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Glucuronidation of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs): Identifying the Enzymes Responsible in Human Liver Microsomes

Gwendolyn E. Kuehl, Johanna W. Lampe, John D. Potter, and Jeannette Bigler

Cancer Prevention Program (G.E.K, J.W.L., J.D.P., J.B.)
Fred Hutchinson Cancer Research Center
Seattle, Washington

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Running Title: Glucuronidation of NSAIDs by UGTs and Human Liver Microsomes

Corresponding Author: Jeannette Bigler, 1100 Fairview Ave. N., M5-A864, P.O. Box 19024,

Seattle, WA. 98109; phone: (206) 667-5077; fax: (206) 667-2537; email: jbigler@fhcrc.org

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ABBREVIATIONS: COX, cyclooxygenase; DMSO, dimethylsulfoxide; HDCA,

hyodeoxycholic acid; HLM, human liver microsomes; HPLC, high performance liquid

chromatography; LC, liquid chromatography; MS, mass spectrometry; NSAID, non-steroidal

anti-inflammatory drug; PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; UGT,

UDP-glucuronosyltransferase

ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs), used for the treatment of pain and inflammation, are eliminated primarily through conjugation with polar sugar moieties to form glucuronides. Glucuronidation is catalyzed by the UDP-glucuronosyltransferases (UGT) superfamily. An inverse relationship may exist between glucuronidation activity and NSAID efficacy; however, specific UGTs catalyzing conjugation of the structurally diverse NSAIDs have yet to be identified systematically. Therefore, NSAID glucuronidation activity by 12 individually expressed UGTs was investigated by LC-MS/MS. The relative rates of NSAID glucuronidation varied among UGT enzymes examined, demonstrating specificity of the individual UGTs towards selected NSAIDs. Kinetic parameters were determined for expressed UGT Supersomes^{TM} and compared with parameters determined in pooled HLMs. Comparison of K_m values suggested roles for UGTs 1A3 and 2B7 in indene glucuronidation and UGTs 1A9, 2B4, and 2B7 in profen glucuronidation. Inhibitory studies in pooled HLMs support the role of UGTs 1A1, 1A3, 1A9, 2B4, and 2B7 in the glucuronidation of ibuprofen, flurbiprofen and ketoprofen. Bilirubin did not inhibit indomethacin or diclofenac glucuronidation, suggesting that UGT1A1 was not involved in catalysis. Imipramine did not inhibit glucuronidation of sulindac, sulindac sulfone, indomethacin or naproxen in pooled HLMs, suggesting that UGT1A3 was not a principal hepatic catalyst. Nevertheless, multiple UGT enzymes, most notably UGTs 1A1, 1A9, 2B4 and 2B7, appear to be involved in the hepatic catalysis of NSAID glucuronidation.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are an important class of therapeutic agents used in the treatment of pain and inflammatory disease. Recent evidence suggests that NSAIDs may be useful in the chemoprevention of colorectal cancer [for review see (Baron and Sandler, 2000)]. There are inter-individual differences in drug efficacy, which may be a result of variation in the ability to excrete these compounds as non-reactive metabolites. It is conceivable that an increase in the ability to metabolize these drugs results in a decrease in the duration and extent of therapeutic response. A thorough understanding of the enzymes involved in metabolizing these NSAIDs will facilitate our ability to maximize drug efficacy for individual patients.

One of the primary pathways of NSAID metabolism is glucuronidation, in which a polar sugar is conjugated to the NSAID, increasing its rate of elimination from the body. Glucuronidation is catalyzed by a superfamily of enzymes called UDP-glucuronosyltransferases (UGTs) [for review see (Tukey and Strassburg, 2000)]. Many different genes and pseudogenes have been identified in the UGT superfamily and subdivided into the UGT1A and UGT2B families based on sequence identity (Mackenzie et al., 1997). Protein levels are a result of tissue-specific and inducible expression of each UGT. UGTs 1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B15, and 2B17 are expressed in human liver, and the remaining UGTs are extrahepatic [for reviews see (Tukey and Strassburg, 2000; Turgeon et al., 2001)]. Polymorphisms in UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, and 2B15 have been identified in different populations (Ciotti et al., 1997; Levesque et al., 1997; Lampe et al., 1999; Levesque et al., 1999; Lampe et al., 2000; Balram et al., 2002; Gagné et al., 2002; Huang et al., 2002; Villeneuve et al., 2003; Ehmer et al., 2004; Iwai et al., 2004; Verlaan et al., 2004; Yamanaka et al., 2004). Sometimes, these polymorphisms result in altered enzyme expression or activities

towards a variety of endogenous and xenobiotic aglycones (Ciotti et al., 1997; Levesque et al., 1999; Huang et al., 2002; Ehmer et al., 2004; Iwai et al., 2004; Yamanaka et al., 2004).

A wide variety of chemically distinct compounds have been classified as NSAIDs, based on their ability to inhibit prostaglandin G/H synthase, also called cyclooxygenase (COX) -1 and -2. Prostaglandins formed by COX elicit an inflammatory response. NSAIDs are classified based on their ability to inhibit COX as follows; COX-2 specific inhibitors, COX-1 and COX-2 inhibitors, and COX-independent anti-inflammatory drugs (Frölich, 1997). For this study, we focused our investigation on the most commonly used NSAIDs that undergo glucuronidation in the absence of a principal oxidation step. These drugs were separated into three classes based on chemical composition: indene derivatives including sulindac, sulindac sulfone, and indomethacin; propionic acid-containing drugs (profens) including ibuprofen, flurbiprofen and ketoprofen; and others which are structurally distinct including diclofenac and naproxen (Figure 1). These compounds are all primarily nonspecific COX inhibitors (Frölich, 1997), except for sulindac sulfone, which acts in a COX-independent manner (Piazza et al., 1997). These drugs readily undergo glucuronidation at the carboxyl group to form acyl glucuronides.

