1H NMR-based metabolic profiling of naproxen-induced toxicity in rats

Jeeyoun Jung \textsuperscript{a,b}, Minhwae Park\textsuperscript{a}, Hye Jin Park\textsuperscript{c}, Sun Bo Shim\textsuperscript{c}, Yang Ha Cho\textsuperscript{c}, Jinho Kim\textsuperscript{c}, Ho-Sub Lee\textsuperscript{b}, Do Hyun Ryu\textsuperscript{d}, Donwoong Choi\textsuperscript{c,e,*}, Geum-Sook Hwang\textsuperscript{a,e,**}

\textsuperscript{a} Korea Basic Science Institute, Seoul 136-701, Republic of Korea
\textsuperscript{b} Department of Physiology, College of Oriental Medicine, Wonkwang University, Iksan 540-749, Republic of Korea
\textsuperscript{c} National Institute of Food and Drug Safety Evaluation, Seoul, 122-704, Republic of Korea
\textsuperscript{d} Department of Chemistry, Sungkyunkwan University, Suwon 440-746, Republic of Korea
\textsuperscript{e} Graduate School of Analytical Science and Technology, Chungnam University, Daejeon, 305-764, Republic of Korea

\section*{A B S T R A C T}

The dose-dependent perturbations in urinary metabolite concentrations caused by naproxen toxicity were investigated using 1H NMR spectroscopy coupled with multivariate statistical analysis. Histopathologic evaluation of naproxen-induced acute gastrointestinal damage in rats demonstrated a significant dose-dependent effect. Furthermore, principal component analysis (PCA) of 1H NMR from rat urine revealed a dose-dependent metabolic shift between the vehicle-treated control rats and rats treated with low-dose (10 mg/kg body weight), moderate-dose (50 mg/kg), and high-dose (100 mg/kg) naproxen, coinciding with their gastric damage scores after naproxen administration. The resultant metabolic profiles demonstrate that the naproxen-induced gastric damage exhibited energy metabolism perturbations that elevated their urinary levels of citrate, cis-aconitate, creatine, and creatine phosphate. In addition, naproxen administration decreased choline level and increased betaine level, indicating that it depleted the main protective constituent of the gastric mucosa. Moreover, naproxen stimulated the decomposition of tryptophan into kynurenate, which inhibits fibroblast growth factor-1 and delays ulcer healing. These findings demonstrate that 1H NMR-based urinary metabolic profiling can facilitate noninvasive and rapid diagnosis of drug side effects and is suitable for elucidating possible biological pathways perturbed by drug toxicity.

\section*{1. Introduction}

Naproxen is a nonsteroidal anti-inflammatory drug (NSAID) commonly used to reduce fever and pain associated with acute or chronic diseases and inflammation. However, naproxen-induced adverse effects on the gastrointestinal (GI) tract are common; of patients receiving regular NSAID therapy, approximately 40–60% develops gastric erosions, and approximately 15–30% develops full-fledged gastric ulcers (Jaszewski, 1990; Larkai et al., 1987; Silvoso et al., 1979).

The pathogenesis of naproxen-induced damage to the GI tract is incompletely understood, but the depletion of endogenous prostaglandins by naproxen has been clearly documented (Vane, 1971; Whittle, 1981). Most NSAIDs inhibit not only cyclooxygenase (COX)-2, which produces prostaglandins associated with pain induction, but also COX-1, which produces prostaglandins involved in maintaining the integrity of gastrointestinal mucosa. Therefore, as shown by a large cohort study (Bombardier et al., 2000), COX-2-selective NSAIDs are much less likely than non-selective NSAIDS (such as naproxen) to produce adverse GI events. However, the pathogenesis of GI damage by NSAIDs is complicated, followed by a multistage pathogenic event in which intestinal permeability, reactive oxygen species (ROS), gastric motility, luminal contents, neutrophils, and the microcirculation play a role in the development of inflammation and ulcers. (Rainsford and Whitehouse, 1980; Somasundaram et al., 1995; Wallace, 1994; Whittle, 1992).

