Evaluation of Hydroxyimine as Cytochrome P450-Selective Prodrug Structure

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Hydroxyimine derivatives of ketoprofen (**1**) and nabumetone (**2**) were synthesized and evaluated in vitro and in vivo as cytochrome P450-selective intermediate prodrug structures of ketones. **2** released nabumetone in vitro in the presence of isolated rat and human liver microsomes and in different recombinant human CYP isoforms. Bioconversion of **2** to both nabumetone and its active metabolite, 6-methoxy-2-naphthylacetic acid (6-MNA), was further confirmed in rats in vivo. Results indicate that hydroxyimine is a useful intermediate prodrug structure for ketone drugs.

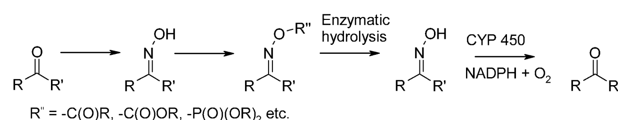
Introduction

The prodrug approach is a valuable tool to overcome limitations of a parent drug that would otherwise hinder its clinical use.[1] So far prodrug structures have mainly been developed for amine, hydroxyl, and carboxyl functionalities of drug molecules.[2] Only a few prodrug structures have been developed for molecules containing ketone functionality, although a ketone group is a common functional group in drug molecules. One promising strategy to apply the prodrug approach to a ketone compound is to synthesize an oxime structure via a hydroxyimine, which is oxidized to the corresponding ketone by microsomal cytochrome P450 (CYP) enzymes.[3] CYP enzymes are versatile xenobiotic metabolizing enzymes[4] and particularly abundant in the liver and intestinal tract, although they are expressed practically in every tissue of the body.[5] This wide substrate specificity of CYP enzymes can be exploited in prodrug technology. CYP enzyme-mediated reactions have been utilized in targeting drugs into the liver,[6] in CYP-based gene therapy,[7] and in activating a prodrug to an active drug during first-pass metabolism in the liver.[8]

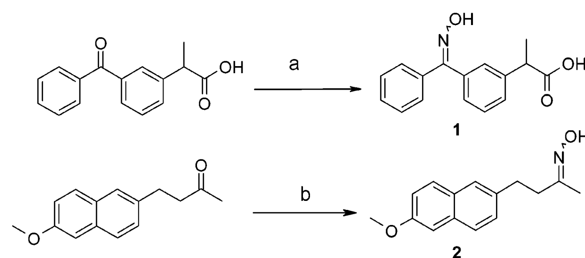
In the prodrug approach, hydroxyimine is especially suitable as an intermediate structure because it can be further derivatized, for example to an ester, phosphate, or carbamate. Thus oxime prodrugs are actually double-prodrugs requiring a two-step bioactivation process (Scheme 1). The oxime structure is first hydrolyzed enzymatically to hydroxyimine, which is further oxidized to the corresponding active ketone drug by CYP enzymes. Because the CYP-catalyzed oxidation reaction of hydroxyimine, which also leads to nitric oxide (NO) biosynthesis in biological systems, occurs in vivo mainly in liver, oximes can especially be used to improve site-specificity, especially liver-targeting, of a parent drug. Previously hydroxyimine structure has been used in very few prodrug molecules,[9−11] and their fundamental in vitro/in vivo-characterization has been lacking.

To evaluate the in vitro and in vivo usefulness of the hydroxyimine moiety as an intermediate prodrug structure for ketones, hydroxyimine derivatives of nabumetone and ketoprofen were synthesized. These compounds were chosen as model compounds of the ketone functionality because of their suitable detection properties and their low amount of other functional groups. In addition, nabumetone and ketoprofen are both nonsteroidal antiinflammatory drugs (NSAID), and the hydroxyimine can act as a NO-donating group. Some NSAIDs, e.g. aspirin, naproxen, and diclofenac, have been coupled to an NO-donating moiety which may have either protective (e.g. GI-sparing effect or protective role in some liver injuries)[12] or harmful effects (in conditions such as liver ischemia,[13] asthma,[14] or rheumatoid arthritis[15]) in the body. In this study, these two hydroxyimine intermediate prodrug structures are extensively characterized with respect to their physicochemical properties, chemical and enzymatic stability, formation of nitric oxide from the hydroxyimine structure, and the release of the parent drug in vitro and in vivo.

