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Hydroxyl Radical Scavenging Activity of Nonsteroidal Anti-Inflammatory Drugs

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The hydroxyl radical ($^{\cdot}OH$) -scavenging activity of d-2-[4-(3-methyl-2-thienyl)phenyl]propionic acid (M-5011), a novel nonsteroidal anti-inflammatory drug (NSAID), and that of several other NSAIDs were investigated by the hyaluronic acid (HA) degradation method and the electron spin resonance (ESR) spin-trapping technique. The superoxide anion ($^{\cdot}O_2^-$) -scavenging activity of M-5011 was also measured by the ESR technique.
(1) M-5011 and the other NSAIDs examined inhibited the degradation of HA induced by the Fenton reaction system in a dose-dependent manner.
(2) M-5011 and the other NSAIDs scavenged $^{\cdot}OH$ directly in a dose-dependent manner.
(3) M-5011 was the most potent drug among the NSAIDs tested regarding the scavenging activity of $^{\cdot}OH$ as follows; M-5011 > indomethacin > ketoprofen = suprofen > aspirin. The $^{\cdot}OH$-scavenging activity of M-5011 was potent in comparison with that of oxidized glutathione (GSSG), an endogenous $^{\cdot}OH$ scavenger.
(4) M-5011 did not scavenge $^{\cdot}O_2^-$; nor did GSSG.
These results suggest that M-5011 acts as a scavenger of $^{\cdot}OH$ at sites with inflammatory lesions.

Keywords: Nonsteroidal anti-inflammatory drug (NSAID), hydroxyl radical, electron spin resonance (ESR), spin-trapping, hyaluronic acid (HA)

INTRODUCTION

Active oxygen species such as hydroxyl radical ($^{\cdot}OH$), superoxide anion ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$) have been implicated in the pathophysiology of inflammation.[1] In inflammation such as that in joints affected by rheumatoid arthritis (RA) or osteoarthritis (OA), active oxygen species are directly involved in tissue injuries,[2–4] and these activated oxygen species indirectly facilitate tissue destruction by inactivating α1-protease inhibitors that form a complex with elastase, a serine proteinase.[2,5,6] Oxygen radicals have been implicated in the damage of many tissues, organs and macromolecules including hyaluronic acid (HA), the major macromolecular species of synovial fluid. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used extensively for the treatment of inflammatory diseases such as RA and OA. These

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drugs probably have multiple actions; they have been suggested to exert their anti-inflammatory effects as inhibitors of prostaglandin biosynthesis by inhibiting cyclo-oxygenase.[7,8] There has been considerable interest in the possibility that some of the NSAIDs might act, in part, as oxidants in vivo. In terms of suppressing inflammation, it is desirable to eliminate the active oxygen species that are formed excessively at inflammatory sites. Many anti-inflammatory agents act as scavengers of active oxygen species.[9–16]

We recently developed a novel NSAID, d-2-[4-(3-methyl-2-thienyl)phenyl]propionic acid (M-5011).

The purpose of the present study was to determine the ·OH-scavenging activity of M-5011 and of several other NSAIDs (indomethacin, ketoprofen, suprofen and aspirin), and to compare their ·OH-scavenging activities with that of oxidized glutathione (GSSG), an endogenous ·OH scavenger, using the electron spin resonance (ESR) spin-trapping method.

MATERIALS AND METHODS

Chemicals

M-5011 was synthesized at Maruho Co. (Osaka, Japan). Indomethacin, ketoprofen, suprofen, aspirin, deferoxamine, reduced glutathione (GSH), 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), hypoxanthine (HPX) and superoxide dismutase (SOD) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Labotec Co. (Tokyo, Japan). GSSG and HA were purchased from Wako Pure Chemical Ind. (Osaka). Ferrous sulfate, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and diethylenetriaminepentaacetic acid (DETAPAC) were obtained from Nacalai Tesque (Kyoto, Japan). Catalase and xanthine oxidase (XOD) were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). H_2O_2 was obtained from Mitsubishi-Edogawa Chemical Co. (Tokyo). All other chemicals used were of reagent grade. All solutions, including the buffer, were prepared with Milli-Q water (Milli-Q SP UF or Milli-Q Labo, Millipore, Tokyo).