In addition, endogenous metabolites are important factors in cellular regulatory and metabolic processes that respond to environmental, pathogenic, and toxicological insults (Goodacre et al., 2004; Nicholson et al., 2002), but few studies of the endogenous metabolic changes caused by NSAIDs have been reported. However, metabolomics, the multi-targeted analysis of endogenous metabolites from biological samples, is increasingly being used as a noninvasive method for the supervision of pathophysiologic processes and toxicity assessment. (Hwang et al., 2009; Nicholson et al., 1999). Um et al. (2009) also reported that a metabolomics approach...
could be used with surrogate biomarkers to predict the adverse effects of NSAIDs.

The present study illustrates the use of a metabolomic approach to understanding biological processes related to metabolite perturbations. Specifically, we used $^1$H NMR spectroscopy-based profiling of urinary metabolites to investigate the dose-dependent effects of naproxen on metabolism and GI integrity in rats. We also examined how these metabolic effects of naproxen might relate to the mechanism underlying its toxicity.

2. Materials and methods

2.1. Animals and treatment

Male Sprague–Dawley (SD) rats (body weight: 50–300 g) were kept in an animal facility accredited by the Korea Food and Drug Administration (KFDA, Unit No. 000996; Seoul, Korea) in accordance with the International Animal Care Policies of the Association for Assessment and Accreditation of Laboratory Animal Care. All animals were provided ad libitum with a standard irradiated Chow diet (Purina Mills Inc., Seoul, Korea) and water. Upon commencement of the study, the rats were housed in a specified pathogen-free state at 23 ± 1 °C at a relative humidity of 50 ± 10% under a 12 h light/12-h dark cycle. Before dosing, the rats were fasted overnight and transferred to metabolic cages designed specifically for separate collection of urine and feces. Each rat then received an oral dose of vehicle (0.5% Tween-80 in autoclaved tap water) or naproxen (10, 50, or 100 mg/kg body weight; Sigma, USA). There were 10 rats in the control group and in each dosage group. The experimental protocol was approved by the Institutional Animal Care and Use Committees of KFDA.

2.2. Sample collection

After drug administration, urine samples were collected from the rat cages during the 7 h period, then immediately aliquoted and stored at −74 °C until analysis. The animals were euthanized with CO$_2$, and their stomachs were removed and scored for hemorrhagic damage. Scoring was performed by considering the size and depth of all the lesions, and the value was added to give an overall gastric damage score for each rat (Kim et al., 1998). All processes relating to the animals were performed as outlined in A Good Practice Guide to the Administration of Substances and Removal of Blood Including Routes and Volumes (Diehl et al., 2001).

2.3. $^1$H NMR experiments

Urine samples were centrifuged (20 min at 15,000 g) to remove any solid debris. Aliquots (200 µl) of the supernatant fractions were removed to microcentrifuge tubes containing 60 µl of 5 mM DSS (3-[trimethylsilyl]-1-propanesulfonic acid sodium salt) in D$_2$O and 340 µl of 0.2 M sodium phosphate buffer (pH 7.0) containing 0.0185% NaN$_3$. After mixing, samples were adjusted to pH 7.0 and analyzed by $^1$H NMR spectroscopy on a 600-MHz Varian NMR System (Varian Inc., Palo Alto, CA, USA).

Solvent suppression of residual water signals was achieved via the NOESYPRE-SAT pulse sequence, in which the residual water peak is irradiated during the relaxation delay (1.5 s) and during the mixing time (0.1 s). For each sample, $^1$H NMR spectra were collected using 64 scans containing 67,568 data points at a spectral width of 8445.9 Hz with an acquisition time of 4.0 s and a relaxation delay of 2.0 s.

2.4. Data analysis

The $^1$H NMR spectra were phased and baseline-corrected using Chenomx NMR Suite 5.1 (Chenomx, Inc., Edmonton, Canada). Spectrum was referenced to DSS. Signal intensities in each spectrum were calculated by integrating 0.005 ppm sections. After the spectral regions containing the water and urea peaks (4.66–5.04 and 5.45–6.30 ppm, respectively) were removed, the spectra from 0.58–10.45 ppm were acquired as shown in Table 1. Among these metabolites, kynurenate, pantothenate, alanine, and glycine, whereas those in naproxen-treated rats indicate that naproxen toxicity induced metabolic changes in rats. The dominant metabolites in vehicle-treated rats were N-isovalerylglutamic acid, 2-oxoglutarate, 3-hydroxybutyrate, TMAO/betaine, creatine, creatine phosphate, phenylacetilglutamine, glycine, creatine, creatine-phosphate, phencylcytglycine, hippurate, adipate, TMAO/betaine, taurine, and kynurenate, in the samples.

