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Free Radical Scavenging Properties of Sulfinpyrazone

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Sulfinpyrazone, a potent uricosuric drug, was tested *in vitro* for its scavenging action against oxygen free radicals. In this study, sulfinpyrazone was able to scavenge 1,1-diphenyl-2-picrylhydrazyl radical with IC_{50} value of 29.82 μg/ml compared to butylated hydroxytoluene (BHT, IC_{50} value = 20.15 μg/ml) and Trolox (IC_{50} value = 16.01 μg/ml). It was able to scavenge superoxide anion with IC_{50} value of 27.72 μg/ml compared to Trolox (IC_{50} value = 22.08 μg/ml) and ascorbic acid (IC_{50} value = 14.65 μg/ml). The hydroxyl radical scavenging activity of sulfinpyrazone is in a concentration-dependent fashion. In the range of concentrations used, sulfinpyrazone was not a scavenger toward H_2O_2. However, the intracellular H_2O_2-induced 2′,7′-dichlorofluorescin diacetate (DCF-DA) fluorescence in HL-60 cells was significantly reduced by sulfinpyrazone during 30–60 min of incubation. Finally, phorbol-12-myristate-13-acetate induced-lucigenin chemiluminescence in whole blood was markedly inhibited by sulfinpyrazone. Our results suggest a new direction for the pharmacological actions of sulfinpyrazone in free radical scavenging properties.

Keywords: Sulfinpyrazone; Free radicals; Radical scavenging capacity; Uricosuric drug

INTRODUCTION

Gout is an arthritic disorder and/or condition in which deposits of urate are formed in connective tissue (tophi). Hyperuricemia is a biochemical state in which abnormally high serum uric acid levels occurs.[1] Proctor reported that urate promotes the free radical-mediated autooxidation of epinephrine, and free radical mechanisms may play a major role in the etiology of hyperuricemic syndromes such as gouty arthritis and the Lesch-Nyhan Syndrome.[2]

Sulfinpyrazone, a pyrazoline derivative, is a renal tubular reabsorption blocking agent. This drug competitively inhibits active reabsorption of uric acid at the proximal convoluted tubule, thus promoting uric acid excretion and reducing serum urate concentrations. Previous studies pointed out that sulfinpyrazone was a platelet cyclooxygenase inhibitor and elicit its antithrombotic efficacy *in vivo*.[3,4] It can also prevent the development of atherosclerosis by inhibiting platelet adhesiveness in animal model.[5] In a randomized, double-blind, multicenter trial, sulfinpyrazone had been shown to reduce cardiac mortality during the second through the seventh month after myocardial infarction.[6] Whether benefits can be expected after this period is still unknown. The exact mechanism by which this is achieved is unclear. Müller-Peddinghaus *et al.* suggested that the beneficial effect of sulfinpyrazone on experimental infarction might be related to ROS reduction by invaded polymorphonuclear leukoctyes (PMNL).[7]

F-Met-Leu-Phe is chemotactic for leukocytes and promotes cell adhesion, secretion of granule content, production of oxygen radicals and arachidonate metabolites.[8] Sulfinpyrazone has been reported to behave as a functional antagonist of f-Met-Leu-Phe-induced superoxide anion release by neutrophils.[9] Nevertheless, its free radical scavenger properties have not yet been fully addressed.

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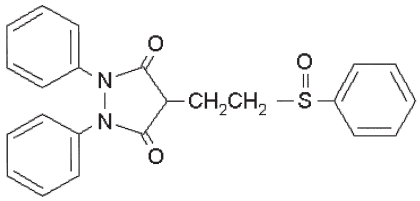

FIGURE 1 Molecular structure of sulfinpyrazone.

In the present paper, sulfinpyrazone was studied for its activity as scavenger of free radicals in different acellular and cellular assay systems.

MATERIALS AND METHODS

Materials

Sulfinpyrazone, a pyrazoline derivative (Fig. 1), was obtained from Taiwan Tung Yang Chemical Ind. Co. Ltd, Taipei. Its chemical purity reaches over 99%. According to the pharmacokinetic reports of sulfinpyrazone, the peak plasma concentrations found in patients after a single 200 mg oral dose of sulfinpyrazone are 10–30 μg/ml, and plasma concentrations remain at 15–20 μg/ml for 1–6 h.[10,11] Mean plasma concentrations of 120–160 μg/ml have been reported in patients receiving 800 mg of oral sulfinpyrazone daily.

