DSC measurements under nearly anaerobic conditions, the radical-scavenging activity of ebselen was low but detectable. Although ebselen was a poor radical scavenger against \( \text{PhCOO}^- \) or \( \text{R}^- \), it strongly suppressed growing MMA radicals. In particular, growing MMA radicals derived from thermal decomposition of BPO were strongly suppressed by high concentrations of ebselen (Figure 2, BPO-f). This suggests that radical-oxidation products derived from ebselen might preferentially suppress growing MMA radicals. We have demonstrated that quinones derived from BHT are weaker inhibitors but more potent retarders compared with BHT itself (8). Similarly, some polyphenol quinones possess radical-scavenging activity at least as great as that of the parent polyphenols (18).

\[ k_{\text{inh}} \]

Next, we examined the \( n \) and \( k_{\text{inh}}/k_p \) values calculated from Eq. (1) and Eq. (4), respectively. The \( k_{\text{inh}}/k_p \) values for ebselen, BHA and MMI at 0.1 mol% were 94, 11 and 80, respectively, from which \( k_{\text{inh}} \) was estimated to be 3.8x10^4 M^−1 s^−1, 4.5x10^3 M^−1 s^−1 and 3.2x10^4 M^−1 s^−1, respectively. (Note: the \( k_p \) of MMA at 70°C, 405 M^−1 s^−1, was extrapolated from values of 143 M^−1 s^−1 at 30°C and 367 M^−1 s^−1 at 60°C (19).) Reduction of lipid peroxide levels has previously been found in rats treated with oral ebselen, suggesting that ebselen may be an effective scavenger of organic hydroperoxides, in particular of lipid hydroperoxides (20). In vivo experiments are too complex to be amenable to simple interpretation, which is why we have developed a physicochemical model for the PUFA radical-scavenging activity of antioxidants, namely the induction period method in the radical polymerization of MMA. We believe that the present study is the first to report the \( k_{\text{inh}} \) of ebselen with lipid radicals, and the relatively high \( k_{\text{inh}} \) value found may provide an explanation for the ability of ebselen to reduce lipid peroxide levels \( \textit{in vivo} \). The \( k_{\text{inh}} \) value found for ebselen is of a similar order of magnitude to that of phase II gene inducers such as green tea polyphenols (21).

**References**


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