Few studies have been carried out to examine the glucuronidation of a variety of NSAIDs by heterologously expressed UGTs. UGT2B7 has been suggested as the principal catalyst of the glucuronidation of a variety of NSAIDs such as naproxen, ketoprofen, ibuprofen, fenoprofen, tiaprofenic acid, benoxprofen, zomepirac, diflunisal and indomethacin (Jin et al., 1993). Expressed UGT1A3 has been reported to catalyze the glucuronidation of profens including ibuprofen and ketoprofen (Green et al., 1998). Sabolovic and coworkers proposed that along with UGTs 1A3 and 2B7, 1A6 expressed in Caco-2 cells may also catalyze the glucuronidation of profen NSAIDs (Sabolovic et al., 2000). More recently, Owens and coworkers have demonstrated that UGTs 1A3, 1A7, 1A8 and 1A10 catalyze the glucuronidation of several NSAIDs including flurbiprofen, diclofenac, and indomethacin (Basu et al., 2004). While these

studies represent a significant contribution to our understanding of NSAIDs glucuronidation, a systematic comparison of the ability of individual members of the UGT superfamily to catalyze the glucuronidation of a variety of NSAIDs has not been accomplished. To this end, we developed a liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method to measure the glucuronidation of the NSAIDs mentioned previously, determined and compared the apparent kinetic parameters for glucuronidation catalyzed by selected UGTs, and measured the inhibition of NSAID glucuronidation in pooled HLMs in the presence of UGT-selective probes to confirm the role of individual enzymes in the hepatic catalysis of specific compounds.

MATERIALS AND METHODS

Materials: All reagents were purchased from Sigma-Aldrich (St. Louis, MO.) unless otherwise stated. A crude solution of β-glucuronidase from *Helix pomatia* was used (134,400 units/ml). UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 Supersomes[TM] and pooled HLMs were obtained from BD Biosciences Gentest (Woburn, MA.). Morphine was purchased from Cerilliant Corporation (Round Rock, TX.). All reagents were of the highest grade commercially available.

LC-MS/MS Analysis of NSAID Glucuronides: Metabolites were separated on a Luna C_5 column (5 μ, 100 x 2 mm) from Phenomenex (Torrance, CA.) isocratic at 30% TFA (0.05%) and 70% acetonitrile (0.15 ml/minute) with post-column infusion of ammonium hydroxide (7%) at a flow rate of 1 μl/minute. The high performance liquid chromatography (HPLC) system consisted of 2 Shimadzu LC-10AD pumps with a gradient controller and a Shimadzu SIL-10ADvp autoinjector or a Waters Alliance 2695 Separation Module. Glucuronides were quantified using a Micromass Quattro II Mass Spectrometer or a Micromass Quattro Micro Mass Spectrometer (Waters Corporation, Milford, MA.) with electrospray ionization (ESI) in negative ion mode. ESI source

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conditions on the Quattro II Mass Spectrometer were set as follows: capillary voltage, 3.5 kV;

cone voltage, 30 V; collisional energy, 30 V; source block temperature, 100° C; capillary

temperature, 250° C; and collisional gas, 3×10^{-3} mbar. ESI source conditions on the Quattro

Micro Mass Spectrometer were set as follows: capillary voltage, 3.5 kV; cone voltage, 20 V;

collisional energy, -16 V; source temperature, 120 ºC, desolvation temperature, 500 ºC. Elution

times and parent and daughter ions used in the quantification of NSAID glucuronides are given

in Table 1 along with their limits of detection. Fragmentation patterns were consistent with

previously reported literature (Bolze et al., 2002). Rates of glucuronidation were quantified by

comparison with known concentrations of glucuronide standards. The response of the MS/MS

detector was linear over the concentration ranges for NSAID glucuronides. Sample aliquots

were analyzed before and after addition of NSAID glucuronide standards at concentrations

consistent with those produced by each enzyme to confirm the accuracy of quantification.

Generation of ibuprofen glucuronide: The ibuprofen glucuronide was isolated from overnight

urinary metabolites excreted by a single individual dosed with 200 mg Advil®. The Institutional

Review Board of the Fred Hutchinson Cancer Research Center approved the study and informed

written consent was obtained from this individual. Urine pH was adjusted to between 3 and 4

using concentrated phosphoric acid. Urinary metabolites were concentrated on a C_{18} SepPak

SPE cartridge (Waters Corporation, Milford, MA.) and eluted with methanol. Eluates were dried

under nitrogen and resuspended in nanopure water. Fractions were analyzed with and without β-

glucuronidase treatment. Metabolites were analyzed and purified by HPLC using an Hewlett-

Packard series 1050 separation module with a ultraviolet detector and autoinjector (Agilent

Technologies, Palo Alto, CA.) eluted from a Waters C_{18} Nova-Pak column (5 µm, 3.9 x 300 mm)

using a gradient of 0.1% trifluoroacetic acid (TFA), A, and methanol, B, as follows: 100% A to

50% B over 40 minutes. Ibuprofen and glucuronide conjugate were detected by UV absorbance

at 235 nm. Under these conditions, the ibuprofen glucuronide eluted at 33 minutes. The

ibuprofen glucuronide eluate was dried under nitrogen. The glucuronide was resuspended in

nanopure water and aliquots treated with β-glucuronidase in triplicate. No residual ibuprofen

glucuronide peak was detected by HPLC-UV after treatment with β-glucuronidase. The

concentration was determined by comparison of the aglycone released with known

concentrations of ibuprofen standard. Mass spectrometry (MS) and MS/MS analysis was also

used to confirm the identity of the ibuprofen glucuronide standard.

Biosynthesis of sulindac, sulindac sulfone, indomethacin, diclofenac, flurbiprofen, ketoprofen,

and naproxen glucuronide standards: HLMs have been used previously to produce authentic

glucuronide standards (Soars et al., 2002). Glucuronides of each NSAID were biosynthesized in

pooled HLMs under the following conditions; 2 mg/ml protein, 50 µg alamethicin/mg protein,

500 µM aglycone (in 1% DMSO), 2 mM UDP-glucuronic acid, 3 mM saccharolactone, and 10

mM $MgCl_2$ in 50 mM potassium phosphate (pH 6). Reactions were carried out overnight at 37°

C in a shaking water bath and terminated with half volumes of methanol. Mixtures were

vortexed and centrifuged at 13,000 x g to precipitate protein. Supernatants were transferred to

fresh tubes and dried completely under nitrogen. Metabolites were then resuspended in nanopure

water for purification of the glucuronides by HPLC. HPLC purification was carried out as

described previously for ibuprofen glucuronide standard production except that the follow

gradients were used; 100% TFA (0.1%) to 50% acetonitrile in 25 minutes (sulindac, sulindac

sulfone and indomethacin) or 100% TFA (0.1%) to 67% methanol in 30 minutes (diclofenac,

flurbiprofen, ketoprofen and naproxen). UV detection of sulindac, sulindac sulfone, and

indomethacin was carried out at 300 nm (Figure 2), while other NSAIDs were detected at 235

nm. Concentrations of each purified glucuronide were determined by β-glucuronidase treatment

as described above. Residual glucuronide peaks were not observed by HPLC when NSAID

glucuronides were treated with β-glucuronidase. MS and MS/MS analysis was carried out to confirm the identity of each NSAID glucuronide standard.