Degradation of Hyaluronic Acid

M-5011 and the other NSAIDs were dissolved in 100 mM Na_2HPO_4, and the solutions were then adjusted to pH 7.5 with 100 mM NaH_2PO_4 just before use. HA was dissolved in 100 mM sodium phosphate buffer (pH 7.5). The Fenton reaction system containing Fe^{2+}-EDTA and H_2O_2 was used as a ·OH generating system. The reaction mixture contained 0.3% HA, 5.0 μM $FeSO_4$-EDTA, 80 mM sodium phosphate buffer (pH 7.5), 20 mM H_2O_2 and one of various concentrations of the test samples. After incubation at 37°C for 45 min, the degradation was stopped by the addition of DMSO. The degradation of HA was determined by the decrease in viscosity (mPa.s) of a 1.0 ml reaction mixture. The viscosity was measured in a VISCONIC ED viscometer (Tokyo Keiki Co., Tokyo). Each determination was carried out in triplicate.

Spin-trapping of Hydroxyl Radical

M-5011 and the other NSAIDs were dissolved in 50 mM K_2HPO_4, and the solutions were then adjusted to pH 7.6 with 50 mM KH_2PO_4 just before use. The spin-trapping agent used in these experiments was DMPO, which forms secondary radicals (spin adduct) with ·OH. The Fenton reaction system containing $FeSO_4$ and H_2O_2 was used as the ·OH generating system. Spectra recording started 60 sec after the mixing of 0.34 mM of $FeSO_4$, 0.84 mM (or 0.1–10 mM) of DMPO, 11.4 mM potassium phosphate buffer (pH 7.6), 0.68 mM H_2O_2 and one of various concentrations of the test samples was completed. The ESR spectra were recorded on a JES-TE300 spectrometer equipped with an ESPRIT-425 computer system (JEOL Co., Tokyo) using a flat quartz cell (Labotec Co.). Measurements were carried out at

room temperature under the following conditions; magnetic field: 335.8 ± 5.0 mT, microwave power: 8.0 mW, frequency: 9.423 GHz, modulation amplitude: 0.079 mT, sweep time: 2.0 min, response time: 0.1 sec and received gain: ×125. The intensity of the DMPO-OH (DMPO spin adduct of ·OH) signal was measured as a ratio of the signal intensity at the lowest magnetic field to that of manganese oxide used as an internal standard. A TEMPOL solution (1.0 μM) was used for the primary standard of ESR absorption. All measurements were performed in triplicate.

Spin-trapping of Superoxide Anion

M-5011 was dissolved in DMSO before use. The HPX-XOD system was used as a $\cdot O_2^-$ generating system. The spin-trapping agent used in these experiments was DMPO, which forms secondary radicals (spin adduct) with $\cdot O_2^-$. The spectra recording started 60 sec after the mixing of 0.5 mM HPX, 0.96 mM DETAPAC, 1.3 M DMSO, 69 mM DMPO, 15 mM potassium phosphate buffer (pH 7.6), 0.1 U/ml XOD and the test sample was completed. The ESR instrument conditions were the same as those used for measuring the ·OH.

