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Mechanism of Hydroxyl Radical Scavenging by Rebamipide: Identification of Mono-hydroxylated Rebamipide as a Major Reaction Product

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Rebamipide, an antiulcer agent, is known as a potent hydroxyl radical (·OH) scavenger. In the present study, we further characterized the scavenging effect of rebamipide against ·OH generated by ultraviolet (UV) irradiation of hydrogen peroxide (H_2O_2), and identified the reaction products to elucidate the mechanism of the reaction. Scavenging effect of rebamipide was accessed by ESR using DMPO as a ·OH-trapping agent after UVB exposure (305 nm) to H_2O_2 for 1 min in the presence of rebamipide. The signal intensity of ·OH adduct of DMPO (DMPO-OH) was markedly reduced by rebamipide in a concentration-dependent fashion as well as by dimethyl sulfoxide and glutathione as reference radical scavengers. Their second order rate constant values were 5.62×10^{10}, 8.16×10^{9} and $1.65 \times 10^{10}\,M^{-1}\,s^{-1}$, respectively. As the rebamipide absorption spectrum disappeared during the reaction, a new spectrum grew due to generation of rather specific reaction product. The reaction product was characterized by LC-MS/MS and NMR measurements. Finally, a hydroxylated rebamipide at the 3-position of the 2(1*H*)-quinolinone nucleus was newly identified as the major product exclusively formed in the reaction between rebamipide and the ·OH generated by UVB/H_2O_2. Specific formation of this product explained the molecular characteristics of rebamipide as a potential ·OH scavenger.

Keywords: Rebamipide; ·OH scavenger; UVB/H_2O_2; LC-MS/MS; NMR; 3-Hydroxylated rebamipide

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMSO, dimethyl sulfoxide; ESR, electron spin resonance; GSH, glutathione; LC-MS/MS, HPLC-coupled to tandem mass spectrometry; UV, ultraviolet

INTRODUCTION

Hydroxyl radical (·OH) is known as one of the extremely highly reactive and most harmful oxygen-derived free radicals in living organisms.[1] It reacts with various cellular components including lipid, protein and DNA to oxidatively modify or decompose them.[2,3] Therefore, it appears to play an important role in the pathogenesis of various diseases, including gastritis and peptic ulcer diseases.[4] Indeed, *Helicobacter pylori* and nonsteroidal antiinflammatory agents, that are widely known as major causes of gastroduodenal ulcer, are reported to promote generation of oxygen-derived free radicals from inflammatory cells infiltrated into gastric mucosa, and also from the endothelium during gastric ischemia–reperfusion.[5,6]

Rebamipide (2-(4-chlorobenzoylamino)-3-[2(1*H*)-qunolinone-4-yl] propionic acid) (CAS 11911-87-6) is an antiulcer agent clinically used in Southeast Asian countries including Japan and Korea.[7,8] Pretreatment with rebamipide significantly inhibited the gastric mucosal injury induced by ischemia–reperfusion or intragastric instillation of H_2O_2.[9,10] Since rebamipide has been reported to suppress the elevation of gastric mucosal lipid peroxide level in rat gastric mucosal lesions such as caused by indomethacin,[11,12] diethyldithiocarbamate[13] and platelet activating factors.[14] Therefore,

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the antiulcer activity of rebamipide may be, in part, related to its suppressive effect on oxygen-derived free radical generation in gastric mucosa. Mainly, two mechanisms of rebamipide against free radicals have been reported. One is an inhibition of superoxide anion radical ($O_2^{\cdot-}$) production by activated neutrophils,[13,15] and the other is $^{\cdot}OH$ scavenging activity.[16,17] Yoshikawa *et al.* found that rebamipide was one of the most potent $^{\cdot}OH$ scavengers, with the second order rate constant for the reaction as $2.24 \times 10^{10}\,mol^{-1}\,s^{-1}$.[16] Consecutively, Naito *et al.* investigated the structure/activity relationship for the $^{\cdot}OH$ scavenging reaction among seven rebamipide analogues, and reported that the 3,4-double bond of the 2-quinolinone nucleus of rebamipide and the acid amide bond carrying *para*-chlorobenzoyl group were important determinants of the $^{\cdot}OH$ scavenging property.[17] In all these studies, however, the Fenton reaction was used for $^{\cdot}OH$ generating system. It is well known that iron in the Fenton system frequently reacts with the reactant (including test scavengers) to make the reaction system complex, such that $^{\cdot}OH$ generation is enhanced rather than inhibited by some scavenging molecules.[18] Moreover, catechols were reported to suppress $^{\cdot}OH$ generation in the Fenton reaction because they mask metal ion by chelate formation.[19,20] The Fenton reaction is thus not adequate to study precise inhibitory action of $^{\cdot}OH$ scavenger candidate molecules, especially when they are expected to react with iron.