The spectral data from the urine of vehicle- and naproxen-treated rats indicate that naproxen toxicity induced metabolic alterations in the rats. The dominant metabolites in vehicle-treated rats were N-isovalerylglutamic acid, 2-oxoglutarate, 3-hydroxybutyrate, kynurenate, creatine, creatine phosphate, phenylacetilglutamine, glycine, creatine, creatine-phosphate, phencylcytglycine, hippurate, adipate, TMAO/betaine, taurine, and kynurenate, in the samples.

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Fig. 1. Changes in histopathology of gastric lesions (A–D) and gastric damage scores (E) after naproxen administration in rats. (A) Vehicle-treated rats. (B) Naproxen-treated rats (low dose: 10 mg/kg body weight). (C) Naproxen-treated rats (moderate dose: 50 mg/kg). (D) Naproxen-treated rats (high dose: 100 mg/kg). *P < 0.05, tested by one-way ANOVA test and Tukey’s multiple comparison test. (E) Columns represent means ± S.E.M.

Notably, levels of citrate, cis-aconitate and kynurenate rose in response to naproxen treatment in a dose-dependent manner, and the levels of creatine phosphate, choline, citrate, cis-aconitate, and kynurenate varied significantly between the vehicle-treated and high-dose naproxen-treated groups.

4. Discussion

4.1. Naproxen-induced disturbances in energy and choline metabolism

To examine the mechanism by which naproxen induced gastric damage, we used ¹H NMR-based metabolomics to identify dose-dependent changes in urinary metabolic profiles following naproxen treatment. Results indicated that levels of citrate and cis-aconitate were significantly elevated, in a dose-dependent manner, following naproxen dosing. Because these two compounds are intermediates in the citric acid cycle, these changes may be indicative of altered energy metabolism. Additionally, the levels of phosphocreatine and creatine were significantly higher in naproxen-dosed groups than in the vehicle-treated group. Because these two compounds act as emergency energy regulators (Morvan and Demidem, 2007), increased levels may suggest competition for ATP availability in response to the naproxen-induced changes in levels of citrate and cis-aconitate.

NSAID administration has been reported to decrease mitochondrial function of jejenum tissue in a pro-inflammatory state...
Fig. 2. Representative 600-MHz $^1$H NMR spectra of urine from rats treated with vehicle (A) or naproxen at a low (B), moderate (C), or high (D) dose.

Fig. 3. PCA score plot (A) and mean score trajectory (B) of urinary $^1$H NMR spectra obtained from rats treated with vehicle (violet circles), low-dose (10 mg/kg) naproxen (green diamonds), moderate-dose (50 mg/kg) naproxen (blue squares), and high-dose (100 mg/kg) naproxen (red triangles). Metabolic differences between the groups are clearly evident. $R^2 = 0.591; Q^2 = 0.35$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Concentrations of metabolites (normalized to creatinine) in rat urine observed after administration of vehicle or low-dose (10 mg/kg), moderate-dose (50 mg/kg), or high-dose (100 mg/kg) naproxen.