RESULTS

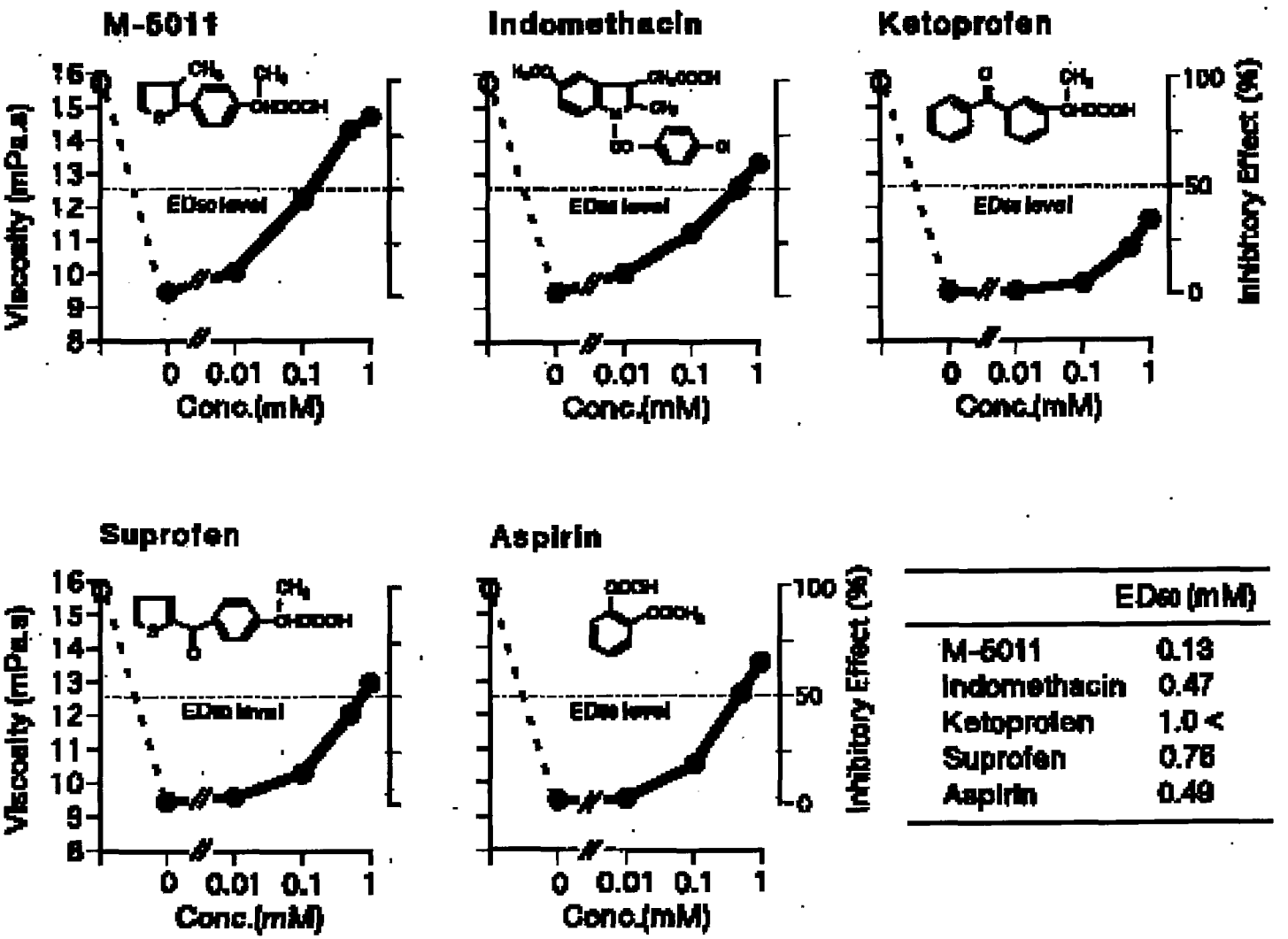
Degradation of Hyaluronic Acid

With the Fenton reaction system, the viscosity of the reaction mixture was reduced to approximately 40% of the initial value (control) at 45 min after the initiation of the ·OH flux (Figure 1). All NSAIDs tested inhibited the reduction of the viscosity of the mixture in a dose-dependent manner. The inhibitory effects of M-5011 and several other NSAIDs on the HA degradation are expressed as the ED_{50} (50% effective dose), and can be summarized as follows; M-5011 > indomethacin = aspirin = suprofen > ketoprofen. The ED_{50} was calculated by the interpolation of a log concentration vs. inhibitory effects (%).