Screening UGT Supersomes^{TM} for NSAID Glucuronidation Activity: Glucuronidation activity by UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 Supersomes^{TM} was measured. The assay conditions were as follows: 0.05-2 mg/ml protein, 50 μg alamethicin /mg protein, 2 mM UDP-glucuronic acid and 500 μM concentrations of each NSAID, and 10 mM MgCl$_2$ in 50 mM potassium phosphate (pH 6). Optimal alamethicin/protein ratios have been determined previously for UGT Supersomes^{TM} (Kuehl and Murphy, 2003) and were kept constant throughout the study.   Reactions were allowed to progress for 2 hours in a shaking water bath at 37° C before being terminated with equal volumes of methanol. Samples were vortexed and centrifuged at 13,000 x g for 5 minutes. The supernatants were then removed and dried completely under nitrogen. The dried supernatant was resuspended in nanopure water and glucuronides were quantified by LC-MS/MS analysis. Control Supersomes^{TM} not transfected with any of the human UGT cDNAs did not catalyze the glucuronidation of any NSAID examined. The rates of NSAID glucuronidation were linear from 30 to 120 minutes over the protein concentration ranges examined.

Kinetics of NSAID Glucuronidation by Selected UGTs: The mean plasma concentrations for several NSAIDs have been determined previously as follows: indomethacin, 1.4 μM; ibuprofen, 38.8 μM (low dose), 111.0 μM (high dose); flurbiprofen, 53.2 μM; ketoprofen, 9.4 μM; diclofenac, 6.1 μM; naproxen, 1.3 μM (Cryer and Feldman, 1998).   Rates of glucuronidation with 1 μM, 10 μM, 50 μM, 100 μM, 500 μM, and 1 mM concentrations of each NSAID by UGTs 1A1, 1A3, 1A9, and 2B7 for sulindac, sulindac sulfone, and indomethacin or UGTs 1A3, 1A9, 2B4 and 2B7 for the remaining NSAIDs were measured under the following conditions; 0.5

mg/ml protein, 50 µg alamethicin/mg protein, 8.5 mM saccharolactone, 1 mM

phenylmethylsulfonyl fluoride (PMSF), 10 mM $MgCl_2$, 50 mM potassium phosphate (pH 7) and

2 mM UDP-glucuronic acid. Reactions were allowed to progress for 30 minutes before being

terminated with equal volumes of ice-cold methanol, vortexed and centrifuged. Glucuronides

were quantified by LC-MS/MS as described previously.

Kinetics of NSAID Glucuronidation in Pooled HLMs: Rates of glucuronidation in pooled HLMs

were measured under the following conditions: 1 mg/ml protein, 50 µg alamethicin/mg protein,

8.5 mM saccharolactone, 1 mM PMSF, 10 mM $MgCl_2$, 50 mM potassium phosphate (pH 7) and

2 mM UDP-glucuronic acid with 1 µM, 10 µM, 50 µM, 100 µM, 500 µM, and 1 mM

concentrations of each NSAID. Reactions were allowed to progress for 20 minutes before being

terminated with equal volumes of ice-cold methanol, vortexed, and centrifuged. Glucuronides

were quantified by LC-MS/MS as described previously.

Glucuronidation of NSAIDs in HLMs in the Presence of UGT-Selective Probes: The

glucuronidation of each NSAID catalyzed by pooled HLMs was measured in the absence and

presence of UGT-selective probes. Bilirubin, estradiol, imipramine, propofol, hyodeoxycholic

acid (HDCA) and morphine were used to determine the contribution of UGTs 1A1, 1A1/2B7,

1A3, 1A9, 2B4/2B7, and 2B7, respectively, to the glucuronidation of each NSAID (Pillot et al.,

1993; Bosma et al., 1994; Coffman et al., 1997; Ethell et al., 2002; Nakajima et al., 2002b;

Mackenzie et al., 2003; Lepine et al., 2004). These aglycones have been used previously to

determine whether individual UGTs contribute to aglycone glucuronidation in HLMs (Miners et

al., 1988; Hanioka et al., 2001; Nakajima et al., 2002a; Nakajima et al., 2002b; Nakajima et al.,

2002c; Kuehl and Murphy, 2003). To determine that these UGT-selective probes acted as

competitive inhibitors, rates of sulindac and flurbiprofen glucuronidation were measured in the

absence and presence of 10 µM, 20 µM, and 50 µM concentrations of estradiol, imipramine, propofol, hyodeoxycholic acid (HDCA) and morphine. Rates of glucuronidation were quantified by LC-MS/MS analysis. Once we had determined that each inhibitor acted in a competitive fashion, the percent inhibition observed upon addition of each UGT-selective probe to reactions was measured at 10 µM and 500 µM concentrations of the NSAIDs with 5 µM bilirubin or 50 µM estradiol, imipramine, propofol, HDCA, or morphine.

Data Handling and Analysis: Reactions were carried out in duplicate and are reported as the average of determinations varying by less than 10 percent. Rates of glucuronidation were fit to the Michaelis-Menten equation using GraphPad Prism 3.03 software (GraphPad Software Inc., San Diego, CA.).

RESULTS

Screening UGT Supersomes^TM for NSAID Glucuronidation Activity

Individually expressed UGT enzymes were screened for their ability to catalyze the glucuronidation of a variety of NSAIDs. Rates of glucuronidation of different NSAIDs by an individual UGT enzyme were compared. These rates were not adjusted for levels of expressed protein or active enzyme in each of the Supersomes^TM preparations.