All materials of cell culture were obtained from Gibco BRL (Rockville, MD). Trolox was purchased from Aldrich (Milwaukee, WI). 2′,7′-dichlorofluorescin-diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR). All other chemicals were obtained from the Sigma Chemical Company (St Louis, MO).

Stable Free Radical Scavenging Action

Reduction of a stable nitrogen-centered free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), was determined according to Schinella[12] with some modifications. An ethanol DPPH solution (200 μM) was mixed with different concentrations of sulfinpyrazone and the absorbance at 517 nm was determined spectrophotometrically after 10 min. Butylated hydroxytoluene (BHT) and Trolox were used as reference compounds.

Scavenging of the Superoxide Radical Anion Generated by the Phenazine Methosulphate-NADH System

Superoxide anions were generated in a non-enzymatic system, phenazine methosulphate-NADH system, and assayed by the reduction of nitroblue tetrazolium (NBT).[13] Briefly, the reaction mixture contained 10 μM phenazine methosulphate, 78 μM NADH and 25 μM NBT in 0.1 M phosphate buffer (PBS) pH 7.4, with or without sulfinpyrazone. After 2 min of the incubation at room temperature, the absorbance was read at 560 nm against blank samples which contained no phenazine methosulphate. Trolox and ascorbic acid were used as reference compounds. The effect of sulfinpyrazone on the activity of xanthine oxidase was determined by measuring uric acid formation at 295 nm using the previous method.[14]

Scavenging of Hydrogen Peroxide

Assay for H_2O_2 was conducted using horseradish peroxidase-mediated oxidation of phenol red as previously described.[15] The reaction mixture contained 200 μM of phenol red solution, 10 μM of H_2O_2 and different concentrations of sulfinpyrazone. After 5 min incubation at room temperature, the reaction was started by 0.1 unit/ml of horseradish peroxidase and incubated for another 5 min. Finally, the reaction was terminated by adding 10 μl of 1 N NaOH. The purplish complex of oxidized phenol red was quantitated at 610 nm. Trolox and ascorbic acid were used as reference compounds.

Scavenging of Hydroxyl Radical

The hydroxyl radical was generated by incubation for 60 min at 37°C in a reaction mixture containing 20 μM $FeCl_3$, 1.4 mM H_2O_2, 2.8 mM deoxyribose, 2 mM EDTA and 50 μM ascorbate in 1 ml 20 mM KH_2PO_4–K_2HPO_4 buffer, pH 7.4. Deoxyribose degradation by hydroxyl radical was estimated using the thiobarbituric acid method.[16] Mannitol (50 mM) was used as a reference compound.

Scavenging of Intracellular Oxidants Generated by H_2O_2 Stimulated HL-60 Cells

Human promyelocytic leukemia cell line (HL-60) was cultured in RPMI 1640 containing 10% heat-incubated fetal bovine serum and 100 U/ml penicillin–streptomycin. Cells were cultured in a humidified atmospheric incubator at 37°C in 5% CO_2. Cell numbers were counted using a hemocytometer, and cell viability was >98%, as evidenced by trypan blue staining.

A fluorescent probe, DCF-DA, was used to detect production of the free radicals. The fluorescence of these cell-permeable agents significantly increased after oxidation, particularly by reactive oxygen species (ROS).[17] The stock solutions of DCF-DA (50 mM) in DMSO was stored at −20°C. Exponentially growing HL-60 cells (2×10^5 cells/ml) were loaded with 50 μM of DCF-DA at 37°C incubator for

