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Original Article

Photoproducts of indomethacin exhibit decreased hydroxyl radical scavenging and xanthine oxidase inhibition activities

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ABSTRACT

Indomethacin (IN) is a widely used nonsteroidal anti-inflammatory drug. In this study, four photoproducts of IN (IN1—IN4) were produced and isolated from photoirradiated IN. This study investigated the abilities of IN and its photoproducts to scavenge hydroxyl radicals and inhibit xanthine oxidase (XO). The hydroxyl radical-scavenging activity was measured *in vitro* by electron spin resonance spectrometry using 5,5-dimethyl-1-pyrroline-N-oxide as a spin trapping agent. Enzyme activity was measured by continuous monitoring of uric acid formation, using xanthine as a substrate. The results showed that, among all the related products, IN has the strongest hydroxyl radical-scavenging ($IC_{50} = 65$ μM) and XO inhibitory ($IC_{50} = 86$ μM) effects. To further understand the stereochemistry of the reactions between these IN derivatives and XO, we performed computer-aided molecular modeling. IN was the most potent inhibitor with the most favorable interaction in the reactive site. Various photoproducts exhibited affinity toward XO as a result of the absence of hydrogen bonding with molybdopterin domain.

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1. Introduction

Reactive oxygen species (ROS), including superoxide anion $O_2^{\cdot-}$, hydroxyl radical (HO·), and hydrogen peroxide (H_2O_2), can initiate inflammation, which contributes to a number of pathological processes [1,2]. Xanthine oxidase (XO), an essential enzyme that catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid, is a critical biological source of ROS [3]. An antioxidant with dual roles of ROS-scavenging and XO inhibition activities can be beneficial as a

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protective agent in diseases associated with ROS and XO, such as gout. Indomethacin (IN) has widely been used as a nonsteroidal anti-inflammatory drug (NSAID) because of its cyclooxygenase (COX) inhibitory activity [4]. Treatment with NSAIDs is often accompanied by adverse effects such as gastrointestinal damage and platelet dysfunction [5]. Recent reports have noted the ability of NSAIDs to eliminate and inhibit free radicals [6]. For example, nimesulide and its metabolite 4-hydroxynimesulie have been found to protect cartilage against oxidative stress through their ROS-scavenging activity. IN has demonstrated ROS-scavenging activity [7] but has not been proved effective in XO inhibition.

Photoproducts of NSAIDs may have unexpected effects on biological systems [8,9]. In our previous studies, photoproducts of IN, including γ-lactone (IN1), decarboxylated product (IN2), methyl ester (IN3), and ethyl ester (IN4), were produced through photoirradiated synthesis [10]. IN3 inhibited prostaglandin $(PGE)_2$ and nitric oxide (NO) production, and expressed inducible NO synthase (iNOS) and COX-2 in lypopolysaccharide (LPS)-stimulated macrophage more effectively than IN [11]. However, light energy absorbed by IN may convert to heat, and photolysis may be accompanied by a thermal reaction when the activation energy is reached. Amber or light-resistant containers are commonly used to prevent a photochemical reaction. This study evaluated the HO·-scavenging and XO inhibition activities of IN and its photoproducts. Structure-based molecular modeling was also performed to further understand the stereochemistry of these IN derivatives and XO.

2. Methods

2.1. Chemicals and reagents

IN, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), ferrous sulfate (FeSO4·7H2O), XO (EC 1.2.3.2.), and xanthine were obtained from Sigma Chemical Company (St Louis, MO, USA). Hydrogen peroxide (H_2O_2) was purchased from Acros Chemical Company (Morris Plains, NJ, USA) and EDTA from Merck (Darmstadt, Germany). Methanol and ethanol used in the study were of HPLC grade (JT Baker, Phillipsburg, NJ, USA).