Scheme 1. The Two-Step Process of the Synthesis and Enzymatic Activation of an Oxime Prodrug to the Corresponding Ketone



Scheme 2[a]



[a] Reagents: (a) $NH_2OH\cdot HCl$, Pyr, dioxane, 75%, b) $NH_2OH\cdot HCl$, Pyr, EtOH, 94%.

Results and Discussion

Chemistry. The syntheses of ketoprofen and nabumetone hydroxyimines (**1** and **2**, respectively) were obtained in good yields by treating nabumetone or ketoprofen with hydroxylamine hydrochloride in the presence of pyridine (Scheme 2). The *E*- and *Z*-isomers of **2** could be isolated by column chromatography. However, all the assays described below were made using the mixture of *E*- and *Z*-isomers.

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Table 1. Aqueous Solubilities and Distribution Coefficients (log D) of Ketoprofen, Nabumetone, and Their Hydroxyimines (**1**, **2**) in Buffer Solutions (mean ± SD, $n = 3$ unless otherwise mentioned)

	aqueous solubility (μg/mL)		log D	
compd	pH 5.0	pH 7.4	pH 5.0	pH 7.4
ketoprofen	740 ± 30	465 ± 76 mg/mL	2.1 ± 0.01	0.008 ± 0.02
1	1800 ± 300	116 ± 9.2 mg/mL[a]	2.0 ± 0.01	−0.04 ± 0.01
nabumetone	6.4 ± 0.2	4.3 ± 0.4	3.2 ± 0.04	3.3 ± 0.04
2	3.0 ± 0.1	2.0 ± 0.1	3.6 ± 0.04	3.6 ± 0.04

[a] $n = 2$.

Aqueous Solubility and Lipophilicity. Since hydroxyimines are designed to be intermediate structures, not final prodrugs, and be further derivatized, it may be desired that their physicochemical properties do not differ from parent molecules. However, it is important to find out the effects of hydroxyimine structure on physicochemical properties of a parent drug. Therefore, the aqueous solubilities and distribution coefficients (log D) of **1** and **2** and their parent compounds at pH 7.4 and 5.0 were determined (Table 1). The aqueous solubilities of both hydroxyimines were lower than their parent compounds at pH 7.4. At pH 5.0 the hydroxyimine structure enhanced the aqueous solubility of ketoprofen but diminished the aqueous solubility of nabumetone. The distribution coefficients of **1** and **2** were similar compared to their parent compounds at both pH 7.4 and 5.0. In conclusion, the differences in aqueous solubility and lipophilicity between both **1** and **2** compared to their parent drugs were not significant.

Stability in Buffer Solutions and in 80% Human Serum. Chemical degradation of both **1** and **2** followed first-order kinetics at pH 1.0−9.0 and the half-lives are presented in Table 2. The hydrolysis of **2** was faster than that of **1** at all pH values, probably due the aromatic nature around the hydroxyimine structure of ketoprofen, which makes the hydroxyimine nitrogen less basic than that of **2**. Also the bulk aromatic rings around the carbonyl carbon of **1** increase the steric hindrance, thus slowing down the nucleophilic attack of the water molecule, which can be understood by examining the mechanism of hydrolysis illustrated in Scheme 3. The nitrogen of hydroxyimine is first protonated, which leads to the nucleophilic attack of the water molecule to the carbonyl carbon. Second, protonation of the nitrogen makes NH_2OH a good leaving group, the C−N bond breaks, and a double bond of the corresponding ketone is formed. The chemical stability of **2** increases with pH (Table 2), and in the pH-range of pH 5.0 to 9.0 the half-lives of **2** are over 16 h.