Overall, rates of NSAID glucuronidation catalyzed by UGTs 1A1, 1A3, 1A9, 2B4, and 2B7 Supersomes^TM were higher than the rates of NSAID glucuronidation catalyzed by all other UGT enzymes examined (Figure 3, note different scales). UGT1A1 catalyzed the glucuronidation of sulindac sulfone, 184 pmol/min/mg protein, at the highest rate when compared with other NSAIDs examined (Figure 3). Rates of sulindac, flurbiprofen, and naproxen glucuronidation by UGT1A1 were less than 2-fold lower than the rate of sulindac sulfone glucuronidation. Unlike UGT1A1, UGT1A3 catalyzed flurbiprofen at the highest rate,

393 pmol/min/mg protein. Ibuprofen glucuronidation by UGT1A3 was catalyzed at the lowest rate. UGT1A9 catalyzed the glucuronidation of indomethacin at the highest rate, 361 pmol/min/mg protein. Rates of glucuronidation of the remaining NSAIDs were more than 4-fold lower than indomethacin glucuronidation. UGT2B4 was a better catalyst of profen glucuronidation than indene glucuronidation. Flurbiprofen and ketoprofen glucuronidation by UGT2B4 was higher than all other NSAIDs investigated. UGT2B7 catalyzed diclofenac glucuronidation at the highest rate, 748 pmol/min/mg protein. Like UGT2B4, UGT2B7 catalyzed the glucuronidation of profens, diclofenac, and naproxen at higher rates than indenes.

Kinetics of NSAID Glucuronidation by UGT Supersomes^{TM}:

Kinetics of indene glucuronidation catalyzed by UGTs 1A1, 1A3, 1A9, and 2B7 were determined and fit to the Michealis-Menten equation (Table 2). UGT1A1 had the highest affinity for sulindac and sulindac sulfone glucuronidation, as demonstrated by a low binding constant (K_m). UGT1A3 had the highest apparent affinity for indomethacin. Among the indenes investigated, sulindac sulfone glucuronidation was catalyzed with the highest maximal rates by UGTs 1A1, 1A3, and 2B7. UGT1A9 had a higher V_{max} for indomethacin glucuronidation than sulindac or sulindac sulfone glucuronidation. UGTs 1A1, 1A3, and 2B7 were more efficient catalysts (higher V_{max}/K_m values) of sulindac sulfone glucuronidation than of sulindac or indomethacin glucuronidation. UGT1A9 was more efficient catalyst of indomethacin glucuronidation than of sulindac or sulindac sulfone glucuronidation. Rates of sulindac and sulindac sulfone glucuronidation by UGT2B7 and sulindac sulfone by UGT1A1 decreased when NSAID concentrations were increased from 500 to 1000 µM. Rates of glucuronidation by UGTs 1A1, 1A3, 1A9, and 2B7 fit Michaelis-Menten kinetics only from 1 µM to 100 µM indomethacin, decreasing at higher NSAID concentrations.

Kinetics of profen glucuronidation catalyzed by UGTs 1A3, 1A9, 2B4, and 2B7 were determined and fit to the Michealis-Menten equation (Table 2). UGT2B7 had the highest affinity for profen glucuronidation (flurbiprofen > ketoprofen > ibuprofen). The relative V_{max} values for profen glucuronidation were enzyme-specific; for example, UGTs 1A3, 2B4, and 2B7 had a higher V_{max} and were more efficient catalysts of ketoprofen glucuronidation than ibuprofen or flurbiprofen glucuronidation (ketoprofen > flurbiprofen > ibuprofen). The V_{max} values for UGT1A9-catalyzed flurbiprofen and ibuprofen glucuronidation were similar and higher than the V_{max} for ketoprofen glucuronidation. Decreased rates of glucuronidation by UGTs 1A3 and 1A9 were observed at 1000 µM flurbiprofen compared to 500 µM. No other UGTs showed decreased rates of glucuronidation at high flurbiprofen concentrations.

Kinetics of diclofenac and naproxen glucuronidation by UGTs 1A3, 1A9, 2B4, and 2B7 were also investigated and fit to the Michealis-Menten equation (Table 2). UGT2B4 demonstrated the highest affinity for diclofenac (Table 2). Unlike diclofenac glucuronidation, naproxen glucuronidation was catalyzed with the highest affinity by UGT2B7. UGTs 1A3 and 2B7 catalyzed the glucuronidation of diclofenac and naproxen at similar maximal rates. However, the V_{max} of UGTs 1A9 and 2B4 for naproxen glucuronidation was 3-fold higher than the V_{max} for diclofenac glucuronidation. UGTs 1A3, 1A9, and 2B4 catalyzed naproxen glucuronidation less efficiently than diclofenac glucuronidation, whereas the reverse was true for UGT2B7. Rates of diclofenac glucuronidation fit Michaelis-Menten kinetics from 1 µM to 100 µM concentrations; however, glucuronidation rates decreased above 100 µM. Rates of naproxen glucuronidation catalyzed by UGT2B7 decreased above 500 µM, but no decrease in rates was observed for naproxen glucuronidation catalyzed by UGTs 1A3, 1A9, and 2B4.

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Kinetics of NSAID Glucuronidation in Pooled HLMs

Rates of glucuronidation catalyzed in pooled HLMs were determined from 1 µM to 1000 µM of each NSAID. In pooled HLMs, sulindac sulfone and indomethacin glucuronidation was catalyzed with the highest affinities, K_m values of 34 µM and 30 µM, respectively (Table 3). Pooled HLMs had comparable affinities for sulindac, diclofenac, and naproxen glucuronidation and had the lowest affinity for ibuprofen glucuronidation with a K_m of 188 µM. The highest V_{max} and efficiency (V_{max}/K_m) was observed when diclofenac and naproxen glucuronidation was measured. Rates of glucuronidation fit the Michaelis-Menten equation from 1 µM to 500 µM of each NSAID. However, rates of sulindac, indomethacin, flurbiprofen, diclofenac, and naproxen glucuronidation in pooled HLMs decreased above 500 µM.