2.2. Photodegradation of IN

An amount of 200 mg of IN was weighed and placed in a 100-mL volumetric flask. Methanol was added to make a concentration of 5.6 mM (2.0 mg/mL). The solution was transferred to a 100-mL quartz sample vial. A Philips 400 W UV lamp was used as a light source (HPA 400/30S UV lamp, Philips, Bruxelles, Belgium). The sample was irradiated for 14 days with the UV lamp mounted horizontally overhead, 30 cm from the sample. IN1–IN3 were isolated from the irradiated sample in methanol and IN4 from the irradiated sample in ethanol [8]. Chemical structures of IN and its photoproducts are shown in Fig. 1 {IN, 1-(4-chloro-benzoyl)-5-methoxy-2-methyl-indol-3-acetic acid; IN1, 8-(4-chloro-benzoyl)-3a-hydroxy-5-methoxy-8a-methyl-2-oxo-2,3,3a,8a-tetrahydro-1H-furo-[2,3-b]-indole; IN2, 1-(4-chloro-benzyl)-5-methoxy-2,3-dimethyl-indol; IN3, [1-(4-chloro-benzoyl)-5-methoxy-2-methyl-indol-3-yl]-acetic
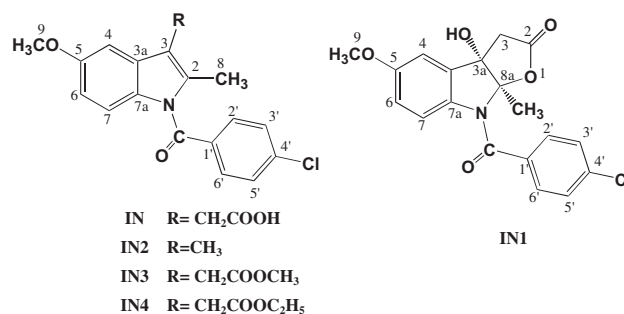

Fig. 1 – Structures of IN and its photoproducts (IN1–IN4). IN = indomethacin.

acid methyl ester; and IN4, [1-(4-chloro-benzoyl)-5-methoxy-2-methyl-indol-3-yl]-acetic acid ethyl ester} [11].

2.3. HO·-scavenging activity

HO·-scavenging activity was measured using the electron spin resonance (ESR) spin-trapping method. The reaction mixture contained 20 μL of 500 mM DMPO, 20 μL of 0.5 mM FeSO4, 20 μL of 1 mM EDTA, and 20 μL of the sample solution. After rapid stirring, the reaction mixture was placed into a capillary ESR tube. Recording of the ESR spectrum was started 40 seconds after the addition of 20 μL of 1 mM H2O2. Deionized water was used instead of the sample solution for control experiments. Intensities of the DMPO–OH spin signal (secondary peak) in electron paramagnetic resonance (EPR) spectrometry were used to evaluate the scavenging activity of the isolated compounds. HO·-scavenging activity was calculated according to the following formula:

$$\text{Scavenging activity}(\%) = \left(1 - I_{\text{sample}}/I_{\text{control}}\right) \times 100 \qquad (1)$$

where I_{control} and I_{sample} represent the intensity of the signal in the absence and presence of the sample, respectively.

2.4. ESR spectrometry

The DMPO–OH spin adduct was detected by ESR spectroscopy at room temperature using a Bruker EMX-6/1 ESR spectrometer equipped with Bruker WIN-EPR Sim-Fonia program (Billerica, MA). ESR measurement conditions were as follows: magnetic field, 347.5 ± 5.0 mT; microwave power, 2.0 mW; modulation frequency, 100 kHz; modulation amplitude, 5 G; and time constant, 0.6 seconds [12].

2.5. XO activity assay

Enzyme activity was determined spectrophotometrically by measuring uric acid formation continuously at 295 nm, using xanthine as a substrate. The XO assay consisted of a reaction mixture containing 20 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 38 μM EDTA, 3 u/L XO, and 50 μM xanthine. The assay was started by adding the enzyme to the reaction mixture, with or without inhibitors. The assay mixture was incubated for 3 minutes at 37 °C, and absorbance was noted every 5 seconds. Data obtained for the enzyme assays were plotted using Microsoft Office Excel 2010 [13].