Both **1** and **2** showed high stability toward enzymatic hydrolysis in 80% human serum (pH 7.4) with the half-life of approximately 800 and 600 h, respectively (Table 2).

Enzymatic Degradation. The oxidation of hydroxyimines was determined in liver microsomes from humans, untreated rats, and rats treated with CYP-inducing agents (dexamethasone (CYP3A), phenobarbital (CYP2B), 3-methylcholantrene (CYP1A)).[16] The half-lives of the oxidation reactions were determined for both **1** and **2** and the formation of ketoprofen also for **1**. During the oxidation of **2**, two major metabolites, nabumetone and 6-methoxy-2-naphthylacetic acid (6-MNA), were formed but were not quantified due to their fast metabolism to several other metabolites.

The oxidation of **2** occurred more than 1000-fold faster than that of **1** with half-lives of 2 to 58 min and 72 to 167 h, respectively (Table 3). The degradation of **2** was fastest with 3-methylcholanthrene-treated microsomes with a half-life of 2 min followed by phenobarbital- and dexamethasone-treated rat microsomes, respectively. For both compounds **1** and **2**, the reaction was slowest in control rat microsomes ($t_{1/2}$ values were 10020 and 58 min, respectively). The formation of ketoprofen from **1** was also affected by enzyme induction, the most efficient reaction being mediated by dexamethasone-pretreated rat liver microsomes (Table 4). These oxidation reactions did not occur without microsomes or NADPH, proving that the oxidation reactions are catalyzed by microsomal oxidative enzymes.

The difference in degradation rates between **1** and **2** is significant (Table 3). This is likely due to CYP substrate specificity of the two compounds. Since the active site of CYP enzymes is negatively charged, **1** bearing a negative charge is suggested to be poor substrate of CYPs. Also the aromatic rings enhance the steric hindrance around the hydroxyimine structure and may also explain the slow oxidation rate. Since the oxidation of **2** was much more effective compared to **1**, the metabolism of **2** was investigated in detail using human recombinant CYP enzymes in vitro.

Formation of Nabumetone in Human Recombinant CYP Enzymes. Incubation of 100 μM **2** was performed with eight different human CYP enzymes. All tested CYP forms (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) metabolized **2** to nabumetone (Table 5). The nabumetone formation rate was most rapid with CYP3A4 and CYP2A6 with rates of 5.6 and 5.4 nmol of metabolite (mg of protein)$^{-1}$ (60 min)$^{-1}$, respectively. According to these results it is obvious that the enzymatic oxidation of hydroxyimine is catalyzed by several human CYP enzymes in vitro.

All but one CYP form (CYP2C9) followed Michaelis−Menten kinetics and the parameters K_m and V_{max} could be determined. Kinetic studies indicated that CYP3A4 possessed the highest V_{max} (1600 min^{-1}) and CYP2B6 the lowest (290 min^{-1}) (Table 6). K_m values of **2** varied between 40 μM (CYP2E1) and 200 μM (CYP3A4). According to the specificity constant (V_{max}/K_m), the most efficient enzymes were CYP2A6 and CYP2E1 with specificity constants of 11.9 μM^{-1} min^{-1} and 11.0 μM^{-1} min^{-1}, respectively.

Determination of Nitrite. The release of nitric oxide from **2** during incubation in microsomes from dexamethasone-induced rats was determined as nitrite via nitrate reductase by the standard Griess reaction.[3] Nitric oxide was formed during the 4 h incubations at almost equimolar quantities, as nabumetone was formed (11.0 ± 0.8 and 13.0 ± 0.24 nmol ($n = 2$), respectively) and it was not formed without the presence of microsomes or without **2**. Although small quantities of other metabolites were formed during the incubation and these were not quantified, this study does demonstrate that nitric oxide is formed simultaneously with nabumetone during incubation.