We compared the relative kinetic parameters determined for NSAID glucuronidation in HLMs with those determined by expressed UGT Supersomes[TM]. In HLMs, the affinities for sulindac sulfone and indomethacin glucuronidation were comparable, but higher than the affinity for sulindac. This is consistent with affinities for indene glucuronidation catalyzed by UGTs 1A3 and 2B7. Differences in the apparent V_{max} values for indene glucuronidation (sulindac ≈ indomethacin < sulindac sulfone) is consistent with UGT2B7 as a principal catalyst of glucuronidation. Among the profens, enzymes in pooled HLMs catalyzed flurbiprofen glucuronidation with the highest affinity followed by ketoprofen and ibuprofen. This is comparable to the affinities for profen glucuronidation by expressed UGTs 1A9, 2B4, and 2B7. However, the V_{max} of ketoprofen glucuronidation in pooled HLMs was higher than ibuprofen and flurbiprofen glucuronidation. The differences in the V_{max} of glucuronidation between profens were more pronounced in Supersomes[TM] than in pooled HLMs. The affinities of the UGTs for diclofenac and naproxen differ between the two assay systems used. In HLMs, diclofenac glucuronidation was catalyzed at a higher V_{max} than naproxen glucuronidation. This is different

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from the maximal rates of diclofenac and naproxen glucuronidation catalyzed by the expressed UGT Supersomes[TM].

NSAID Glucuronidation by Pooled HLMs in the Presence of UGT-Selective Probes

Multiple UGT Supersomes[TM] catalyzed the glucuronidation of each structurally distinct NSAID. Expressed UGTs 1A1, 1A3, 1A9, and 2B7 were catalysts of indene glucuronidation. Along with UGT2B4, these enzymes were also significant catalysts of the glucuronidation of profens, diclofenac, and naproxen. UGTs 1A1, 1A3, 1A9, 2B4, and 2B7 are expressed in the liver, suggesting that they might contribute to hepatic glucuronidation. Therefore, we examined the role of these enzymes on NSAID glucuronidation in pooled HLMs by measuring effects of the addition of the UGT-selective probes bilirubin (1A1), estradiol (1A1/2B7), imipramine (1A3/1A4), propofol (1A9), hyodeoxycholic acid (HDCA) (2B4/2B7), and morphine (2B7) to reaction mixtures.

To determine whether these UGT-selective probes acted as competitive inhibitors, sulindac and flurbiprofen glucuronidation kinetics were measured at increasing concentrations of inhibitors. Rates of glucuronidation were fit to Lineweaver-Burk plots. A positive correlation was observed between line slopes and 1-50 µM estradiol, imipramine, propofol, hyodeoxycholic acid and morphine for each UGT-selective aglycone examined suggesting that these probes were competitively inhibiting NSAID glucuronidation. The contribution of UGT1A1 to NSAID glucuronidation was determined by addition of bilirubin at a concentration of 5 µM consistent with concentrations used previously (Nakajima et al., 2002c).

Estradiol and HDCA inhibited sulindac, sulindac sulfone, and indomethacin glucuronidation (Table 4). Percent inhibition of glucuronidation was dependent on NSAID concentrations. For example, addition of estradiol decreased the rate of sulindac glucuronidation by 55% and 34% at 10 µM and 500 µM sulindac, respectively. Conversely, addition of propofol

decreased the glucuronidation rate by 22% and 51% at 10 µM and 500 µM sulindac, respectively. Bilirubin did not decrease indomethacin glucuronidation, suggesting that UGT1A1 was not involved in hepatic catalysis. Imipramine failed to inhibit the glucuronidation of these indenes, suggesting that UGT1A3 was not involved in catalysis. Propofol did inhibit sulindac and indomethacin but not sulindac sulfone glucuronidation. Finally, morphine inhibited the glucuronidation of 10 µM sulindac and sulindac sulfone but failed to inhibit indomethacin glucuronidation. Therefore, UGT2B7 may not be involved in the glucuronidation indomethacin or of sulindac sulfone at high concentrations. However, inhibition was observed with both estradiol and HDCA, UGT-selective aglycones that likely also inhibit glucuronidation by UGT2B7, suggesting a role for UGTs 1A1, 1A9, 2B4, and 2B7 in hepatic indene glucuronidation.

Ibuprofen, flurbiprofen, and ketoprofen glucuronidation was inhibited by bilirubin, estradiol, imipramine, propofol and HDCA (Table 4), whereas morphine inhibited only ibuprofen and flurbiprofen glucuronidation. However, both estradiol and HDCA resulted in decreases in ketoprofen glucuronidation, still implicating UGT2B7 in catalysis. UGT1A1 may not be a major player in flurbiprofen glucuronidation, because bilirubin did not inhibit glucuronidation at 500 µM flurbiprofen. Ketoprofen glucuronidation at 500 µM was not inhibited by imipramine or propofol indicating that UGTs 1A3 and 1A9, respectively, may not play a role in catalysis.

Diclofenac and ketoprofen glucuronidation was inhibited in the presence of estradiol, propofol, and HDCA (Table 4). Bilirubin and morphine did not inhibit diclofenac glucuronidation, and imipramine did not inhibit naproxen glucuronidation. Again, while morphine failed to inhibit diclofenac glucuronidation, both estradiol and HDCA addition resulted in decreased glucuronidation rates, suggesting that UGT2B7 may still be involved in catalysis.

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The inhibition studies suggested a role for UGTs 1A3, 1A9, 2B4, and 2B7 in diclofenac

glucuronidation and UGTs 1A1, 1A9, 2B4, and 2B7 in naproxen glucuronidation.

DISCUSSION

This work is a comprehensive evaluation of the glucuronidation of structurally distinct

groups of NSAIDs by heterologously expressed UGTs and pooled HLMs. UGTs 1A3, 1A6,

1A7, 1A8, 1A10, and 2B7 have been reported as catalysts of the acyl glucuronidation of

carboxylic acid-containing drugs (Jin et al., 1993; Green et al., 1998; Sabolovic et al., 2000;

Basu et al., 2004), but a thorough comparison of the activities of all members of the UGT family

towards these aglycones has not previously been done. Therefore, we quantified the

glucuronidation of three classes of commonly used NSAIDs: indene derivatives (sulindac,

sulindac sulfone, and indomethacin), profens (ibuprofen, flurbiprofen, and ketoprofen), and

others (diclofenac and naproxen) by heterologously expressed UGTs and in pooled HLMs. The

glucuronidation of these NSAIDs by UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4,

2B7, 2B15, and 2B17 was quantified by LC-MS/MS to identify the principal catalysts. These

studies were complemented with the measurement of NSAID glucuronidation in pooled HLMs

in the absence or presence of UGT-selective substrates.