2.6. Modeling of 3D computational docking

The X-ray crystal structure of bovine XO was retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb, PDB code 3NVY), and the C-chain of this protein was used for docking studies of IN and its photoproducts. Small molecules were removed from the C-chain, hydrogen atoms were added, and the resultant protein structure was used in the docking simulation. Three-dimensional structures of compounds were built and optimized by energy minimization using the MM2 force field and a minimum RMS gradient of 0.05 in the software Chem3D 6.0 (CambridgeSoft Corp., Cambridge, MA, USA). Docking simulation was performed using the GOLD 5.0 program on an HP xw6600 workstation with Intel Xeon E5450/ 3.0 GHz Quadcores as the processors. The GOLD program utilizes a genetic algorithm (GA) to perform flexible ligand docking simulations. In the present study, for each of the 30 independent GA runs, a maximum number of 100,000 GA operations were performed on a single population of 100 individuals. Operator weights for crossover, mutation, and migration were set to be 95, 95, and 10, respectively. The GoldScore fitness function was applied for scoring the docking poses of compounds. The docking region was defined to encompass the active site of XO. The best docking solution (with the highest GOLD fitness score) for a compound was chosen to represent the predicted binding mode to the active site of XO [14].

3. Results

Among the oxygen radicals, HO· is most reactive and damages adjacent biomolecules severely. We investigated the HO·-scavenging abilities of the four photoproducts (IN1–IN4) using ESR spectrometry. HO·, which was generated by the Fenton reaction, exhibited a four-line ESR spectrum (DMPO-OH adducts). Fig. 2A shows the HO· suppression activity of the isolated photoproducts IN1–IN4 at a concentration of 100 μM. IN1 showed comparable activity to IN, whereas IN2–IN4 did not exhibit substantial suppressive activity in this assay. Formation of the DMPO–OH spin adduct was inhibited significantly by 150 μM IN and completely by 250 μM IN. IN and its photoproducts demonstrated HO·-scavenging activity in a dose-dependent manner (Fig. 2B). The results are shown in terms of the inhibition scavenging activity (%). IC_{50} values were determined from the regression line obtained using the least-squares method, as shown in Table 1. IN had a stronger HO·-scavenging effect, with $IC_{50} = 65$ μM, than its photoproducts. IC_{50} values of IN1, IN3, and IN4 were 84, 110, and 120 μM, respectively. IN2 did not display a significant inhibitory activity against HO· ($IC_{50} > 500$ μM; Fig. 2C).

Table 1 shows the IC_{50} values for XO inhibition by IN and its photoproducts. IN had the most potent inhibitory effect on XO, with $IC_{50} = 86$ μM. IC_{50} values of IN2 and IN3 were 93.3 μM and 88.8 μM, respectively; however, IN1 and IN4 did not exhibit a significant inhibitory effect ($IC_{50} > 100$ μM). All the photoproducts exhibited a weaker inhibitory effect than IN.