Spin-trapping of Hydroxyl Radical

As shown in Figure 2, the ESR signal of DMPO-OH (DMPO spin-adduct of ·OH), four characteristic signal lines (1:2:2:1), was detected after the addition of H_2O_2 to the reaction mixture containing $FeSO_4$ and DMPO in the potassium phosphate buffer. M-5011 inhibited the DMPO-OH formation in a dose-dependent manner, and the inhibition was almost complete at 1.0 mM. We observed that the other NSAIDs also decreased the signal intensity of DMPO-OH in a dose-dependent manner (Figure 3).

When the decrease of the ESR signal intensity is due to the scavenging of a ·OH by a sample, the inhibition rate should be dependent on the relative concentration of the sample vs. DMPO, because DMPO and the sample compete in the reaction with ·OH. Conversely, when the inhibition is due to the inhibition of the Fenton reaction, the inhibition rate should be dependent on the absolute concentration of the sample and independent of the concentration of DMPO. Therefore, to separate the ·OH-scavenging activities of M-5011 from its direct effects on the Fenton reaction, we employed different concentrations of DMPO (Figure 4). The effects of M-5011 and some inhibitors on DMPO-OH formation were investigated at several concentrations (M-5011 and DMSO: 0.01 mM–1.0 mM, catalase: 0.64 mM–64 mM (10–1000 U/ml), deferoxamine: 1.0 μM–0.1 mM). The molar ratio of the sample (S) to DMPO (D) is expressed as S/D. The direct scavenger of ·OH should have the same regression curves between the inhibition rate of DMPO-OH and S/D if the sample has no inhibitory effect on the ·OH-generating system. Catalase and deferoxamine decreased the signal intensity of DMPO-OH, and the inhibition rate was dependent on the concentration of the samples but independent of the concentration of DMPO, indicating that the decrease of DMPO-OH was due to the inhibition of the Fenton reaction. DMSO, a well-known ·OH



	EDso (mM)
M-5011	0.13
Indomethacin	0.47
Ketoprofen	1.0 <
Suprofen	0.76
Aspirin	0.49

FIGURE 1 Effects of M-5011 and Several NSAIDs on HA Degradation by the Fenton System. Degradation was measured by the decrease in viscosity of the reaction mixture. HA degradation was stopped by the addition of DMSO at 45 min after the H_2O_2 addition. Open circles indicate the initial viscosity of the reaction mixture. Each point represents the mean ± SD (n = 3).