Table 1

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Chemical shift (ppm) and multiplicity</th>
<th>Vehicle Low dose</th>
<th>Middle dose</th>
<th>High dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-isovaleroylglycine</td>
<td>δ 0.92(d), 1.39(m), 2.16(d), 3.76(d), 7.96(br)</td>
<td>0.06 ± 0.024</td>
<td>0.061 ± 0.013</td>
<td>0.066 ± 0.014</td>
</tr>
<tr>
<td>2-Hydroxyisobutyrate</td>
<td>δ 1.35(s)</td>
<td>0.01 ± 0.001</td>
<td>0.0064 ± 0.0017</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>Putrescine</td>
<td>δ 1.75(m), 3.04(t)</td>
<td>0.066 ± 0.027</td>
<td>0.061 ± 0.016</td>
<td>0.074 ± 0.028</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>δ 2.43(t), 3.03(t)</td>
<td>0.14 ± 0.16</td>
<td>0.15 ± 0.1</td>
<td>0.31 ± 0.29</td>
</tr>
<tr>
<td>Kynurenate*</td>
<td>δ 3.71(d), 4.15(t), 6.80(t), 7.39-7.44(m), 7.94(d)</td>
<td>0.017 ± 0.001</td>
<td>0.064 ± 0.01</td>
<td>0.093 ± 0.03</td>
</tr>
<tr>
<td>1-Methylisocitramide</td>
<td>δ 4.47(s), 8.17(t), 8.88(d), 8.95(d), 9.26(s)</td>
<td>0.16 ± 0.046</td>
<td>0.17 ± 0.028</td>
<td>0.20 ± 0.069</td>
</tr>
<tr>
<td>Pantothenate**</td>
<td>δ 0.88(s), 0.92(s), 2.41(t), 3.39(d), 3.43(q), 3.51(d), 3.98(s), 7.99(br)</td>
<td>0.061 ± 0.02</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lactate</td>
<td>δ 1.32(d), 4.10(q)</td>
<td>0.088 ± 0.003</td>
<td>0.075 ± 0.01</td>
<td>0.089 ± 0.03</td>
</tr>
<tr>
<td>Alanine</td>
<td>δ 1.47(d), 3.78(q)</td>
<td>0.036 ± 0.006</td>
<td>0.043 ± 0.01</td>
<td>0.031 ± 0.008</td>
</tr>
<tr>
<td>Adipate</td>
<td>δ 1.54(m), 2.18(m)</td>
<td>0.11 ± 0.06</td>
<td>0.12 ± 0.04</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>Succinate</td>
<td>δ 2.41(s)</td>
<td>0.27 ± 0.12</td>
<td>0.28 ± 0.09</td>
<td>0.27 ± 0.16</td>
</tr>
<tr>
<td>Citrate*</td>
<td>δ 2.55(d), 2.70(d)</td>
<td>1.06 ± 0.94</td>
<td>0.8 ± 0.29</td>
<td>1.85 ± 1.52</td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>δ 2.72(s)</td>
<td>0.16 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>N,N-Dimethylglycine</td>
<td>δ 2.91(s), 3.72(s)</td>
<td>0.062 ± 0.029</td>
<td>0.061 ± 0.035</td>
<td>0.064 ± 0.056</td>
</tr>
<tr>
<td>Creatine*</td>
<td>δ 3.02(s), 3.92(s)</td>
<td>0.053 ± 0.013</td>
<td>0.11 ± 0.21</td>
<td>0.13 ± 0.18</td>
</tr>
<tr>
<td>Creatine phosphate*</td>
<td>δ 3.03(s), 3.93(s)</td>
<td>0.05 ± 0.02</td>
<td>0.13 ± 0.048</td>
<td>0.17 ± 0.06</td>
</tr>
<tr>
<td>cis-Aconitate*</td>
<td>δ 3.12(s), 5.79(s)</td>
<td>0.32 ± 0.12</td>
<td>0.29 ± 0.06</td>
<td>0.45 ± 0.14</td>
</tr>
<tr>
<td>Choline*</td>
<td>δ 3.19(s), 3.50(br), 4.05(m)</td>
<td>0.016 ± 0.004</td>
<td>0.011 ± 0.004</td>
<td>0.014 ± 0.003</td>
</tr>
<tr>
<td>Betaine*</td>
<td>δ 3.26(s), 3.89(s)</td>
<td>0.049 ± 0.02</td>
<td>0.078 ± 0.02</td>
<td>0.076 ± 0.03</td>
</tr>
<tr>
<td>Taurine</td>
<td>δ 3.26(t), 4.44(t)</td>
<td>0.82 ± 0.69</td>
<td>1.4 ± 1.0</td>
<td>0.82 ± 0.64</td>
</tr>
<tr>
<td>Trimethylamine N-oxide</td>
<td>δ 3.27(s)</td>
<td>0.098 ± 0.052</td>
<td>0.11 ± 0.08</td>
<td>0.11 ± 0.06</td>
</tr>
<tr>
<td>4-Hydroxyphenylacetate</td>
<td>δ 3.44(s), 6.83-6.87(m), 7.13-7.17(m)</td>
<td>0.06 ± 0.03</td>
<td>0.06 ± 0.05</td>
<td>0.046 ± 0.01</td>
</tr>
<tr>
<td>Glycine</td>
<td>δ 3.55(s)</td>
<td>0.21 ± 0.24</td>
<td>0.11 ± 0.04</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>Phenylacetylglucose</td>
<td>δ 3.67(s), 3.75(d), 7.32-7.37(m), 7.41(t), 7.98(br), 7.15(m), 3.04(t)</td>
<td>0.21 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.25 ± 0.07</td>
</tr>
<tr>
<td>Hippurate</td>
<td>δ 3.96(d), 7.51-7.57(m), 7.63(t), 7.80-7.85(m), 8.52(br)</td>
<td>0.26 ± 0.12</td>
<td>0.26 ± 0.1</td>
<td>0.29 ± 0.13</td>
</tr>
<tr>
<td>Allatoin</td>
<td>δ 3.38(s), 6.03(s)</td>
<td>6.84 ± 0.77</td>
<td>6.88 ± 1.0</td>
<td>7.78 ± 1.1</td>
</tr>
<tr>
<td>3-Indoxylsulfate</td>
<td>δ 7.18(t), 7.26(t), 7.36(s), 7.49(d), 7.69(d)</td>
<td>0.11 ± 0.047</td>
<td>0.11 ± 0.044</td>
<td>0.12 ± 0.034</td>
</tr>
</tbody>
</table>