To provide a further insight into the inhibitory effects of IN on XO and to explain the structure–activity relationships, a
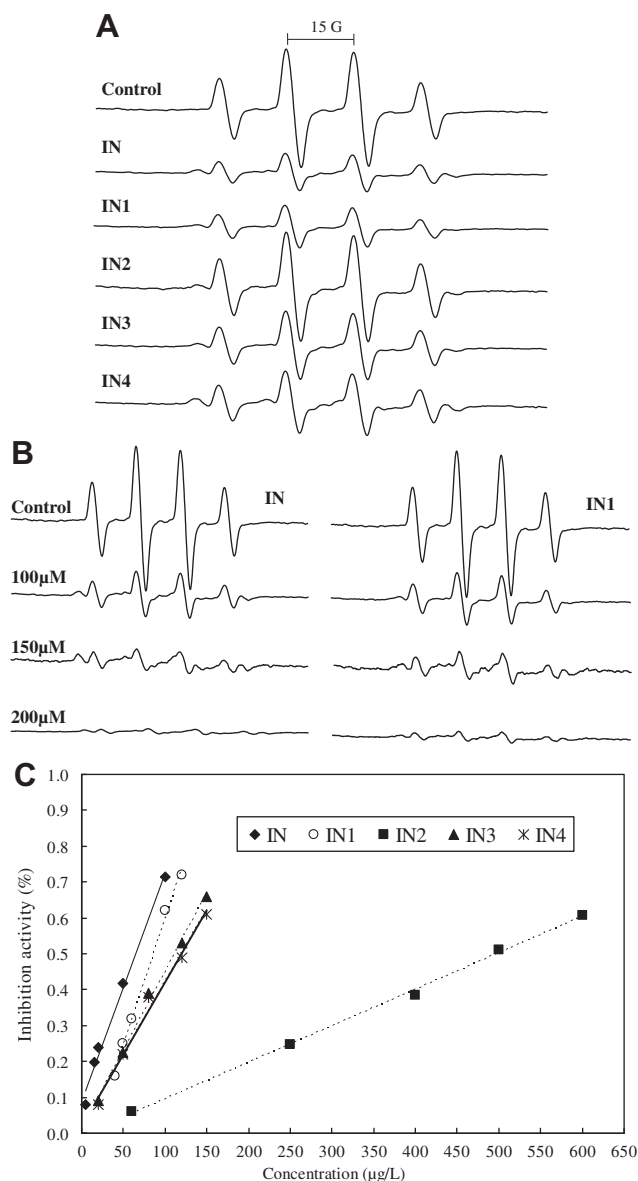

Fig. 2 – (A) Scavenging activity of IN and its photoproducts against HO·. Equal amounts of IN and the photoproducts IN1–IN4 (100 μM) were used in the Fenton reaction for ESR spectrometry. (B) ESR spectra of DMPO–OH adduct obtained from the Fenton reaction with IN and IN1 at various concentrations (0–200 μM). (C) Inhibitory activities of IN and its photoproducts against the Fenton reaction generated hydroxyl radicals. DMPO = 5,5-dimethyl-1-pyrroline-N-oxide; ESR = electron spin resonance; IN = indomethacin.

molecular model was created for IN docking on XO (Fig. 3). We focused on IN dockings in the molybdopterin domain of XO. In the co-crystal structure of salicylate–XO [15], both carboxylated atoms of salicylate were close to the guanidinium group of Arg880, to the hydroxyl side chain of Thr1010, and to Glu1261 via water. The aromatic inhibitor was aligned parallel to the ring of Phe914, whereas the phenyl group of Phe 1009 was aligned perpendicular to the ring of the inhibitor or

Table 1 – Hydroxyl radical-scavenging and XO inhibition activities of IN and its photoproducts.

Compounds	Scavenging activity (IC$_{50}$, μM)	XO inhibition activity (IC$_{50}$, μM)
IN	65	86
IN1	84	>100
IN2	500	93.3
IN3	110	88.8
IN4	120	>100

IN = indomethacin; XO = xanthine oxidase.

substrate. Molecular docking of IN and its photoproducts in the active site of XO was performed using the docking program GOLD 5.0 and the crystal structure of bovine XO (PDB code 3NVY). IN docking in the molybdopterin domain of XO displayed a similar position and interaction to salicylate binding. The *p*-chlorobenzoyl of IN had a stacking force with Phe 914, and an indole ring stretched to the space surrounded by several residues including Phe1009, Val1011, Leu648, Leu873, and Leu1014. The carboxylate group of IN can interact with Arg880 by forming a salt bridge that is a combination of electrostatic interactions and hydrogen bonding (Fig. 3A). The binding energy of IN was also proved to be more potent in XO docking than other photoproducts (Fig. 3B), which is consistent with the results of enzyme activity. The photoproducts might have shown less affinity for XO than IN because of the absence of salt bridge between IN and the molybdopterin domain.