Based on initial screening experiments with UGT Supersomes[TM], UGTs 1A1, 1A3, 1A9,

2B4, and 2B7 were candidate catalysts involved in NSAID glucuronidation. However, a role for

the other UGT enzymes could not be excluded based on the screening data. Currently there exists

no reliable method to quantify active UGT enzymes in cell preparations and rates of

glucuronidation cannot be compared directly. In experiments with pooled HLMs and UGT-

selective competitive inhibitors, we observed that estradiol and HDCA inhibited glucuronidation

of all NSAIDs, supporting a role for UGTs 2B4 and 2B7 in hepatic glucuronidation of all

NSAIDs examined. However, inhibition of UGTs 1A3, 1A6, and 1A9 has also been observed by

Hanioka and coworkers (Hanioka et al., 2001), albeit at higher concentrations, suggesting that

HDCA may not be a selective inhibitor for UGTs 2B4 and 2B7. Significant inhibition of

sulindac sulfone, indomethacin, flurbiprofen, ketoprofen, and diclofenac glucuronidation by

morphine was not observed. This lack of inhibition may be due to the high K_m of morphine

glucuronidation (Stone et al., 2003), suggesting that morphine may not be a good competitive

inhibitor of NSAID glucuronidation. Therefore, these results do not rule out a role for UGT2B7

in NSAID glucuronidation, especially since both estradiol and HDCA acted as inhibitors of

NSAID glucuronidation. Indomethacin and diclofenac glucuronidation was catalyzed by

expressed UGT1A1; however, bilirubin did not inhibit glucuronidation in pooled HLMs. Even

though expressed UGT1A3 was a relatively good catalyst of indene glucuronidation, imipramine

failed to inhibit glucuronidation of sulindac, sulindac sulfone, indomethacin, and naproxen in

pooled HLMs. This result may be due to the relatively low UGT1A3 expression levels in human

liver (Green et al., 1998). Imipramine did inhibit profen glucuronidation suggesting that even at

low expression levels, UGT1A3 still contributed to profen glucuronidation.

These discrepancies in glucuronidation between expressed UGTs and UGTs in HLMs

show that simple screening of UGT activity in heterologous expression systems is not sufficient,

by itself, to identify the principal catalysts of drug glucuronidation *in vivo*. Our results

emphasize the need to compare glucuronidation activity of heterologously expressed UGT

enzymes with inhibitory experiments in microsomes from human tissues in order to obtain a

more accurate representation of the UGTs involved in drug glucuronidation. These studies also

emphasize the need for competitive inhibitory antibodies to test the individual contribution of

UGT enzymes to glucuronidation in microsomes from human tissues.

Rates of sulindac, sulindac sulfone, indomethacin, flurbiprofen, diclofenac, and naproxen

glucuronidation catalyzed by multiple UGTs decreased at increasing aglycone concentrations.

The most pronounced decreases were observed for indomethacin and diclofenac glucuronidation.

Indomethacin and diclofenac glucuronidation by UGT2B7 decreased 3% and 8%, respectively,

when concentrations were increased from 100 µM to 500 µM and further decreased 51% and

5%, respectively, when concentrations of NSAIDs were increased from 500 µM to 1000 µM.

The inhibition of glucuronidation activity at increasing aglycone concentrations has been

observed previously for entacapone glucuronidation by UGT1A9 (Lautala et al., 2000), (S)-

oxazepam glucuronidation by UGTs 1A9, 2B15, and in HLMs (Court et al., 2002), and for

catechol estrogen glucuronidation catalyzed by UGTs 1A1 and 1A3 (Lepine et al., 2004). The

cause of this decrease in glucuronidation rates at higher substrate concentrations is unknown.

Acyl glucuronides have been reported to react with proteins (Bailey and Dickinson, 2003). It is

possible that decreases in glucuronidation activity at higher substrate concentrations may be a

result of acyl glucuronide reactivity with UGT enzymes. At higher NSAID concentrations, we

also suspect that the membrane fluidity may be altered resulting in decreased enzymatic activity.

Substantial decreases in the rates of NSAID glucuronidation were not observed in pooled HLMs

and glucuronidation rates fit Michaelis-Menten kinetics from 1 µM to 1000 µM. The fact that

such decreases in glucuronidation rates were not observed in HLMs suggests that this effect is

unique to Supersomes[TM].

Pooled HLMs demonstrated the highest affinity for sulindac sulfone and indomethacin

glucuronidation. Results were consistent with the contribution of multiple UGT enzymes to the

catalysis of NSAID glucuronidation in hepatic tissue. UGTs 1A3 and 2B7 were implicated in

indene glucuronidation and UGTs 1A9, 2B4, and 2B7 in the glucuronidation of profen NSAIDs.

Here we report the identification of UGTs 1A1, 1A3, 1A9, 2B4, and 2B7 as important

catalysts of the glucuronidation of many structurally distinct NSAIDs. Consistent with our

findings, Sakaguchi and coworkers reported that expressed UGTs 1A3, 1A9, and 2B7 catalyzed

the glucuronidation of ibuprofen, flurbiprofen, ketoprofen, and diclofenac (Sakaguchi et al.,

2004). However, UGT1A1 expressed in HK 293 cells did not catalyze the glucuronidation of

NSAIDs (Sakaguchi et al., 2004). This difference probably is a result of higher UGT1A1 expression in the Sf9 cells than the HK 293 cells as well as the lower limits of detection of LC-MS/MS analysis used in our study compared to thin layer chromatography of radiolabeled compounds. Further support for the role of UGT1A1 in the glucuronidation of diclofenac, indomethacin, ketoprofen and naproxen has recently been suggested (Mano et al., 2005).