An ultraviolet (UV)/H_2O_2 system is an alternative method for generating $^{\cdot}OH$ and is more appropriate to investigate direct scavenging ability of test compounds toward $^{\cdot}OH$.[18,20] In the present study, we reevaluated the $^{\cdot}OH$-scavenging activity of rebamipide using the UVB/H_2O_2, in order to provide better rationale of protective mechanisms proposed for antiulcer action of rebamipide.

MATERIALS AND METHODS

Chemicals

A spin trapping agent, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Dojindo Co. (Kumamoto, Japan) and used without further purification. Hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO) and glutathione (GSH) were obtained from Wako Pure Chemical (Osaka, Japan), Kanto Chemical (Tokyo, Japan) and Kohjin Co. (Tokyo, Japan), respectively. Rebamipide, OPC-12959, OPC-22285 and OPC-22001 were synthesized in Otsuka Pharmaceutical Co. (Tokushima, Japan; see Fig. 1). All other chemicals used were of reagent grade.

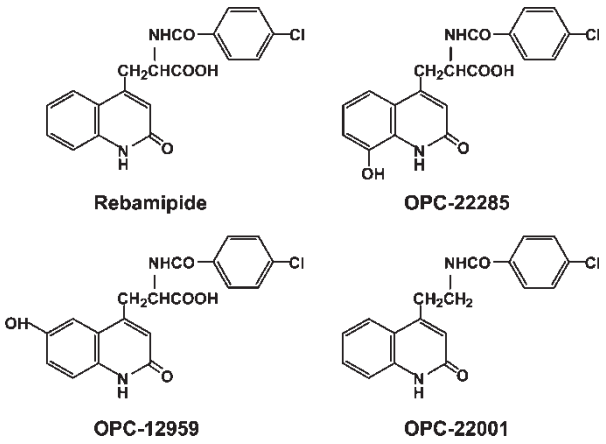

FIGURE 1 Chemical structures of rebamipide and related analogues.

Scavenging Activity against $^{\cdot}OH$ Generated by UV Irradiation of H_2O_2

Reaction mixture containing 20 mM DMPO, 40 mM H_2O_2 and test samples in 300 μl with or without rebamipide (0.1–1 mM), DMSO (0.5–5 mM), or GSH (0.5–5 mM) was prepared in a glass test tube (total 300 μl).

For preparation of rebamipide stock solution, (≈20 mM) rebamipide saturated solution was prepared in 0.02N NaOH. After adjusting the pH to neutral, the concentration of rebamipide solution was determined from the OD at 293 and 329 nm using the equation as follows; rebamipide concentration (mol) = $(A_{329} - A_{293})/4.6 \times 10^3$.

DMSO and GSH (reference antioxidant) were directly diluted in distilled water. The reaction mixtures were taken into a hematocrit capillary (25 μl, Drummond Scientific Company, PEN) and illuminated at 305 nm using PAN UV lamp (Toshiba, Japan). One min after the UV-irradiation, DMPO-OH signal was measured by JEOL-JES-TE 200 ESR spectrometer (X-Band Microwave Unit, JEOL, Tokyo, Japan). ESR measurement conditions were the following: microwave power 8 mW, microwave frequency 9.20 GHz, modulation amplitude 0.1 mT, time constant 0.03 s, sweep time 1 min, center field 331.6/321.6 mT. The signal intensity of DMPO-OH was normalized against Mn^{2+} signal, which was used as an internal standard. The 50% inhibition concentration (IC_{50}) of each scavenger was calculated from the linear regression plot of DMPO-OH signal intensity versus scavenger concentration. The second order rate constant for the reaction between each scavenger and $^{\cdot}OH$ was calculated as previously reported.[16]