Conversion of 2 to Nabumetone in Vivo. The conversion of **2** to nabumetone and 6-MNA, the main metabolite of nabumetone, was demonstrated in vivo in rats after per os administration. Other minor metabolites formed were not assayed. Since 6-MNA is partially conjugated with glucuronic acid in its metabolism,[17,18] the amount of 6-MNA was determined after the alkaline hydrolysis[19] to break the ether bond of the glucuronide. The amounts of **2** and nabumetone were determined without hydrolysis, since **2** hydrolyzes to nabumetone under alkaline hydrolysis conditions.

The recovery of the extractions from urine, determined by using naproxen as an internal reference, was high with an average of 97% (from 70 to 100%). The variance of urine volumes between rats was substantial with an average of 17.9 ± 8.5 mL (from 8.9 to 39.9 mL). In general, the variation between rats was quite high, whether assessed as nmol/total urine sample or as nmol/mL of urine.

Table 2. Rates of Hydrolysis of Nabumetone (**1**) and Ketoprofen Hydroxyimine (**2**) in Buffer Solutions and Human Plasma at 37 °C (mean ± SD, n = 3 unless otherwise mentioned)

compd	$t_{1/2}$ (min) pH 1.0	$t_{1/2}$ (min) pH 3.0	$t_{1/2}$ (min) pH 5.0	$t_{1/2}$ (min) pH 7.4	$t_{1/2}$ (min) pH 9.0	$t_{1/2}$ (min) 80% human serum
1	360.0 ± 1.8[a]	n.d.[b]	–[c]	–[c]	–[c]	47760 ± 4157
2	6.0 ± 0.15[d]	11 ± 0.6[d]	980 ± 153[d]	43200 ± 7200	–[c]	35460 ± 6660

[a] n = 2. [b] n.d. = not determined. [c] No degradation was observed during the four weeks' incubation, [d] n = 4.

Scheme 3. The Mechanism of the Chemical Hydrolysis of Hydroxyimine to Ketone in Acidic Conditions

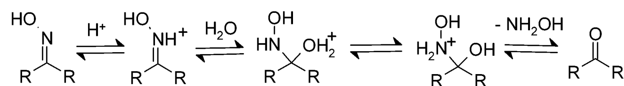

Table 3. Half-Lives of the Oxidation of **1** and **2** in Liver Microsomes from Untreated (CTRL), Dexamethasone (DEX)-, 3-methylcholanthrene (3-MC)-, and Phenobarbital (PB)-Treated Rats and from Humans (mean ± SD, n = 3)

microsomes	$t_{1/2}$ of **1** (min)[a]	$t_{1/2}$ of **2** (min)
without microsomes	–[b]	–[b]
CTRL	10020	58 ± 3.8
DEX	4320	46 ± 5.0
PB	5040	28 ± 2.7
3-MC	6000	2.0 ± 0.16
human	5016	51± 3.6

[a] n = 1. [b] No oxidation was detected during incubation.

Table 4. Formation of Ketoprofen in Liver Microsomes from Untreated (CTRL), Dexamethasone (DEX)-, 3-methylcholanthrene (3-MC)-, and Phenobarbital (PB)-Treated Rats and from Humans[a]

incubation conditions	**1**[b]	incubation conditions	**1**[b]
CTRL	0.12	3-MC	1.46
− NADPH	0.02	− NADPH	0.14
DEX	6.72	PB	1.39
− NADPH	0.48	− NADPH	0.10
+ketoconazole	2.50	human	0.80[c]
		without microsomes	–[d]

[a] n = 1. [b] Results are expressed as nmol of metabolite (mg of protein)$^{-1}$ (24 h)$^{-1}$. [c] Results are expressed as nmol of metabolite (mg of protein)$^{-1}$ (4 h)$^{-1}$. [d] No formation of ketoprofen was observed.