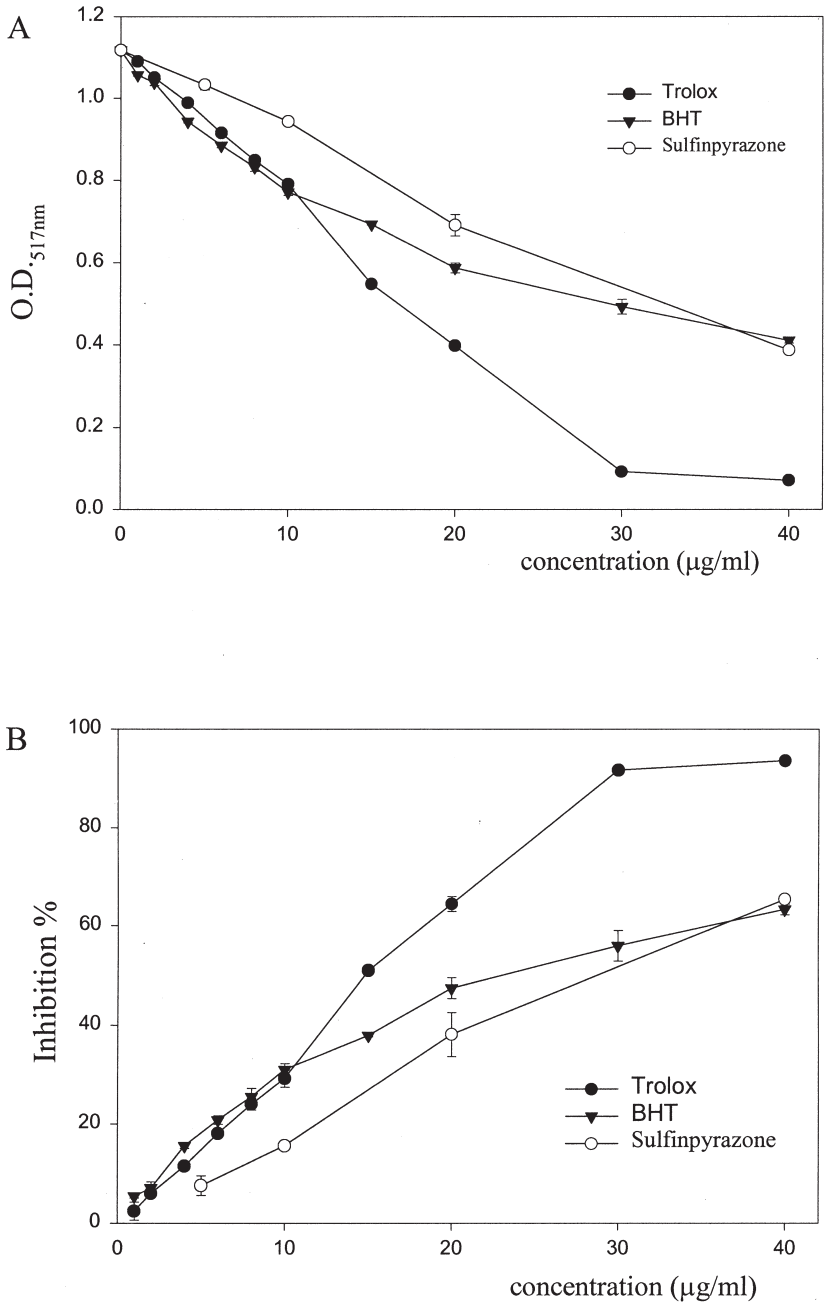

FIGURE 2 DPPH radical scavenging capacity of sulfinpyrazone. A 1 ml aliquot of sulfinpyrazone at various concentrations was mixed with 1 ml of 200 μM DPPH solution (in ethanol) for 10 min incubation and the absorbance at 517 nm was determined (A). Values are expressed as percentage inhibition of DPPH reduction in (B). Each point represents the mean of three determinations with the standard deviation.

1 h. After DCF-DA was removed, the cells were washed with PBS and incubated (or not, for negative controls) with 100 μM H_2O_2 for 1 h. In some assays, cells were incubated for 30 min or 1 h with various concentrations of sulfinpyrazone prior to loading with DCF-DA. Samples were harvested at regular intervals and immediately analyzed (10,000 cells/-sample) by flow cytometry using a 488 nm excitation beam. The mean fluorescence intensity was determined by CELL Quest software (Becton-Dickinson) analysis of the recorded histograms.

Blood Sample Preparation and Chemiluminescence Determination

Whole blood samples were obtained with heparinized plastic syringes in the early morning after 12 h fasting. The tubes of heparinized blood were immediately wrapped in aluminium foil to avoid light exposure and kept in the ice box until testing for superoxide radical anion, which in general was done within 2 h. For each measurement, 0.2 ml of heparinized blood was incubated with 0.1 ml of