Spectroscopic Analysis of Rebamipide Decomposition Induced by UVB/H_2O_2

For a concentration-dependent experiment, a reaction mixture containing 0.05 mM rebamipide and 0–5 mM H_2O_2 in a quartz cell was exposed to UVB light (305 nm) for 2 min. Then, UV spectra were recorded in the wavelength range from 400 to 200 nm, using a Hitachi model U-3000 UV/VIS spectrophotometer (Tokyo, Japan). For a time-dependent experiment, the spectra were recorded periodically, with the reaction solution containing 0.05 mM rebamipide and 10 mM H_2O_2, after UVB irradiation for 0–2 min.

Analysis of Reaction Products by LC-MS/MS

Reaction mixture containing 500 mM H_2O_2 and 5 mM rebamipide in a quartz cell (2 ml) was exposed to UVB light (305 nm) for 2 h at room temperature. Then, the reaction products were analyzed by HPLC coupled to tandem mass spectrometry (LC-MS/MS). LC-MS/MS conditions were as follows: [HPLC condition] HPLC, LC-10A HPLC system (Shimazu, Tokyo, Japan); analytical column, symmetry C_{18} (5 μm, 50 × 2.1 mm I.D., Waters); mobile phase A, H_2O:acetic acid = 100:1 (by v/v); mobile phase B, CH_3CN:acetic acid = 100:1 (by v/v); gradient B/A (0/100–64/36, linear for 40 min, hold for 10 min), B/A (0/100–50/50, linear for 30 min, for MS/MS); detector, UV detector (280 nm); flow rate, 0.2 ml/min; column temperature, 30°C. [MS, MS/MS condition] Tandem mass spectrometry (MS/MS): TSQ-7000 (Thermo Electron) triple-stage quadruple MS/MS system; ionization method, electrospray ionization (ESI); ionization mode, positive; electron multiplier voltage, 1.2 kV; sheath gas pressure, N_2-10 unit to 70 psi, auxilliary gas flow rate; N_2-10 units; capillary temperature, 200°C; collision gas pressure, ≈2.0 mTorr (argon); collision energy, −15 or −25 eV. For MS/MS analysis, reaction mixtures were diluted10 times with 80% CH_3CN. Rebamipide, OPC-12959, OPC-22285 and OPC-22001 were used as standard compounds (Fig. 1).

Isolation and Determination of Major Reaction Product by NMR

In order to determine the chemical structure of the major product, reaction mixture containing 500 mM H_2O_2 and 5 mM rebamipide in a glass flask (100 ml) was exposed to UVB light (305 nm, 7.70 mW/cm^2, CFL-20B, Cosmo Bio, Tokyo, Japan) for 2 h at room temperature. This was done to obtain enough amount of the major product. To 1 ml of reaction mixture, 0.2 mmol/l of acetic acid buffer (pH 5.0) and 5 ml of ethyl acetate were added. The mixture was shaken for 10 min and centrifuged at 1500*g* for 10 min, and the organic layer was removed. The organic layer obtained by duplicate procedures was combined and evaporated to dryness under a stream of nitrogen gas and dissolved in methanol for preparative HPLC analysis. Preparative HPLC was performed under the following conditions: HPLC, separation module 2690 (Waters); analytical column, [the first step] TSK GEL ODS-80 Ts column (5 μm, 150 × 4.6 mm I.D., Tosoh, Japan), [the second step] CAPCELL PACK C_{18} MG column (5 μm, 150 × 3 mm I.D., Shiseido, Japan); mobile phase A, H_2O:acetic acid = 100:1 (by v/v); B, CH_3CN:acetic acid = 100:1 (by v/v); A:B = 60:40; detector, UV detector (280 nm); flow rate, 1.0 ml/min. Separated and collected fractions by the first step column were concentrated and further purified by the second step column. The purified fractions were evaporated to dryness under a stream of nitrogen gas and dissolved in dimethylsulfoxide-d_6 (D99.95%, Acros) for ^{1}H-NMR analysis. The ^{1}H-NMR spectra were obtained on a JNM-A500 (JEOL, Tokyo, Japan) Fourier transform spectrometer at 500 MHz.