UGTs 1A7, 1A8, and 1A10 were catalysts of NSAID glucuronidation when examined by individually expressed UGTs. Consistent with these findings, Basu and coworkers reported that UGTs 1A7, 1A8, and 1A10 catalyzed the glucuronidation of ibuprofen, flurbiprofen, and indomethacin (Basu et al., 2004). These enzymes are expressed in extrahepatic tissues only, most notably, the intestine and colon (Mojarrabi and Mackenzie, 1998; Cheng et al., 1999). Acyl glucuronides are highly unstable entities that readily undergo acyl migration and esterase cleavage *in vivo* (Spahn et al., 1989; Shipkova et al., 2003). It is possible that glucuronides formed in the liver are cleaved at other organ sites and the parent compounds then undergo enterohepatic circulation. It is therefore likely that extrahepatic UGT enzymes play a role in NSAIDs metabolism. A role for UGTs 1A7, 1A8, and 1A10 in the glucuronidation of NSAIDs in these tissues still warrants further investigation.

The relative efficacy of the drugs in target tissues is probably a combined effect of the effective concentrations of each NSAID, the level of UGT protein expression, and/or the existence of polymorphisms affecting glucuronidation activity. Polymorphisms in the hepatic UGTs 1A1, 1A3, 1A9, 2B4, and 2B7 have been reported in different populations; however, their role in the altered metabolism of NSAIDs remains to be investigated. The identification of the UGTs involved in glucuronidation now allows for further studies examining the variability in metabolism among populations to optimize the therapeutic benefits of NSAIDs use.

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FOOTNOTES

FIGURE LEGEND

Figure 1. Structures of commonly used NSAIDs (glucuronidation occurs at the carboxyl group)

Figure 2. HPLC-UV separation of A) sulindac glucuronide, B) sulindac sulfone glucuronide, and

C) indomethacin glucuronide biosynthesized using pooled HLMs

Figure 3. Rates of NSAID Glucuronidation by Individual UGT Supersomes[TM]: S, sulindac; SS,

sulindac sulfone; In, indomethacin; Ib, ibuprofen; F, flurbiprofen; K, ketoprofen, D,

diclofenac; N, naproxen. Rates of glucuronidation were determined at 500 µM

concentrations of each NSAID as described in Materials and Methods. Rates are reported

as pmol/min/mg protein (note: different scales).

Table 1. LC-MS/MS Parameters for Analysis of NSAID Glucuronides

Glucuronide	Elution Time (min)	Parent Ion (*m/z*)	Daughter Ion (*m/z*)	Detection Limit
Sulindac	2.4	531	193	0.2 ng
Sulindac Sulfone	2.9	547	193	0.2 ng
Indomethacin	4.1	532	193	0.5 ng
Ibuprofen	3.4	381	193	0.5 ng
Flurbiprofen	2.3	419	193	2 ng
Ketoprofen	2.2	429	193	1 ng
Diclofenac	2.1	405	193	0.5 ng
Naproxen	2.4	470	193	0.5 ng

Table 2. Kinetics of NSAID Glucuronidation by Selected UGT Supersomes[TM]

NSAID	UGT	Apparent K_m (µM)	Apparent V_{max} (pmol/min/mg protein)	V_{max}/K_m (µl/min/mg protein)	Fit (R^2)
Sulindac					
	1A1[a]	5.2 (1.3)	31.7 (1.1)	6.1	0.92
	1A3[a]	19.6 (3.6)	99.2 (3.4)	5.1	0.97
	1A9[a]	68.0 (8.7)	8.0 (0.3)	0.1	0.98
	2B7[b]	124.4 (25.6)	229.2 (17.8)	1.8	0.96
Sulindac Sulfone					
	1A1[b]	5.2 (1.4)	459.6 (18.9)	88.3	0.95
	1A3[a]	15.0 (4.7)	429.8 (23.6)	28.7	0.91
	1A9[a]	44.6 (10.3)	7.5 (0.4)	0.2	0.93
	2B7[b]	34.1 (9.1)	1211 (76.5)	35.6	0.98
Indomethacin					
	1A1[c]	13.2 (2.8)	118.3 (6.3)	9.0	0.98
	1A3[c]	3.1 (0.7)	10.2 (0.4)	3.3	0.97
	1A9[c]	68.5 (16.5)	127.1 (15.0)	1.9	0.99
	2B7[c]	17.7 (4.7)	229.4 (16.9)	13.0	0.97
Ibuprofen					
	1A3[a]	413.2 (28.7)	146.5 (4.0)	0.4	1.0
	1A9[a]	98.4 (19.6)	219.4 (12.9)	2.2	0.96
	2B4[a]	171.0 (30.2)	57.5 (3.1)	0.3	0.98
	2B7[a]	30.0 (6.9)	266.5 (12.8)	8.9	0.95
Flurbiprofen					
	1A3[b]	201.8 (40.2)	472.9 (39.6)	2.3	0.98
	1A9[b]	42.7 (6.4)	263.1 (11.3)	6.1	0.98
	2B4[a]	86.4 (16.0)	213.2 (10.6)	2.5	0.96
	2B7[a]	6.1 (1.2)	217.6 (5.9)	35.7	0.96
Ketoprofen					
	1A3[a]	136.5 (17.6)	688.7 (25.9)	5.0	0.99
	1A9[a]	76.4 (15.3)	163.3 (8.6)	2.1	0.95
	2B4[a]	126.3 (10.7)	404.2 (9.9)	3.2	0.99
	2B7[a]	11.7 (2.5)	789.9 (30.1)	67.5	0.96
Diclofenac					
	1A3[c]	16.7 (6.2)	715.6 (73.0)	42.9	0.95
	1A9[c]	7.7 (1.8)	375.7 (19.9)	48.8	0.97
	2B4[c]	4.7 (0.9)	370.5 (13.0)	78.8	0.98
	2B7[c]	19.9 (4.1)	1323 (79.8)	66.7	0.98
Naproxen					
	1A3[a]	322.5 (67.6)	778.9 (60.0)	2.4	0.98
	1A9[a]	96.0 (20.9)	909.0 (54.3)	9.5	0.95
	2B4[a]	195.3 (32.3)	960.6 (50.3)	4.9	0.98
	2B7[b]	7.0 (1.9)	1613 (75.7)	230.4	0.95

a, rates fit Michaelis-Menten equation from 1 µM to 1000 µM concentrations of NSAID; b, rates fit Michaelis-Menten equation from 1 µM to 500 µM concentrations of NSAID; c, rates fit Michaelis-Menten equation from 1 µM to 100 µM concentrations of NSAID. Rates determined at NSAID concentrations from 1 µM to 1 mM under the following conditions: 0.5 mg/ml protein, 50 µg/mg alamethicin, 1 mM PMSF, 8.5 mM saccharolactone, 2 mM UDP-glucuronic acid, and 10 mM MgCl₂ in 50 mM potassium phosphate (pH 7). Kinetic parameters are reported as best-fit value (standard error).