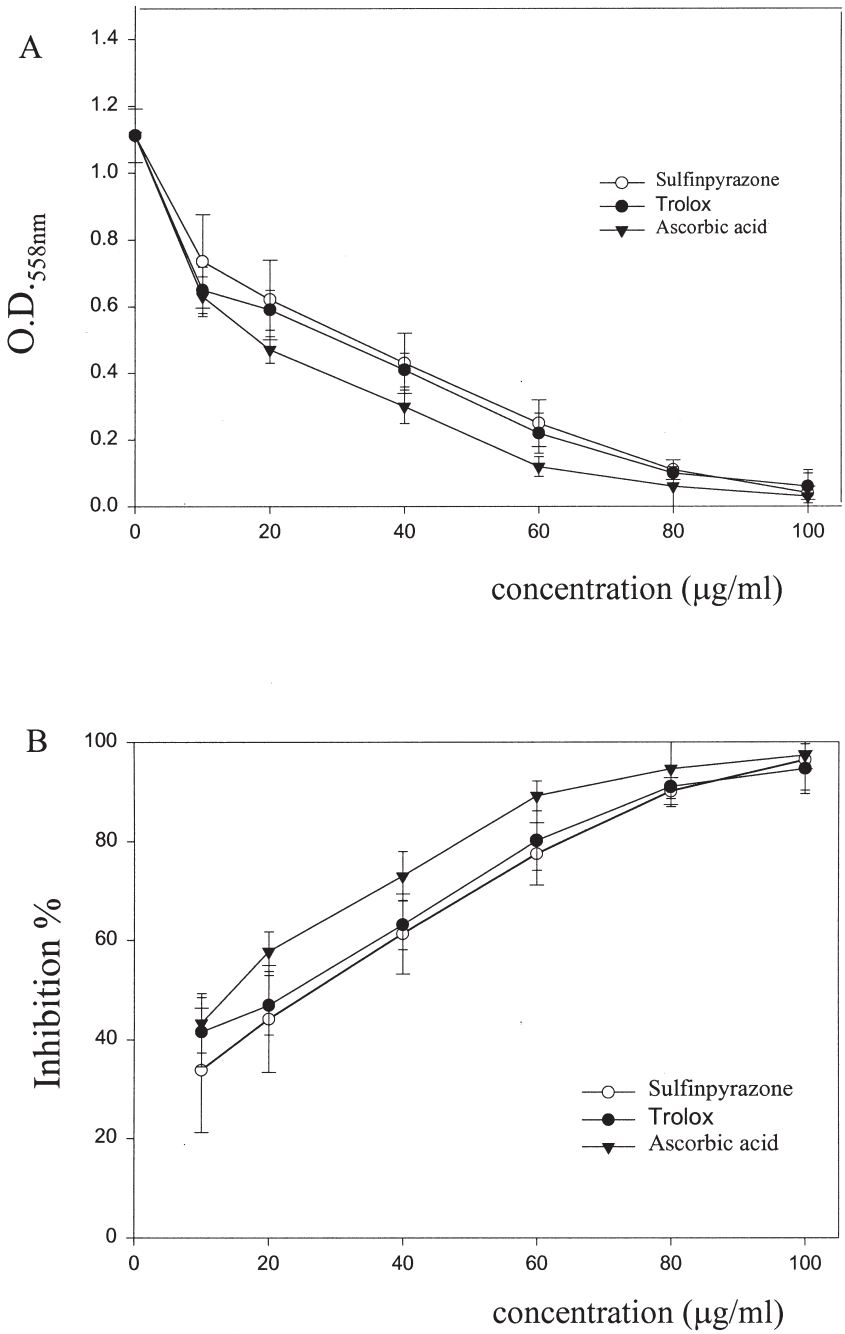

FIGURE 3 The effect of sulfinpyrazone on reduction of nitroblue tetrazolium (NBT) by superoxide anions generated by phenazine methosulphate and NADH. Values are expressed as absorbance at 558 nm in (A) and as percentage inhibition of NBT reduction in (B). Each point represents the mean of three determinations with the standard deviation.