RESULTS

Scavenging Effect of Rebamipide on ·OH Generated by UVB/H_2O_2

Four characteristic signal lines were observed 1 min after UVB irradiation of H_2O_2 with DMPO. The *g*-value ($g = 2.013$) and hyperfine coupling constant ($a_N = a_H = 1.49$ mT) could be assigned to a DMPO-OH spin adduct. The effect of rebamipide on DMPO-OH signal intensity was investigated in quadruplicate at the concentrations from 0.01 to 1 mM. Rebamipide inhibited DMPO-OH signal formation in a concentration-dependent fashion and the IC_{50} value was determined as 0.69 mM from the linear regression plot of DMPO-OH signal intensity versus rebamipide concentration (Fig. 2). DMSO and GSH also suppressed DMPO-OH signal intensity in a concentration-dependent fashion from 0.5 to 5 mM. The IC_{50} values obtained were 3.12 and 1.70 mM for DMSO and GSH, respectively. The values of second order rate constant calculated from the slope of the linear regression plot and the previously reported rate constant k_{DMPO} ($2.1 \times 10^9\ M^{-1}\ s^{-1}$) of DMPO toward the hydroxyl radical were 5.62×10^{10}, 8.16×10^9 and 1.65×10^{10} for rebamipide, DMSO and GSH, respectively. The scavenging activity of rebamipide against ·OH was much stronger than that of DMSO, as previously reported.[16] Thus, potential scavenging activity of rebamipide against ·OH was confirmed.

UV Spectra

It was previously suggested that two structural parts are important for the reaction: (1) the 3,4-double

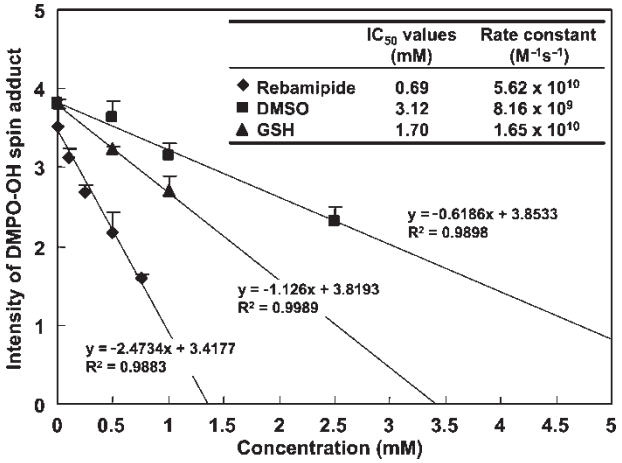

FIGURE 2 Dose dependent inhibition of DMPO-OH formation by rebamipide, DMSO and GSH. ESR signal of DMPO-OH was measured at 1 min after UVB irradiation of reaction mixture containing 20 mM DMPO and 40 mM H_2O_2 at room temperature in the presence or absence of test compounds. Each data point represents the mean ± SD of quadruplicate experiments for rebamipide and triplicate for DMSO and GSH. Fifty percent inhibitory concentrations (IC_{50}) and the second order rate constants were calculated from the linear regression plot of the data in each experiment.

bond of the 2(1*H*)-quinolinone and (2) the acid amide bond carrying para-chlorobenzoyl group. However, any reaction product has been identified to rationale these assumption.[17]

We measured UV absorption spectra of rebamipide solution after UVB irradiation in the presence of H_2O_2. A new absorption peak appeared at around 295 nm with the decrease of rebamipide absorption both in H_2O_2 and irradiation time dependent manner (data not shown). Since the spectral changes seemed to have an isosbestic point at 310 nm, it was suggested that single or a major reaction product was formed from rebamipide under UVB/H_2O_2 reaction (Fig. 3). Indeed, HPLC separation of the reaction mixture using a reverse phase column showed a generation of a new peak with the loss of rebamipide peak when monitored at 280 nm absorbance (data not shown).