Table 5. Formation of Nabumetone from Nabumetone Hydroxyimine (**2**) in Human Recombinant Enzymes (mean ± SD, n = 2)

recombinant enzyme	**2**[a]	recombinant enzyme	**2**[a]
CYP1A2	2.71 ± 0.78	CYP2C19	4.5 ± 0.52
CYP2A6	5.42 ± 0.20	CYP2D6	3.96 ± 0.06
CYP2B6	4.65 ± 0.09	CYP2E1	4.02 ± 0.01
CYP2C9	1.43 ± 0.04	CYP3A4	5.62 ± 0.14

[a] Results are expressed as nmol of metabolite (mg of protein)$^{-1}$ (60 min)$^{-1}$.

The amount of 6-MNA in Group 1 administered with **2** was 23% compared to that of in Group 2, which were administered with nabumetone (Table 7). The combined quantity of the nabumetone and 6-MNA in the total urine samples of Group 1 was 37% of the quantity of 6-MNA in Group 2 (8.4 ± 2.9 and 23.0 ± 8.3 nmol, respectively), revealing that oxidation of **2** to nabumetone occurs also in vivo. This bioconversion is thought to be enzymatic after absorption, not chemical in GI-tract. Since the pH of the fed rat stomach is approximately 4.5[20] and the relatively large volume of oil administered can increase the gastric pH, **2** is suggested to be stable enough to be absorbed intact ($t_{1/2}$ approximately 16 h at pH 5.0). The reason for unchanged **2** in the rat urine samples of Group 1 is probably due to the high dose of **2**, which may have caused saturation of the CYP enzymes in the liver. Thus a part of prodrug is secreted as intact prodrug. To some extent, the situation is the same in samples of Group 2 rats, in which the unchanged nabumetone was also presented in urine. **2** was also found to be stable enough

Table 6. Enzyme Kinetic Parameters for Nabumetone Hydroxyimine (mean, n = 3)

recombinant enzyme	K_m (μM) of **2**	95% confidence intervals	V_{max} [a]	95% confidence intervals	V_{max}/K_m
CYP1A2	180	0−430	670	140−1190	3.7
CYP2A6	90	60−120	1070	900−1240	11.9
CYP2B6	70	40−110	290	30−350	4.14
CYP2C9	–[b]		–[b]		–[b]
CYP2C19	60	30−90	380	300−450	6.3
CYP2D6	90	30−140	870	630−1110	9.7
CYP2E1	40	30−60	440	390−480	11
CYP3A4	200	30−360	1600	780−2400	8

[a] pmol nabumetone (nmol CYP)$^{-1}$ (min)$^{-1}$. [b] Kinetics does not follow Michaelis−Menten kinetics.

in rat urine at 37 °C ($t_{1/2}$ = 69 ± 14 h (n = 4), data not shown). No 6-MNA and only small amounts of nabumetone was formed during the incubation of **2** in rat urine at 37 °C. In summary, the data shows that **2** is converted to both nabumetone and 6-MNA in rats in vivo.

Determination of ALAT and CRP. NO plays an important physiological role in the liver, the effect being either protective or toxic depending on the source of NO.[13] The levels of alanine aminotransferase (ALAT) and C-reactive protein (CRP) in rat serum samples were assayed to determine whether administration of **2** and the released NO could evoke tissue damage in liver. Damage to liver cells leads to the release of acute phase protein ALAT and inflammation, reflected by elevated CRP levels in serum.[21]

The levels of ALAT and CRP were similar in groups 1−3 and no differences between rats administered nabumetone or **2** and control rats were detected. ALAT values were 70 ± 11, 70 ± 13, and 71 ± 11 U/L for groups 1, 2, and 3, respectively. CRP values were 4.2 ± 0.4 mg/L for each group. This result suggests that no overt acute toxic reaction occurred in the liver of rats fed with **2**.