10 μg/ml phorbol-12-myristate-13-acetate (PMA) (or not, for negative control), and then 0.1 ml of PBS buffer (pH 7.4) or various concentrations of sulfinpyrazone were added in a special chamber unit (Model No. TLU-21, Tohoku Electronic Industrial Co.) including a stainless steel cell with magnetic stirrer and stirrer bar, in a totally dark chamber of the Chemiluminescence Analyzing System (Tohoku Electronic Industrial Co., Sendai, Japan). This system contains a photon detector (Model CLD-110), a chemiluminescence counter (Model CLC-10), a water circulator (Model CH-20), and a 32-bit IBM personal computer system. The cooler circulator was connected to the Model CLD-110 photon detector to keep the temperature at 5°C. The Model CLD-110 is extremely sensitive, which can detect radiant energy as weak as 10^{-15} W. Photon emission from whole blood was counted at 10 s intervals at 37°C under atmospheric conditions. After 100 s, 1.0 ml of 0.01 mM lucigenin in PBS was injected into the cell and the chemiluminescence in the blood sample was continuously measured for a total of 300 s.[18]

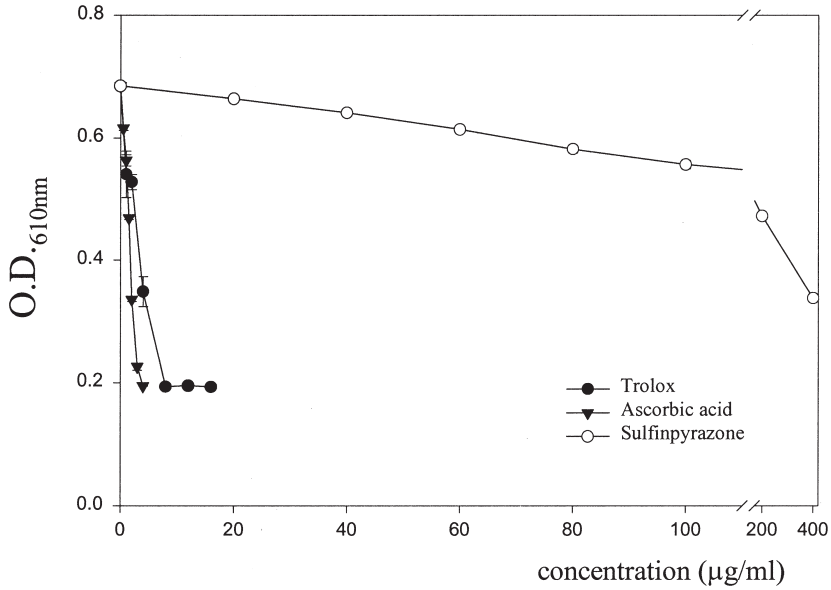

FIGURE 4 Hydrogen peroxide was not scavenged by sulfinpyrazone using horseradish peroxidase-mediated oxidation of phenol red system. Values are expressed as absorbance at 610 nm. Each point represents the mean of three determinations with the standard deviation.

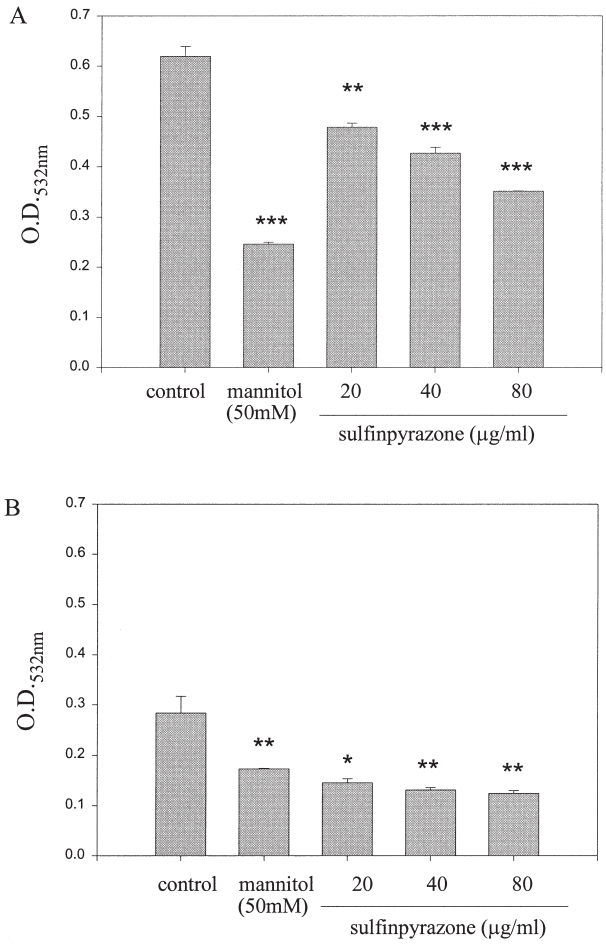

FIGURE 5 Effect of sulfinpyrazone on deoxyribose degradation stimulated with Fe^{3+}–EDTA + H_2O_2 + ascorbate (A), and Fe^{3+}–EDTA + H_2O_2 in the absence of ascorbate (B). Values are expressed as absorbance at 532 nm (mean values ± SD), $n = 3$. *$P < 0.05$, **$P < 0.005$ and ***$P < 0.001$ as compared with the control.