4. Discussion

NSAIDs are the principal therapeutic agents used to treat inflammatory disorders and inhibit COX, which catalyzes the formation of prostaglandin precursors from arachidonic acid
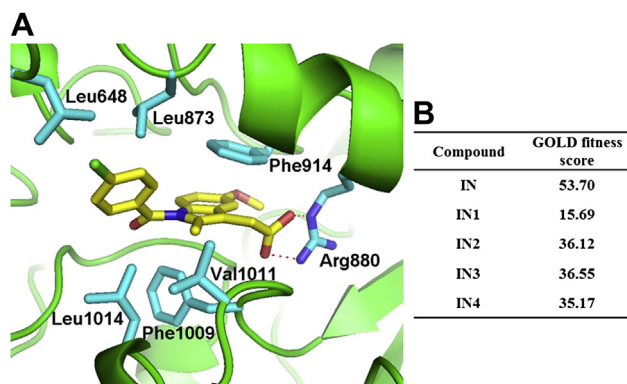

Fig. 3 – (A) Molecular model of indomethacin binding to the active site of xanthine oxidase. Protein structure is represented as a ribbon. IN (yellow) and some interacting amino acid residues (cyan) are shown as sticks. Hydrogen atoms are omitted for clarity. The red broken lines indicate salt bridge interactions. (B) GOLD fitness scores of IN and its photoproducts were obtained using the GOLD 5.0 program. IN = indomethacin.

[16]. ROS has been demonstrated to be a mediator of inflammation [17]. Thus, drugs with inflammatory activity commonly display ROS-eradicating activity, typically by downregulating the production of tumor necrosis factor-α and iNOS or by scavenging ROS directly. Ikeda et al [6] demonstrated the superoxide-scavenging activity of IN, and Costa et al [7] reported the scavenging activity of H_2O_2. The results of our study revealed that IN demonstrates HO·-scavenging activity (Fig. 2, Table 1). Other reactive species, including peroxyl radical (ROO·), HO·, $O_2^{·-}$, H_2O_2, and HOCl, as well as reactive nitrogen species, nitric oxide (NO), and peroxynitrite anion (ONOO$^-$), also play vital roles in the inflammatory process and pathophysiologies [18]. IN counteracts oxidative stress, which accounts for its enhanced efficacy in anti-inflammation.

XO produces uric acid and ROS during purine catabolism [3]. An excess of uric acid can lead to gout, which is responsible for oxidative damage to living tissues. Currently, NSAIDs are the preferred treatment for acute gout attacks, given their effects on the suppression of inflammation coupled with their analgesic properties. Among these drugs, IN is considered to be most potent [19–21]. The main objectives of acute gout therapy are rapid and safe resolution of pain and restoration of functional ability. In the treatment of chronic gout, XO inhibitors, such as allopurinol, are most effective in reducing serum urate in patients who are overproducers or underexcretors of uric acid [22–24]. Experimental data indicated that IN exhibited an inhibitory effect on XO (Table 1). Furthermore, computer-aided molecular modeling provided an insight into this observation. This finding implies that NSAIDs may have polyfunctional curative effects in gout treatment, including those of anti-inflammation, antioxidation, and XO inhibition. The photoproducts exhibited less potent activity than the parental IN. This suggests that IN should not be exposed to light during storage to avoid its photodegradation, because its photoproducts exhibit reduced efficacy.

In conclusion, this study was performed to clarify the beneficial effects of IN and its photoproducts in inflammatory diseases where ROS and XO are pathogenically involved. Our results indicate that the anti-inflammatory and analgesic agent IN is an HO· scavenger. IN also binds to the active site of XO to block its catalytic activity and results in reduced ROS production. Therefore, IN partially accounts for the reduced anti-inflammatory effects of various photoproducts.

Acknowledgments

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