Identification of Products by LC-MS/MS

To characterize the reaction product, the reaction mixture was analyzed by HPLC coupled to tandem mass spectrometry (LC-MS/MS). A HPLC chromatogram obtained for rebamipide solution, illuminated by UVB for 2 h at room temperature without H_2O_2, indicated that rebamipide was not significantly decomposed by UVB alone. The major but tiny degradation product appeared at 20.6 min, and was identified as OPC-22001, decarboxylated product of rebamipide, by the comparison of MS/MS spectra with authentic standard (Table I). In addition, no reaction product was detected when rebamipide was treated with H_2O_2 for 2 h in the absence of UVB. After reacting rebamipide with $^{\cdot}OH$ generated by UVB/H_2O_2, almost ten reactant peaks were detectable both in the HPLC chromatogram and a Q1 total ion scanning mass chromatogram of the reaction mixture, but a single major peak appeared at 19.6 min as we expected above (Fig. 3). From the comparison of HPLC mobility

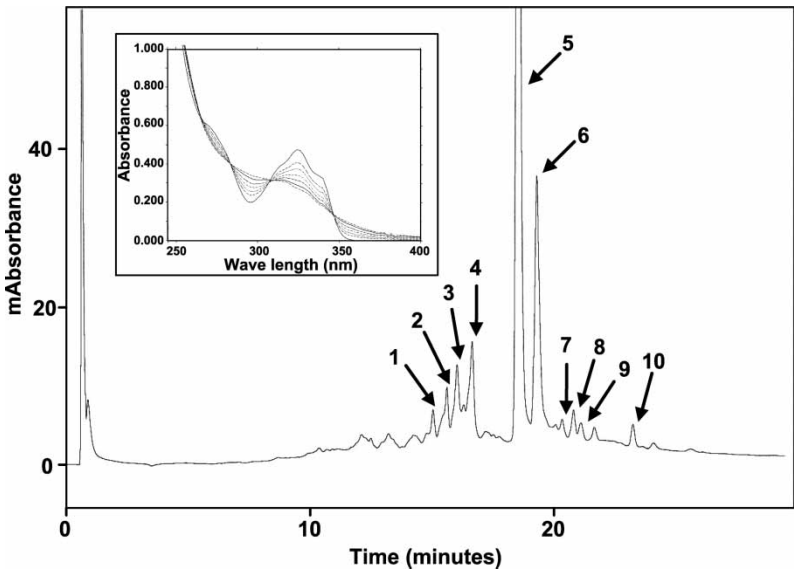

FIGURE 3 HPLC chromatogram of rebamipide after UVB irradiation with H_2O_2. The reaction mixture containing 5 mM rebamipide and 500 mM H_2O_2 was irradiated by UVB (305 nm) for 2 h at room temperature. HPLC conditions are given in the "Materials and methods" section. Numerals in the figure are corresponding to the peak numbers. The inset shows the UVB-exposure-time-dependent spectral change of rebamipide. Reaction mixture containing 10 mM H_2O_2 and 0.05 mM rebamipide was irradiated by UVB (305 nm) for 0.25, 0.5, 0.75, 1.0 and 1.5 min at room temperature.

TABLE I HPLC and LC-MS/MS profiles of rebamipide, OPC-12959, OPC-22285 and OPC-22001

Standard	Retention time (min)	Q1 mass ion (m/z)	Collision energy (eV)	MS/MS product ion (m/z)
OPC-12959	15.8	387	−25	232,139
OPC-22285	16.9	387	−25	232,139
Rebamipide	18.8	371	−25	216,139
OPC-22001	20.6	327	−25	172

and LC-MS/MS spectra of authentic standards, and the product peaks shown in Table I, Peaks 2, 4, 5 and 7 were identified as OPC-12959, OPC-22285, rebamipide and OPC-22001, respectively. OPC-12959 and OPC-22285 are the products of rebamipide being mono-hydroxylated at the 6- and 8-positions of the 2(1*H*)-quinolinone nucleus, respectively, and were determined in rat urine when rebamipide was orally administered.[21,22] Although peak 6, the major reaction product, was also suggested as a mono-hydroxylated analogue of rebamipide due to the molecular weight 387, the Q1 mass ion and MS/MS product ion data were not enough to identify the exact chemical structure (Fig. 4A,B). For further analysis of the structure, the reaction product was isolated and was subjected to NMR spectroscopy.