Data Analysis

The experimental data were shown as means ± SD. IC_{50} was calculated from the concentration/effect regression lines. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test.

RESULTS

The DPPH scavenging activities of sulfinpyrazone, BHT and Trolox were all in a dose-dependent manner with IC_{50} values of 29.82 μg/ml ($r^2 = 0.987$), 20.15 μg/ml ($r^2 = 0.972$) and 16.01 μg/ml ($r^2 = 0.996$), respectively (Fig. 2).

The free radical scavenging ability of sulfinpyrazone towards O_2^- was evaluated using a chemical model system. Superoxide anions were generated by a PMS-NADH system and detected by the reduction of NBT. The sulfinpyrazone proved to be an effective scavenger for the superoxide anion with an IC_{50} value of 27.72 μg/ml ($r^2 = 0.994$) (Fig. 3). And the IC_{50} of sulfinpyrazone was similar with the reference compounds: Trolox ($IC_{50} = 22.08$; $r^2 = 0.994$), ascorbic acid ($IC_{50} = 14.65$; $r^2 = 0.986$) as scavenger of superoxide anion. Sulfinpyrazone (up to 100 μg/ml) did not cause direct reduction of NBT since the absorbance of NBT was not changed by sulfinpyrazone (data not shown). Xanthine oxidase, which is one potential source of free radicals, catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid.[19] Thus, the xanthine–xanthine oxidase system is also frequently used as a

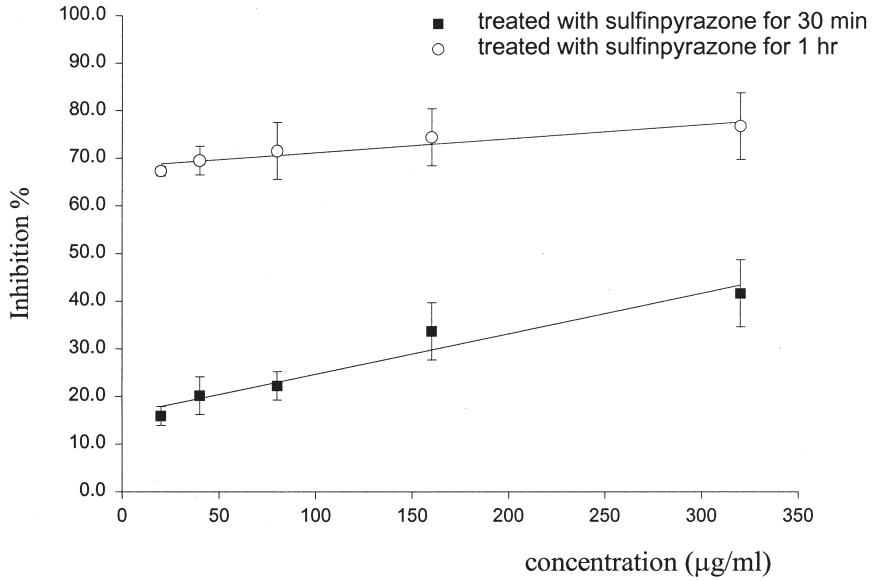

FIGURE 6 Scavenging of H_2O_2-induced intracellular free radicals by sulfinpyrazone in HL-60 cells. After a pre-incubation for 30 min or 1 h with various concentrations of sulfinpyrazone, the HL-60 cells were loaded with DCF-DA, treated for 1 h with 100 μM H_2O_2, and finally analyzed (10,000 events per sample) by flow cytometry. The mean fluorescence intensity values were used to calculate the percentage inhibition of H_2O_2-induced DCF-DA fluorescence. Each point represents the mean of three independent experiments with the standard deviation.