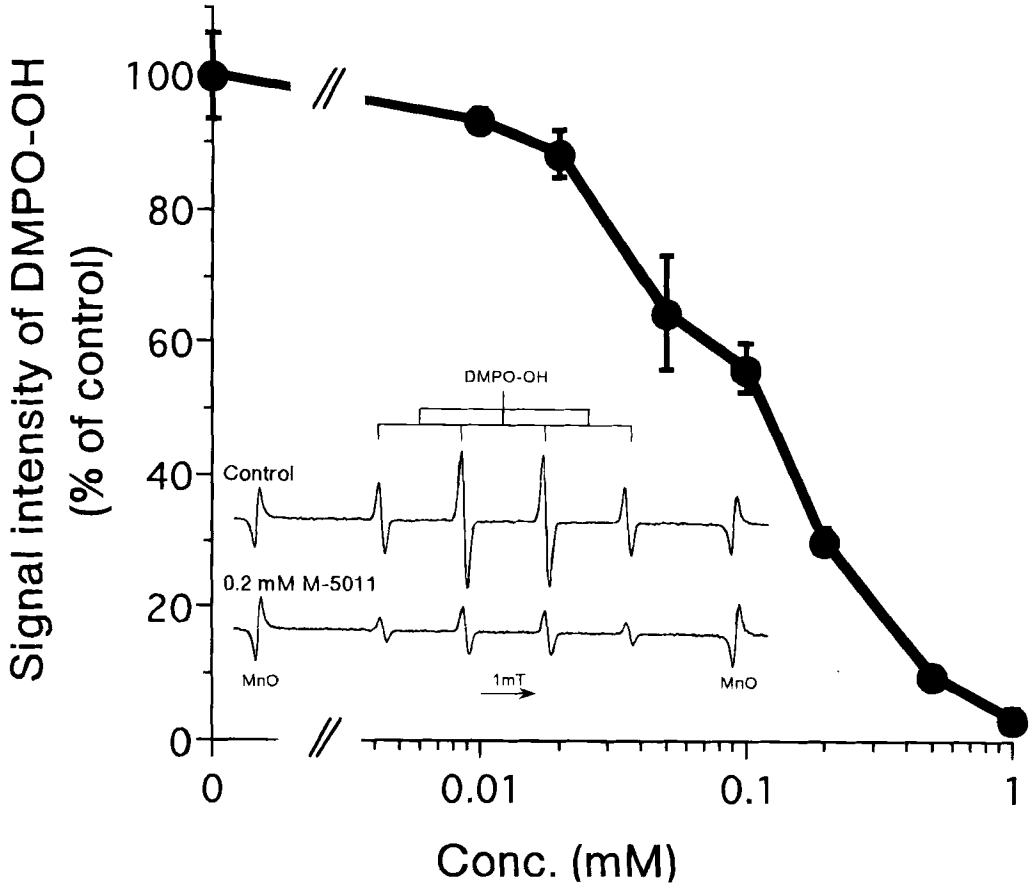


FIGURE 2 Effect of M-5011 on the Formation of the ESR Spin Adducts in the Solution Containing the Fenton System and DMPO. Spectra recorded beginning at 60 sec after the addition of H_2O_2 are shown. The intensity of the first signal of DMPO-OH was corrected as a ratio to the standard signal intensity of the MnO. Each point represents the mean ± SD (n = 3).

scavenger, also reduced the signal intensity of DMPO-OH in the presence of the Fenton reaction system, but the inhibition was dependent on the relative concentration of DMSO vs. DMPO, indicating that the inhibition was due to the competition of DMSO vs. DMPO.[9,17] The inhibitory effect of M-5011 was not due to the inhibition of the Fenton reaction, since the inhibition by the compound was also dependent on the relative concentration of the samples vs. DMPO. M-5011 was the direct scavenger of ·OH, as was DMSO. In contrast, catalase and deferoxamine inhibited the Fenton reaction, as expected. In addition, we confirmed that the NSAIDs indomethacin, ketoprofen, suprofen and aspirin were also direct scavengers of ·OH (data not shown).

We compared the ·OH-scavenging activities of M-5011 and the other NSAIDs with those of the ·OH scavengers such as GSSG. The activities are expressed as the concentration of the complete inhibition of DMPO-OH formation (IC_{100}). The IC_{100} was calculated by the extrapolation of a log concentration vs. inhibition plot. If the IC_{100} value of a compound is small, the scavenging activity is considered to be high. As shown in Table I, the potency of the ·OH-scavenging activities of GSSG and the NSAIDs were in the following order: M-5011 > GSSG = indomethacin > suprofen = ketoprofen > aspirin.