Multiplicity: s, single; d, double; t, triplet; q, quartet; m, multiplet; br, broad.

* Mean ± SD; ND: not detected.

< p < 0.05.

** p < 0.0001.

(Somasundaram et al., 1997), and inactivation of creatine kinase, which is highly sensitive to oxidative stress, is an established marker of NSAID tissue toxicity (Miura et al., 2001). In contrast, which is highly sensitive to oxidative stress, is an established marker of NSAID tissue toxicity (Miura et al., 2001), and inactivation of creatine kinase, which is highly sensitive to oxidative stress, is an established marker of NSAID tissue toxicity (Miura et al., 2001). Moreover, in an acute inflammatory state, the body consumes large amounts of energy while mounting a cytoprotective response (Peters, 2006).

Naproxen-induced elevation of cis-aconitate presumably occurs as a result of activation of aconitase, which catalyzes the conversion of citrate, first to cis-aconitate and then to isocitrate. Neutrophils have also been implicated in the gastric damage associated with NSAIDs (Wallace et al., 1990). These immune cells are recruited to sites of NSAID-induced injury where they amplify the inflammatory response by releasing several chemotaxins, and cause further tissue injury by releasing ROS (Kato and Takeuchi, 2002). This increase in ROS levels, in turn, reduces aconitase activity in rat liver (Andreeshcheva et al., 2004). Thus, systemic energy metabolism is...
modulated in response to oxidative stress in rats with GI damage. Furthermore, in a study of the effects of NSAIDs on mitochondrial respiration and ATP synthesis, the NSAIDs diclofenac, piroxicam, indomethacin, nabumetone, nimesulide, and meloxicam, but not naproxen, stimulated both basal and uncoupled respiration and inhibited ATP synthesis (Moreno-Sánchez et al., 1999). Gastric motility may also be an important factor influencing the pathogenic mechanism of induced gastric lesions in rats (Takeuchi et al., 1986). The non-selective COX inhibitors, naproxen, indomethacin, and diclofenac, inhibit prostaglandin production, increase gastric motility, and induce severe gastric lesions. In contrast, the selective COX-2 inhibitor rofecoxib, which does not cause gastric damage, has no effect on either gastric motility or mucosal prostaglandin E2 content (Tanaka et al., 2001). Thus GI hypermobility following exposure to non-selective COX inhibitors might increase the production of phosphocreatine and creatine.