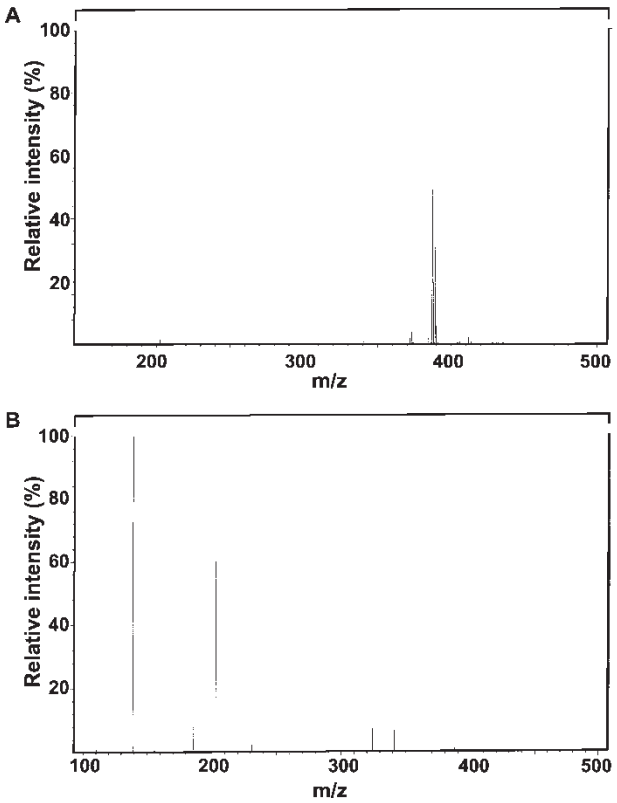

FIGURE 4 Q1 mass spectra and MS/MS product ion spectra for purified major reaction product, peak 6. (A) Q1 mass spectra, (B) MS/MS product ion spectra. MS/MS conditions are given in the "Materials and methods" section.

Identification of the Major Product by ^{1}H-NMR

The ^{1}H-NMR spectra of rebamipide and peak 6 were dissolved in deuterated dimethylsulfoxide-d_6 are shown in Fig. 5A,B, respectively. The spectra are the same as rebamipide, except that the proton signal at the 3-position of quinolinone was missing in the reaction product. On the other hand, MH^+ of the reaction product appeared at m/z 370.0758 by high-resolution fast-atom-bombardment mass spectrometry, indicating that the consisted formula was $C_{19}H_{16}Cl_1N_2O_5$ (cf: protonated formula of rebamipide is $C_{19}H_{16}Cl_1N_2O_4$). This formula was

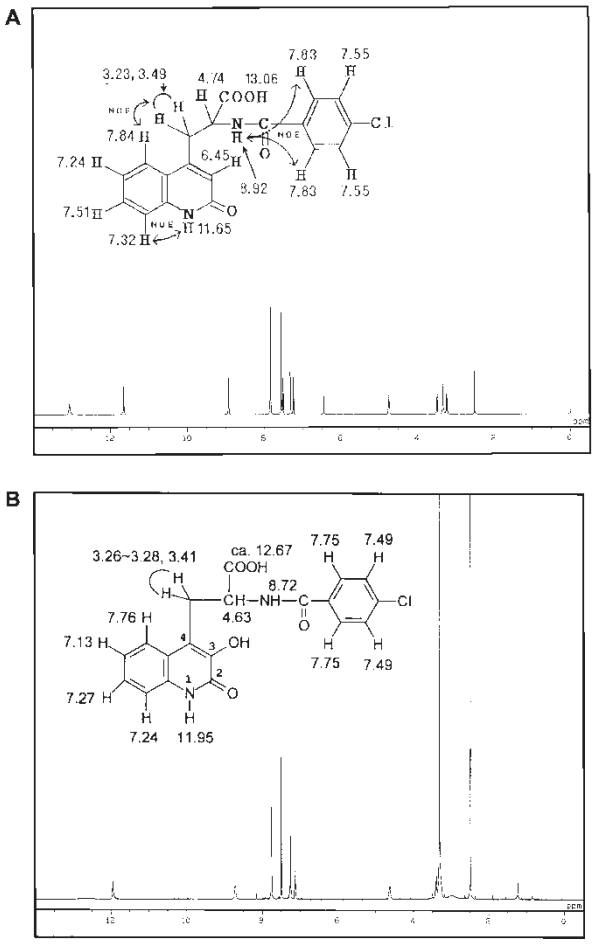

FIGURE 5 NMR spectra of rebamipide and peak 6 and its proposed chemical structure. (A) rebamipide, (B) peak 6. NMR conditions are given in the "Materials and methods" section.

consistent with the structure corresponding to hydroxylated rebamipide at the 3-position.