Conclusions

Hydroxyimine derivatives of ketones offer a feasible tool to modify properties of drugs containing a ketone functionality. The results show that nabumetone hydroxyimine (**2**) was oxidized to nabumetone in vitro by microsomal CYP enzymes. Activation of **2** to nabumetone and other metabolites occurred also in vivo in rats. Urine samples of **2** treated rats contained nabumetone, 6-MNA, and also **2** probably due to treatment with high dose of prodrug causing the saturation of liver enzymes. The present study shows that the hydroxyimine structure is activated to the corresponding ketone both in vitro and in vivo, and the released nitric oxide causes no acute liver toxicity after peroral administration in rats. Detailed studies on each oxime derivative of hydroxyimine are needed for the full characterization of the physicochemical properties and the activation of prodrugs. The present study reveals that the hydroxyimine is a potential intermediate prodrug structure especially for ketone drugs and can easily be further derivatized using appropriate pro-moieties depending upon the purpose of prodrug application.

Experimental Section

Chemistry. All the described reactions were monitored by thin-layer chromatography using aluminum sheets precoated with Merck

Table 7. Formation of Nabumetone and 6-MNA from Nabumetone Hydroxyimine (**2**) in Vivo in Rat (mean ± SD, $n = 3$)

animal group	6-MNA nmol/total urine	6-MNA nmol/mL of urine	**2** nmol/total urine	**2** nmol/mL of urine	nabumetone nmol/total urine	nabumetone nmol/mL of urine
105 mg/kg of **2** (group 1)	5.2 ± 1.7	0.23 ± 0.06	46.7 ± 32.3	1.8 ± 1.0	3.3 ± 1.6	0.15 ± 0.08
100 mg/kg of nabumetone (group 2)	23.0 ± 8.3	1.48 ± 0.75	n.d.[a]	n.d.[a]	9.3 ± 4.9	0.59 ± 0.46
CTRL rats (group 3)	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]

[a] Not detected.

silica gel 60 F_{254}. Samples were visualized by UV-light. Column chromatography was executed on Merck silica gel 60 F_{254} (0.063−0.200 mm mesh). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX500 spectrometer (Bruker, Rheinstetter, Germany) operating at 500 and 125.76 MHz at 25 °C. TMS was used as an internal reference. NMR data is found on the Supporting Information. Electrospray ionization mass spectra were acquired by an LCQ quadrupole ion trap mass spectrometer with an electrospray ionization source (Finnigan MAT, San Jose, CA). Elemental analyses were carried out on a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer. All reagents and enzymes were obtained from commercial suppliers and were used without further purifications.

2-{3-[(*E,Z*)-(hydroxyimino)(phenyl)methyl]phenyl}propionic Acid (1). 2-(3-Benzoylphenyl)propanoic acid (2.0 g, 7.86 mmol), hydroxylamine hydrochloride (1.52 g, 21.9 mmol), and pyridine (1.76 mL, 21.9 mmol) were dissolved in dioxane (50 mL) and refluxed for 24 h. Solvent was evaporated, and the residue was diluted with 40 mL saturated Na_2CO_3. The obtained solution was washed twice with diethyl ether (40 mL). The water phase was acidified with concentrated HCl and extracted with diethyl ether (3 × 30 mL). The organic phase was dried over Na_2SO_4 and evaporated to dryness to give a mixture of **1a** 2-{3-[(*E*)-(hydroxyimino)(phenyl)methyl]phenyl}propanoic acid and **1b** 2-{3-[(*Z*)-(hydroxyimino)(phenyl)methyl] phenyl}propanoic acid as a white solid (1.59 g, 75%, E:Z ratio 50:50). ESI-MS: 268.0 (M − 1). Anal. ($C_{16}H_{15}NO_3$) C, H, N.

(2*E*)-4-(6-Methoxy-2-naphthyl)butan-2-one Oxime and (2*Z*)-4-(6-Methoxy-2-naphthyl)butan-2-one Oxime (2). 4-(6-Methoxy-2-naphthyl)butan-2-one (1 g, 4.38 mmol), hydroxylamine hydrochloride (609 mg, 8.76 mmol), and pyridine (707 μL, 8.76 mmol) were dissolved in ethanol (22 mL) and refluxed for 24 h. Solvent was evaporated, and the residue was diluted with Et_2O. The obtained solution was washed with saturated Na_2CO_3 and water, dried over Na_2SO_4, and evaporated to dryness. Flash chromatography on SiO_2 (petrol ether:ethyl acetate 4:1) gave a mixture of **2a**, (2*E*)-4-(6-methoxy-2-naphthyl)butan-2-one oxime, and **2b**, (2*Z*)-4-(6-methoxy-2-naphthyl)butan-2-one oxime, as a white solid (total yield 1 g, 4.12 mmol, 94%). ESI-MS: 244.0 (M + 1). Anal. $C_{15}H_{17}NO_2$: C, H, N.