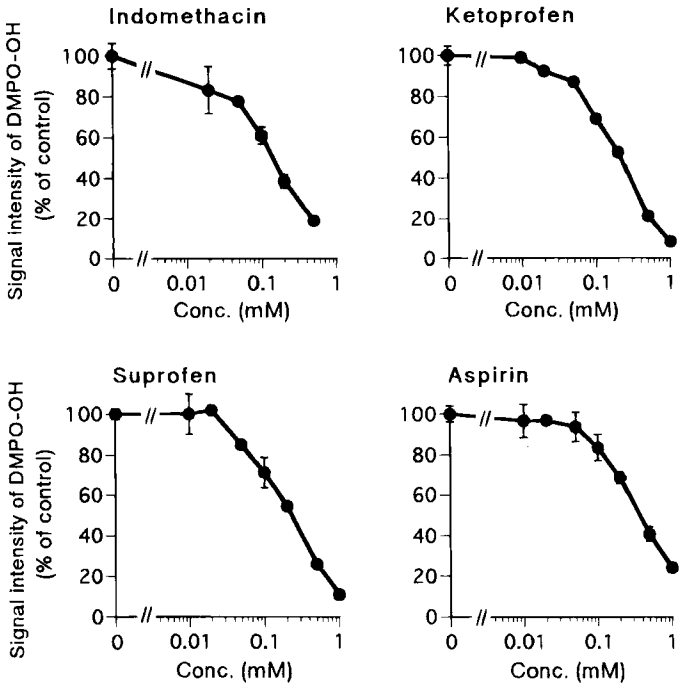


FIGURE 3 Effects of Several NSAIDs on the Relative Intensity of DMPO-OH Spin Adduct Generated from the Fenton System in the Presence of DMPO. The conditions were the same as those described in the legend to Figure 2. Each point represents the mean ± SD (n = 3).

Spin-trapping of Superoxide Anion

The ESR signal of DMPO-OOH (DMPO spin-adduct of $^{\cdot}O_2^-$), twelve characteristic lines, was observed after the addition of XOD to the reaction mixture containing HPX and DMPO in the potassium phosphate buffer (Figure 5). SOD (1.2 U/ml) completely inhibited the DMPO-OOH formation, but M-5011 and GSSG did not inhibit the DMPO-OOH formation.

DISCUSSION

Hydroxyl radical is well known as a very highly reactive oxygen species which reacts rapidly with biological materials, causing oxidative damage.[18,19] Several physiological $^{\cdot}$OH scavengers including ascorbic acid, α-tocopherol and glutathione (GSH) are present in biological systems as part of antioxidative defense mechanisms. Of these scavengers, GSH is an endogenous free radical scavenger, and its biosynthesis is under the regulation of feedback inhibition. Fahim *et al.*[20] showed that the blood GSH content was increased in the acute phase of arthritic inflammation, and its level was decreased during the chronic phase. Therefore, exogenous $^{\cdot}$OH scavengers might be useful for effective scavenging of excessive $^{\cdot}$OH at the site of inflammation. In inflammatory conditions such as RA, leukocytes enter the joints of the body and the oxygen-derived species react with joint components to cause damage. HA, a component of the synovial fluid, is depolymerized by the oxygen-derived species, and the synovial fluid loses its lubricating properties, causing friction in the joint. There are several reports suggesting that $^{\cdot}$OH depolymerizes HA.[21–25] We therefore investigated the inhibitory effects of NSAIDs on HA degradation by $^{\cdot}$OH using viscometry. As expected, the decrease of the viscosity of HA solution was almost completely prevented by DMSO and by catalase (data not shown). All of the NSAIDs tested