DISCUSSION

We have examined the scavenging activity of rebamipide toward ·OH generated from the UVB/H_2O_2 reaction using ESR-spin trapping method. Consequently, reaction products of rebamipide were determined. The Fenton reaction is commonly used to generate ·OH and investigate ·OH scavenging activity of various antioxidative compounds. However, compounds chelating metal ion have been known to modulate ·OH generation in this system. Therefore, we used UV photolysis of H_2O_2 to generate ·OH, which has been used to investigate the direct scavenging activity of antioxidative compounds toward ·OH.[18,20] Another concern is a possibility that rebamipide might react with H_2O_2.[23] However, it could be negligible because no reaction product was detected within the mixture of rebamipide and H_2O_2 by LC-MS/MS analysis. In addition, myeloperoxidase-H_2O_2-KBr-dependent chemiluminescence by a *Cypridina* luciferin analogue (MCLA) was not affected by treatment with rebamipide at doses up to 2.0 mM (data not shown).

As a result, rebamipide dose-dependently suppressed the DMPO-OH signal and the second order rate constant was calculated as $5.62 \times 10^{10}\,M^{-1}\,s^{-1}$, which was greater than those of well-known ·OH scavengers, DMSO and GSH (Fig. 2). The rate constant of rebamipide for ·OH generated from the UVB/H_2O_2 system was approximately two times greater than those determined by Yoshikawa *et al.* and Naito *et al.* previously, using the Fenton reaction as 2.24 and $2.42 \times 10^{10}\,mol^{-1}\,s^{-1}$, respectively.[16,17] Rebamipide might ligate to the iron ion that reacts with H_2O_2 in the Fenton reaction, resulting in the difference in the rate constant.

Occasionally, we found that rebamipide solutions were colored to brownish purple after the reaction with ·OH generated by UVB/H_2O_2. In UV spectra studies, rebamipide absorbance was lost, and a new absorption peak appeared at around 295 nm with an isosbestic point at 310 nm (Fig. 3), when it was exposed to 305 nm UVB light in the presence of H_2O_2, suggesting that a single or a major reaction product was formed. Indeed, HPLC separation of the reaction mixture, using a reverse phase column, showed a generation of a new peak with the loss of rebamipide peak when monitored at 280 nm absorbance (data not shown). To clarify the reaction pathway of rebamipide with ·OH, the reaction mixture was further analyzed by HPLC coupled to tandem mass spectrometry (LC-MS/MS). Almost ten reactant peaks were detectable both in HPLC chromatogram and Q1 total ion scanning mass chromatogram in the reaction mixture but a single peak (peak 6) was major, appeared at 19.6 min as we expected above (Fig. 3). The major reaction product was purified and further studied using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. This technique identified the product as 3-hydroxylated rebamipide (Fig. 5). To rationale the production of 3-hydroxylated rebamipide, a reaction pathway was proposed as shown in Fig. 6, in which one mole rebamipide traps two moles of ·OH at the 3,4-double bond to form diol structure and subsequent decomposition of unstable diol gives rise to 3-hydroxylated rebamipide. This finding supports the previous suggestion that the 3,4-double bond of quinolinone nucleus is important for ·OH scavenging by rebamipide.[17] However, this and other minor reaction products determined in the present study did not indicate the acid amide bond carrying para-chlorobenzoyl group was involved in ·OH scavenging reaction. Moreover, It is quite interesting that the peak 6 production was remarkably exceeded to other hydroxylated products, peak 2 (OPC-22285) and peak 4 (OPC-12959) (Fig. 3), because introduction of ·OH chemically to 3-position is usually more difficult than to 6 or 8.