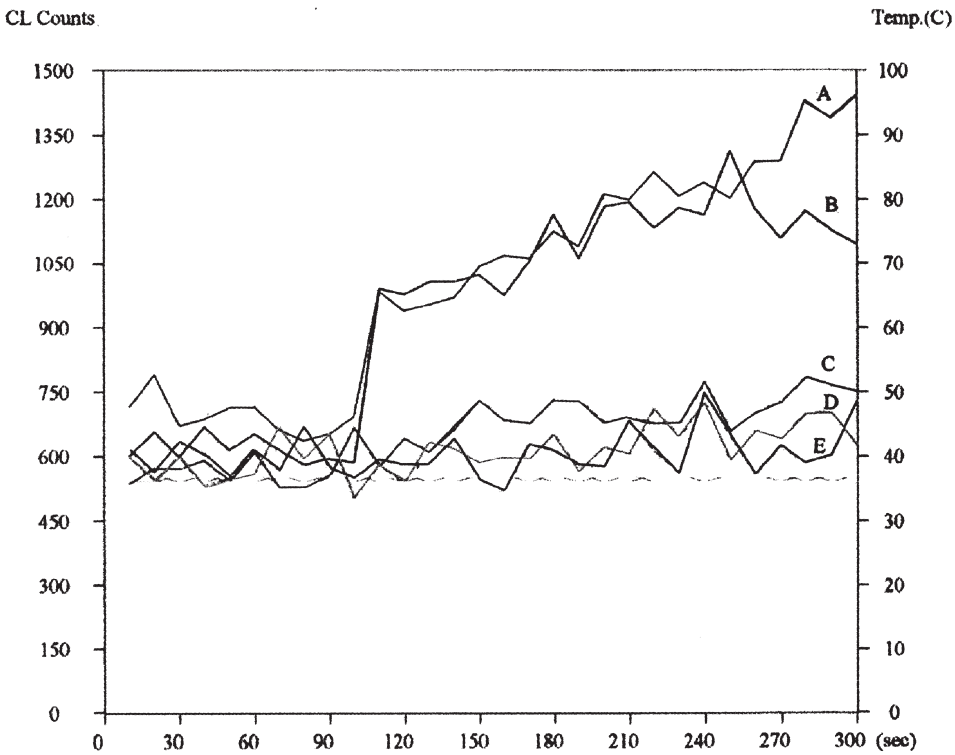

FIGURE 7 Effect of sulfinpyrazone on lucigenin enhanced-chemiluminescence in PMA-activated whole blood cells. Heparinized whole blood sample was incubated with PMA, lucigenin and various concentrations of sulfinpyrazone as described in "Materials and Methods" section. A: actual reading with PMA; B: with PMA and 180 μg/ml sulfinpyrazone; C: with PMA and 360 μg/ml sulfinpyrazone; D: with PMA and 720 μg/ml sulfinpyrazone; E: only whole blood without PMA and sulfinpyrazone. The experiment was repeated three times and a representative data is shown.

generator of superoxide anions. In order to check the inhibitory effect of sulfinpyrazone on xanthine oxidase activity, the enzyme was assayed by measuring the formation of uric acid from xanthine. However, sulfinpyrazone had no inhibitory effect on xanthine oxidase activity up to 200 μg/ml (data not shown).

In our assay system, the IC_{50} values of ascorbic acid and Trolox on scavenging H_2O_2 were 2.20 μg/ml ($r^2 = 0.976$) and 4.89 μg/ml ($r^2 = 0.963$), respectively. And there was no scavenging effect towards H_2O_2 by sulfinpyrazone, even up to 400 μg/ml (Fig. 4).

When ferric-EDTA is incubated with H_2O_2 and ascorbic acid at pH 7.4, hydroxyl radicals are formed in free solution. They can be detected by their ability to degrade the sugar deoxyribose into fragments that, on heating with thiobarbituric acid at low pH, generate a pink chromogen. Figure 5A shows that sulfinpyrazone (20–80 μg/ml) was able to scavenge the hydroxyl radical produced in the Fe^{3+}–EDTA + H_2O_2 system in the presence of ascorbate.

Further, when Fe^{3+}–EDTA is mixed with H_2O_2 in the absence of ascorbate, a slow rate of ·OH generation is noted. Addition of ascorbic acid greatly increases the rate of ·OH generation, presumably by redox cycling iron and maintaining a supply of Fe^{2+}. The ability to reduce Fe^{3+} and stimulate deoxyribose degradation has been adopted as one measure of pro-oxidant properties, and was recently applied to study a variety of antioxidants.[20] Apparently, 20–80 μg/ml of sulfinpyrazone still inhibited the hydroxyl radical-induced deoxyribose degradation in the absence of ascorbic acid (Fig. 5B). Taken together, sulfinpyrazone was able to scavenge DPPH radical, superoxide anion and hydroxyl radical in different acellular assay systems.