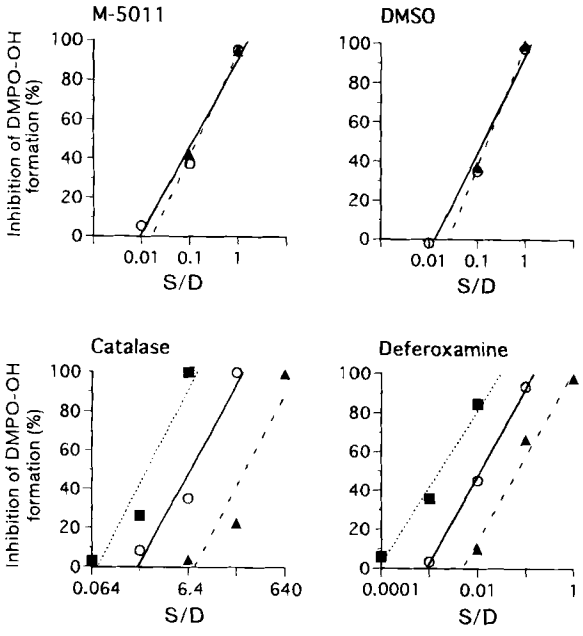


FIGURE 4 Effects of M-5011, DMSO, Catalase and Deferoxamine on the Relative Intensity of DMPO-OH Spin Adduct Generated from the Fenton System in the Presence of DMPO. S/D is the molar ratio of the sample (S) to DMPO (D). Dashed lines with closed triangles, solid lines with open circles and dotted lines with closed squares represent the ·OH-scavenging activities determined at 0.1 mM (▲), 1.0mM (○) and 10mM (■) of DMPO, respectively.
Each point represents the mean value (n = 3).

were also able to inhibit the HA degradation induced by ·OH, indicating their ability to scavenge ·OH (Figure 1). The inhibitory property of M-5011 against HA degradation was stronger than that of the other NSAIDs tested.

We further examined the ·OH-scavenging activities of NSAIDs using the ESR spin-trapping method, which is a definitive method for detecting ·OH. M-5011 and the other NSAIDs inhibited the DMPO-OH formation in the Fenton reaction (Figures 2 and 3), and the inhibition rate was dependent on the relative concentration of the NSAIDs vs. DMPO, indicating that these drugs did not inhibit the Fenton reaction (Figure 4). We used the IC_{100} value as an index of the ·OH-scavenging activity instead of the IC_{50} value, since each NSAID showed a different slope between the inhibition rate of DMPO-OH and the drug concentration. M-5011 was more active in scavenging ·OH than all of the other NSAIDs tested and more active than GSSG, which was used as a control of the ·OH scavenger since GSSG scavenged ·OH more efficiently than did GSH (data not shown). We have confirmed that oral M-5011 (3.0 mg/kg) showed antiinflammatory activity in monosodium urate-induced pleurisy in rats, and that its maximum plasma concentration was 20 μM at 2.0 hr after the administration (unpublished data). The concentration of 20 μM is far below the IC_{100} value (0.68 mM) for the ·OH-scavenging activity of M-5011. However we have no data yet concerning the concentration of M-5011 and the amounts of ·OH at inflammation sites. Since NSAIDs including M-5011 are apparently competitive scavengers of ·OH (Figure 4), the effective concentration of M-5011 to scavenge ·OH would vary with the intracellular amounts of ·OH formed at sites of inflammation; i.e., if the amounts of ·OH were smaller than those in our present system, the concentration of M-5011 needed for ·OH-scavenging would fall below the IC_{100} value. Therefore, we compared the ·OH-scavenging activity of M-5011 with that of GSSG, an endogenous ·OH scavenger.

TABLE I Hydroxyl radical scavenging activities of M-5011 and other NSAIDs

	·OH Scavenging Activity	
	IC_{50} (mM)	IC_{100} (mM)
DMSO	0.12	0.39
GSSG	0.17	0.86
M-5011	0.10	0.68
Indomethacin	0.15	0.88
Ketoprofen	0.17	1.01
Suprofen	0.19	1.01
Aspirin	0.33	2.30

In addition, we examined the effects of M-5011 and GSSG on DMPO-OOH formation, and found that neither M-5011 nor GSSG scavenged $\cdot O_2^-$ (Figure 5).