Melatonin, an endogenously produced indole, is found to be a potent ·OH scavenger.[24] Furthermore, antiulcer activities of melatonin, accompanied by suppression of gastric lipid peroxidation and ·OH generation, have been recently reported against water-restraint stress-induced gastric lesions.[25] The second order rate constant for reaction of melatonin with ·OH was reported as $2.7 \times 10^{10}\,M^{-1}\,s^{-1}$, that was comparable with that of rebamipide (Fig. 2).[16,17] Tan *et al.* identified the product that was formed when melatonin interacted with the ·OH, which was cyclic 3-hydroxymelatonin.[26] They also proposed a reaction mechanism in which melatonin scavenges two ·OH per one molecule, as we proposed for rebamipide. This explains the high efficiency to scavenge ·OH by rebamipide and melatonin. This strong ·OH scavenging activity might be a main frame of the strong antiulcer activity of rebamipide, but other physiological reactions also contribute significantly to the efficacy, such that rebamipide increases gastric prostaglandins level,[27,28] reduces $O_2^{\cdot-}$ generation from activated neutrophils[13,15] and suppresses inflammatory cytokines.[29,30]

In conclusion, rebamipide potently scavenges ·OH generated by UVB/H_2O_2 reaction and the main reaction product is mono-hydroxylation of rebamipide at the 3-potion of the 2(1*H*)-quinolinone nucleus. The present experiments explain how rebamipide works as a potent ·OH scavenger, and this contributes to the development of more potent

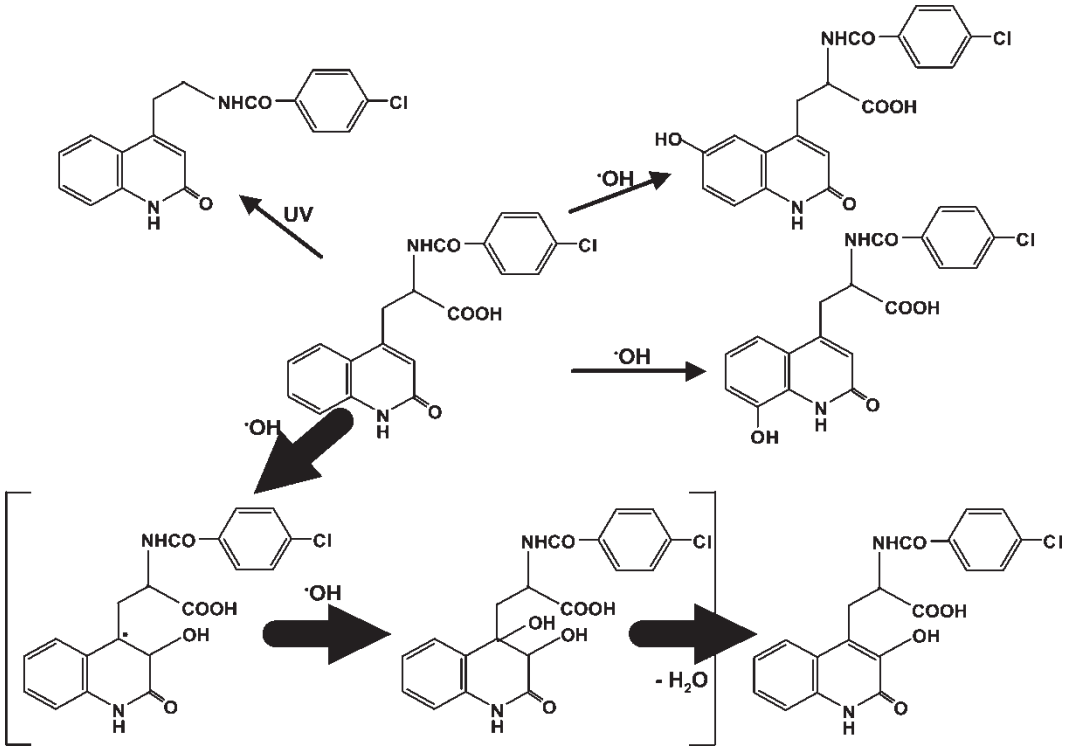

FIGURE 6 Proposed pathways for the reaction of rebamipide with hydroxyl radical.

·OH scavenging antioxidant molecules, as well as to assess how ·OH are involved in pathogenesis of various diseases.

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