We further examined the free radical scavenging effect in cell model. Taking advantage of DCF-DA, which becomes brightly fluorescent after oxidation,[21] we have determined the levels of intracellular free radicals after treatment with 100 μM H_2O_2 in HL-60 cell line. As shown in Fig. 6, the increment of H_2O_2-induced DCF-DA fluorescence was blocked by sulfinpyrazone in dose-dependent and time course-dependent manner.

After adding a stimulant such as PMA, the activated neutrophils, eosinophils, monocyte and macrophages release ROS. Ultra-weak chemiluminescence is widely used in monitoring the formation of ROS. PMA-stimulated lucigenin chemiluminescence was markedly inhibited by sulfinpyrazone (Fig. 7). Lucigenin chemiluminescence was almost completely suppressed by superoxide dismutase (SOD), indicating its specificity for O_2^- (data not shown).

DISCUSSION

Hyperuricemia and hyperlipidemia commonly coexist in clinical practise. Moreover, an increased frequency of coronary artery disease is associated with gout, and hyperuricemia has been suggested as an indicator of coronary risk.[22–26] Although the syndrome is well defined clinically, its basic pathogenesis is still not fully understood. The gouty patients with the multiple risk syndromes are often treated with drug combinations, such as antithrombotic agents for prevention of cardiovascular risk. Recently, the beneficial effects of antioxidant or free radical scavengers are supported by many studies and increasing evidence suggests a decreased risk of several diseases like atherosclerosis, cardiovascular disease, degenerative disease etc.[27,28]

The spins of the electrons in molecular oxygen are parallel, therefore the reduction of oxygen to water must proceed via a series of sequential one-electron transfers, yielding superoxide anion, hydrogen peroxide, and hydroxyl radical as intermediate.[29] In this study, we found that sulfinpyrazone effectively scavenged superoxide anion and hydroxyl radical. Moreover, according to the DPPH assay, sulfinpyrazone acted as a direct free radical scavenger with a potency ($IC_{50} = 29.82$ μg/ml) similar to BHT, a chain-breaking antioxidant. This may explain why sulfinpyrazone is able to interrupt the propagation of an on-going lipid peroxidation of myocardial-membrane phospholipid in the previous report.[30]

Our results showed that sulfinpyrazone can reduce the oxidation of DCFH in H_2O_2-stimulated HL-60 cells. The use of DCFH-DA as a measure of ROS has been widely employed.[17,31] Although the effects of sulfinpyrazone in reducing DCF fluorescence were mainly associated with a decrease in intracellular ROS, we cannot exclude the possibility that sulfinpyrazone can scavenge the DCF semiquinone free radical intermediate (oxygen radical) produced during the formation of the fluorescent product DCF. The possible interaction of fluorescent DCF with intracellular molecules, such as GSH, NADH, or Cu(II) may also reduce the sensitivity of DCFH.[32] Additionally, lucigenin-enhanced chemiluminescence reacts mainly with O_2^-, which is the first primary ROS generated by a phagocyte specific membrane-bound NADPH oxidase. Thus, lucigenin-enhanced chemiluminescence most probably reflects phagocyte activity. In this paper, sulfinpyrazone was able to reduce the cellular release of O_2^-, by using our newly developed assay in whole blood.[18] Therefore, sulfinpyrazone would be expected to prevent injury induced from oxidative stress.

Recently, interest has centered upon the relationship between sulfinpyrazone and multidrug

resistance protein (MRP) because of its inhibitory-organic anion transporter properties.[33,34] And MRP2 is predominantly expressed in apical membrane of the proximal tubules of the kidney. It has been pointed out that the expression of MRP1 can be induced by pro-oxidants and intracellular antioxidant glutathione (GSH) down-regulates MRP1 expression.[35] Our present data suggest that sulfinpyrazone may modulate the MRP gene expression via scavenging intra- and extra-cellular free radicals.

In summary, we have demonstrated that sulfinpyrazone at normal therapeutic plasma concentrations has potent free radical scavenging activity toward ROS, such as O_2^- and ·OH, in acellular and cellular systems. Therefore, we suggest that sulfinpyrazone may benefit gouty patients with other diseases by its uricosuric and free radical scavenging effects.

Acknowledgements

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