Table 3: Kinetics of NSAID Glucuronidation by Pooled HLMs

NSAID	Apparent K_m (µM)	Apparent V_{max} (pmol/min/mg protein)	V_{max}/K_m (µl/min/mg protein)	Fit (R^2)
Sulindac	75.4 (26.5)	382.9 (35.4)	5.1	0.90
Sulindac Sulfone	34.1 (9.1)	663.4 (41.9)	19.5	0.93
Indomethacin	30.0 (6.7)	376.6 (17.3)	12.6	0.96
Ibuprofen	188.3 (16.8)	657.3 (18.5)	3.5	0.99
Flurbiprofen	56.3 (9.7)	446.1 (19.0)	7.9	0.97
Ketoprofen	140.7 (5.7)	742.2 (8.9)	5.3	1.0
Diclofenac	71.6 (23.6)	2685 (150.9)	37.5	0.96
Naproxen	75.3 (15.8)	1708 (93.9)	22.7	0.96

Rates determined at NSAID concentrations from 10 µM to 1 mM under the following conditions: 1 mg/ml protein, 50 µg/mg alamethicin, 1 mM PMSF, 8.5 mM saccharolactone, 2 mM UDP-glucuronic acid, and 10 mM $MgCl_2$ in 50 mM potassium phosphate (pH 7). Rates of glucuronidation were fit to the Michaelis-Menten equation and parameters are reported as best-fit value (standard error).

Table 4. Inhibition of NSAID Glucuronidation in Pooled HLMs by UGT-Selective Probes

[NSAID]	Bilirubin *(1A1)*	Estradiol *(1A1/2B7)*	Imipramine *(1A3)*	Propofol *(1A9)*	HDCA *(2B4/2B7)*	Morphine *(2B7)*
Sulindac						
10 µM	20	55	NI	22	73	66
500 µM	NI	34	NI	51	76	70
Sulindac Sulfone						
10 µM	29	51	NI	NI	53	16
500 µM	13	27	NI	NI	51	NI
Indomethacin						
10 µM	NI	54	NI	25	42	NI
500 µM	NI	31	NI	18	48	NI
Ibuprofen						
10 µM	15	42	37	26	59	21
500 µM	28	67	16	35	50	13
Flurbiprofen						
10 µM	25	61	18	15	55	17
500 µM	NI	60	21	26	35	NI
Ketoprofen						
10 µM	23	54	25	40	44	NI
500 µM	15	42	NI	NI	29	NI
Diclofenac						
10 µM	NI	21	NI	NI	21	NI
500 µM	NI	13	15	14	NI	NI
Naproxen						
10 µM	18	63	NI	32	62	29
500 µM	26	41	NI	27	36	20

NI = no inhibition (inhibition less than 10%). Rates determined at NSAID concentrations from 10 µM to 500 µM under the following conditions: 1 mg/ml protein, 50 µg alamethicin/mg HLM protein, 1 mM PMSF, 8.5 mM saccharolactone, 2 mM UDP-glucuronic acid, and 10 mM $MgCl_2$ in 50 mM potassium phosphate (pH 7) with and without 5 µM bilirubin or 50 µM estradiol, imipramine, propofol, HDCA or morphine. Values are reported as percent inhibition.
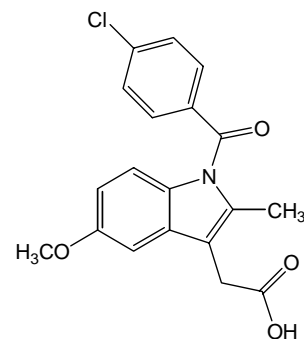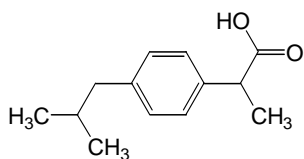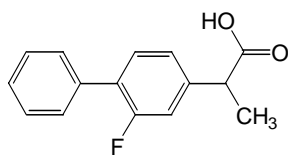
Figure 1

Indenes
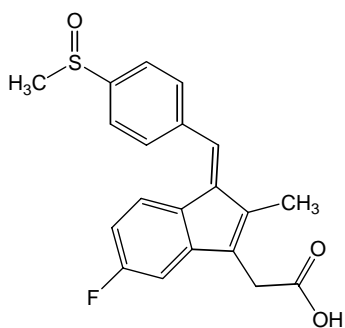
Sulindac
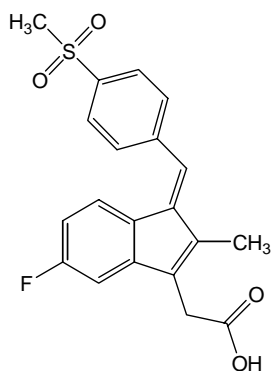
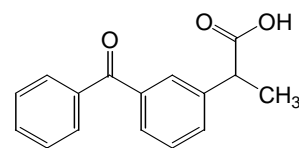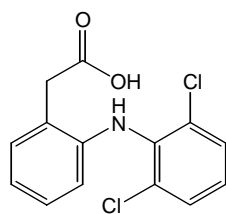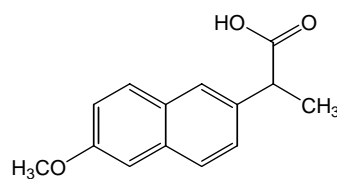Sulindac Sulfone

Indomethacin

Profens

Ibuprofen

Flurbiprofen

Ketoprofen

Others

Diclofenac

Naproxen

Figure 2

Figure 3