HPLC Analysis. HPLC analysis was performed using a Beckman HPLC system, which consisted of a Beckman System Gold Programmable Solvent Module 126, a Beckman System Gold Detector Module 166 (wavelength 254 nm for ketoprofen and **1**; 225 nm for nabumetone and **2**), and Beckman System Gold Autosampler 507e and a Zorbax Eclipse SB−C18 (4.6 mm × 150 mm, 5 μm) analytical column (Agilent Technologies Inc., Little Falls, Wilmington, DE). Separations were performed with isocratic elution by using a mobile phase consisting of either 90% (v/v) acetonitrile and 2.2% (v/v) acetic acid with a ratio of 50:50 (ketoprofen and **1**) or 90% (v/v) acetonitrile and 20 mM phosphate buffer (pH 2.5) with a ratio of 40:60 (nabumetone and **2**) at a flow rate of 1.0 mL/min at 25 °C.

Aqueous Solubility. The aqueous solubilities of nabumetone, ketoprofen, and their hydroxyimines were determined at room temperature in phosphate buffer (0.16 M) at pH 7.4 and in acetate buffer (0.05 M) at pH 5.0 after 48 h of stirring as described previously[23] and analyzed by HPLC.

Distribution Coefficient. The distribution coefficients (log D) were determined at room temperature with a 1-octanol−phosphate buffer system at pH 7.4 and a 1-octanol−acetate buffer system at pH 5.0 after 60 min of stirring as described previously[24] and analyzed by HPLC.

Hydrolysis in Aqueous Solutions. The rates of the chemical hydrolysis of **1** and **2** were determined at 37 °C in borate buffer (0.1 M) at pH 9.0, phosphate buffer (0.16 M) at pH 7.4, acetate buffer (0.05 M) at pH 5.0, citric acid buffer (0.04 M) at pH 3.0 (only for **2**), and HCl buffer (0.14 M) at pH 1.0 as described earlier[23] and analyzed by HPLC. Pseudo-first-order half-lives ($t_{1/2}$) for the hydrolysis of prodrugs were calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug versus time.

Hydrolysis in Human Serum. The rate of enzymatic hydrolysis of prodrugs was determined in 80% human serum diluted with 0.05 mM phosphate buffer (pH 7.4) at 37 °C as described previously[24] and analyzed by HPLC. Pseudo-first-order half-life ($t_{1/2}$) for the hydrolysis of **2** was calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug versus time.

Preparation of Human Hepatic Microsomes. Human liver samples were obtained from the University Hospital of Oulu as surplus from kidney transplantation donors (approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Oulu, Finland). The livers were transferred to ice immediately after surgical excision, cut into pieces, snap-frozen in liquid nitrogen, and stored at −80 °C until the microsomes were prepared by standard differential ultracentrifugation. The metabolic characteristics of these microsomes have been published earlier.[25] A weight-balanced microsomal pool of seven liver microsomal preparations which have been extensively characterized for primary metabolic screening was employed. Baculovirus-insect cells expressing human CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were purchased from Gentest Corp. (Woburn, MA).