We also observed a marked decrease in urinary choline levels in rats treated with naproxen, which may have been related to change in the choline membrane pathway. Generally, choline has two possible fates: oxidation to betaine or incorporation into phosphatidylcholine. Importantly, phosphatidylcholine, a component of the gastric mucosa, has been reported to counteract NSAID-induced gastric injury (Kurinets and Lichtenberger, 1998; Nervi, 2000). In the present study, naproxen treatment resulted in decreased choline levels and increased betaine levels, suggesting that choline was not metabolized to produce phosphatidylcholine and that the gastric mucosa was unable to protect the stomach from naproxen-induced damage.

4.2. Naproxen-induced tryptophan catabolism

A novel metabolite that was altered by naproxen-induced toxicity was kynurenate, an endogenous neuroprotectant that is usually present in the brain at nanomolar concentrations (Moroni et al., 1988). Although kynurenate levels are known to increase following exposure to non-selective NSAIDs, such as indomethacin and naproxen, and to decrease in response to COX-2 selective NSAIDs (Schwieler et al., 2005), the relationship between kynurenate and NSAID-induced gastric damage is poorly understood. Tryptophan, a precursor of kynurenate, is known to protect the gastric mucosa from NSAID-induced damage (Konturek et al., 2010). Lin et al. (2010) reported that tryptophan catabolism was a major pathway in acute intestinal inflammation. Specifically, indoleamine-2,3-dioxygenase, the first enzyme in tryptophan catabolism, is up-regulated by pro-inflammatory stimuli, such as bacterial lipopolysaccharide and the cytokines interferon-γ and tumor necrosis factor (TNF)-α (Mellor and Munn, 2004). Because TNF-α also plays a critical role in the pathogenesis of NSAID-induced gastric injury (Appleyard et al., 1996), the present results suggest that the increased kynurenine levels observed in rats with NSAID-induced gastric damage might stem from NSAID-induced increases in TNF-α levels. Additionally, decreased expression of fibroblast growth factor-1 (FGF-1) inhibits angiogenesis, which is a crucial element in healing from tissue injury, because vascular system provides essential oxygen and nutrients to the site of wound healing (Tarnawski and Jones, 2003). Thus, NSAID inhibition of angiogenesis is regarded as a causal factor delayed ulcer healing. Recently, Serio et al. (2005) reported that at low concentrations, kynurenic acid inhibited the release of FGF-1 in the cell model systems tested and that NSAIDs also inhibited expression of the egr-1 gene, an activator of FGF-1 gene transcription (Wang et al., 1997). Accordingly, FGF-1 inhibition by NSAIDs may be related to NSAID effects on kynurenic acid production from tryptophan.

Lanza et al. (1979) evaluated the effect of low-dose (500 mg/day) and high-dose (750 mg/day) naproxen on gastric mucosal injury in humans. Human gastric damage induced by administration of low-dose (500 mg/day) naproxen for 7 days was similar to damage caused by low-dose (10 mg/kg) naproxen exposure in rats, while high-dose (750 mg/day) naproxen exposure in humans induced slightly less gastric damage than did high-dose (100 mg/kg) naproxen in rats. These results suggest that rat gastric damage at exposure levels of 10 and 100 mg/kg can be extrapolated to human exposure at 500 and 750 mg/day for 7 days. The typical recom-
mended human adult dosage is 500–1000 mg daily. Thus, human naproxen dosing at typical dosage levels over 7 days may result in gastric damage and metabolic perturbation.

In conclusion, the results of this study demonstrate that 1H NMR-based metabolomics can be used to effectively distinguish the metabolic differences between vehicle-treated rats, who suffer no gastric damage, and rats treated with naproxen, who suffer extensive gastric damage. Additionally, this study suggests that the naproxen dose-dependent variation in endogenous metabolite levels is related to the biochemical pathways perturbed by naproxen administration. Furthermore, global and targeted metabolic profiling using 1H NMR-based metabolomics is a noninvasive, novel approach for monitoring drug toxicity and for gaining insight into the biological pathways perturbed in NSAID-induced gastric damage.

Conflict of interest

The author declares that there is no conflict of interest.

Acknowledgments

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