The various NSAIDs including M-5011 were able to protect HA against degradation by ·OH, since these drugs effectively scavenged ·OH. The ·OH-scavenging activity of aspirin was 2- to

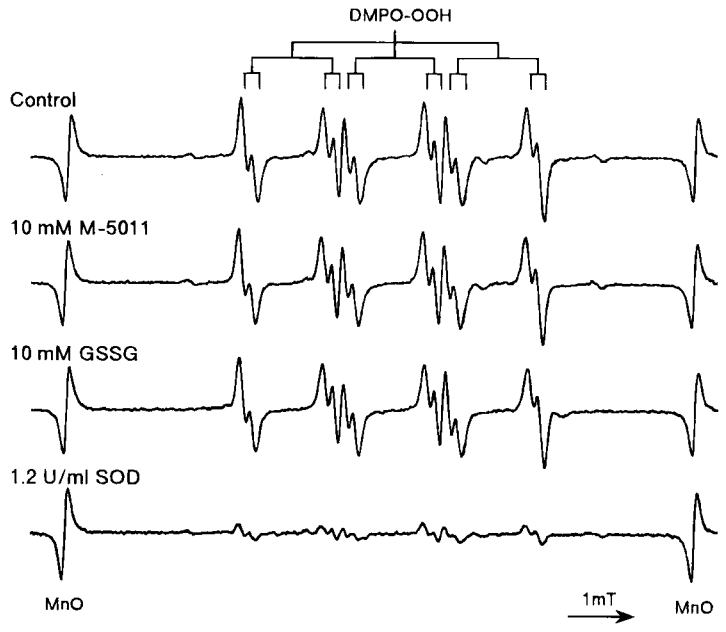


FIGURE 5 ESR Spectra of DMPO-OOH Spin Adducts Formed in the Solution Containing the HPX-XOD System and DMPO. Spectra recorded beginning at 60 sec after the addition of XOD are shown.

3-fold lower than those of the other NSAIDs, including M-5011. The inhibition rate (%) of aspirin against HA degradation at 1.0 mM, however, was nearly the same as those of the other NSAIDs except ketoprofen. Ketoprofen showed a lower protective activity against the HA degradation than those of the other NSAIDs (Figure 1), although the drug inhibited DMPO-OH formation as a direct scavenger of ·OH more potently than did aspirin (Figure 3, Table I). These results suggest that NSAIDs react with ·OH to produce drug-derived radicals[6,12,26,27] which could potentially oxidize molecules, such as protein and lipid, and to cause further tissue damage in vivo. It seems that the drug-derived radical of ketoprofen might be a reactive radical against HA, and the drug-derived radical of aspirin might be less reactive than those of the other NSAIDs.

A possible reaction pathway for the reduction of DMPO-OH is shown in Figure 6. We found that M-5011 inhibited the DMPO-OH production

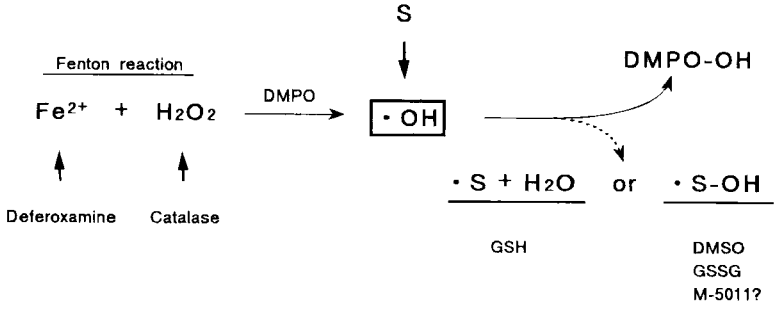


FIGURE 6 A Possible Reaction Pathway for the Inhibition by M-5011 of DMPO-OH Formation by the Fenton System.

with a certain relationship between concentration and scavenging efficacy, as did DMSO, indicating that they had no inhibitory action on the Fenton reaction. Both deferoxamine, an iron chelator, and catalase, an H_2O_2 scavenger, inhibited the iron-catalyzed formation of $^{\cdot}OH$ from H_2O_2, resulting in the decreased formation of DMPO-OH, as expected (Figure 4).

The present results show that M-5011 would act as a $^{\cdot}OH$ scavenger at sites with inflammatory lesions.

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