Preparation of Rat Hepatic Microsomes. Microsomes were prepared as previously reported[26] and stored at −80 °C until use. Protein concentrations were determined by using the Biorad Protein Assay (Bio-Rad, Hercules), and cytochrome P450 contents were determined as reported earlier.[27]

In Vitro Metabolism and Enzyme Kinetic Analyses of Hydroxyimines. The in vitro metabolism of **1** and **2** was evaluated in isolated liver microsomes from humans, untreated rats, and rats treated with inducing agents. A typical incubation mixture, in a final volume of 150 μL-1500 μL, contained 20 μM prodrugs in phosphate buffer for **1** and in ethanol for **2**, 50 mM sodium phosphate buffer (pH 7.4), and 100 μL liver microsomes. Ethanol concentration was 1% or less. The reaction was started by the addition of a NADPH-regenerating system (1.15 mM NADP, 12.5 mM isocitric acid, 56.25 mM KCl, 187.5 mM Tris-HCl, pH 7.4 12.5 mM $MgCl_2$, 0.0125 mM $MnCl_2$, and 1.2 U isocitric acid dehydrogenase) following 2 min of preincubation at 37 °C. In blank solutions liver microsomes and in samples without NADPH, the NADPH solution was replaced with the same volume of water. The reactions were terminated by the addition of the same amount of ice-cold acetonitrile as the sample. The samples were kept on ice and centrifuged for 15 min at 11000 rpm, and the supernatant was analyzed by the HPLC.

Incubations of **2** with human recombinant P450s were performed using conditions described before for liver microsomes, except the mixture contained 5 pmol of P450 (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) instead of microsomes. Enzyme kinetic studies of oxidation of **2** to nabumetone by CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were also conducted in the same conditions using 10 μM−200 μM of **2** and incubating for 60 min.

Half-lives ($t_{1/2}$) for the enzymatic degradation of prodrug at different liver microsomes were calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug against time.

Determination of Nitrite. Determination of nitrite (NO_2^-) was carried out as described previously.[9]

Animal Treatments. Nabumetone and **2** were dissolved in 10.0 mg/mL and 10.5 mg/mL turnip rape oil, respectively. Wistar male rats (aged, weight 221−339 g) were randomly assigned into three groups of five rats. Rats were adapted for 48 h to metabolic cages. After 24 h, urine and feces were collected and rats were treated by administering either **2** (100 mg/kg, Group 1), nabumetone (105 mg/kg, Group 2), or oil only (2 mL/rat, Group 3) by gavage. After 24 h of drug administration, the rats were decapitated, and urine, blood, and liver samples were collected and frozen at −78 °C. The animal experiments were approved by the Ethics Committee for Animal Experiments of the University of Kuopio.

Rat Urine Sample Preparations. Rat urine samples were frozen and stored at −78 °C until analysis. **2**, nabumetone, and 6-MNA were analyzed by diluting 250 μL of urine sample with 210 μL of water and vortex mixed. 40 μL of internal standard solution (naproxen) was added. **2** and nabumetone were applied to the C18 solid-phase extraction cartridges (Discovery DSC-18; Supelco, Bellefonte, PA), dried by aspiration of air, eluted with 3 mL of 40% hexane in ethyl acetate, and evaporated to dryness under a nitrogen stream at 40 °C. 250 μL of 1 M NaOH solution was added to the sample containing 6-MNA, vortex mixed, and allowed to stand for 1 h at room temperature. 250 μL of 1 M HCl solution was added, vortex mixed, and applied to the C18 solid-phase extraction cartridges. After drying, the analytes were eluted with 3 mL of 40% hexane in ethyl acetate and evaporated to dryness under a nitrogen stream at 40 °C. After evaporation, the samples were dissolved to 250 μL of 50% acetonitrile in water and measured by HPLC. The quantities of analytes were determined using external, rat urine spiked standards (nabumetone, **2** and 6-MNA), and the recovery of the extractions was determined with internal standard (naproxen).

Determination of ALAT and CRP from Rat Liver Samples. The analysis of amounts of alanineaminotransferase (ALAT) and C-reactive protein (CRP) was performed at the Laboratory Centre of Kuopio University Hospital. The ALAT assay was performed by a kinetic method (by ECCLS/IFCC guideline) and CRP assay by immunoturbidometrics.

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Supporting Information Available: Elemental analysis data for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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