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The First Bioreversible Prodrug of Metformin with Improved Lipophilicity and Enhanced Intestinal Absorption

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Metformin is a potent antidiabetic agent and currently used as a first-line treatment for patients with type 2 diabetes. Unfortunately, the moderate absorption and uncomfortable gastrointestinal adverse effects associated with metformin therapy impair its use. In this study, two novel prodrugs of a biguanidine functionality containing antidiabetic agent, metformin, were designed, synthesized, and evaluated in vitro and in vivo to accomplish improved lipophilicity and, consequently, enhanced oral absorption of this highly water-soluble drug. These results represent that the more lipophilic prodrug **2a** biotransformed quantitatively to metformin mainly after absorption. The enhanced oral absorption consequently promoted the bioavailability of metformin from 43% to 65% in rats. Thus, this novel prodrug may offer a solution to reduce the required daily doses of metformin, which may decrease the uncomfortable adverse effects associated with metformin therapy.

Introduction

Diabetes mellitus is one of the most seriously growing public health problems, especially in the Western world. The number of people diagnosed with diabetes has increased explosively worldwide during the past decades, and recently it has been estimated that there will be over 300 million people with this disease in 2025.[1,2] Diabetes is an enormous economic burden in the industrialized Western world, where it accounts for huge expenditures of the healthcare costs annually.[3,4] Moreover, diabetes is a leading cause of blindness, kidney failure, cardiovascular disease, and amputations. This serious chronic disease encompasses a wide variety of conditions that are characterized by elevated plasma glucose or hyperglycemia arising as a consequence of a relative or absolute deficiency of insulin secretion, resistance to insulin, or both.[3,5] The diversity splits into two main categories: type 1, insulin-dependent diabetes mellitus (IDDM[a]); prevailing type 2, noninsulin-dependent diabetes mellitus (NIDDM).

Type 2 diabetic patients are treated with lifestyle modification through increased physical activity and dietary management. However, most of the patients require an addition of an oral antidiabetic agent to achieve satisfactory glycemic control. Metformin, *N*,*N*-dimethylimidodicarbonimidicdiamide, is a potent insulin-sensitizing biguanidine used to treat type 2 diabetes[6−8] and usually considered to be a first-line treatment, particularly in obese and/or hyperlipidemic NIDDM patients.[9] The antihyperglycemic effects of metformin are not only the inhibition of intestinal glucose absorption and the improvement of peripheral and hepatic insulin sensitivity but also the reduction of hepatic glucose production and the enhancement of peripheral glucose utilization, although the exact mechanism of action is still uncertain.[8,10,11] In contrast to the other antidiabetic agents, metformin, similar to the other biguanides, does not stimulate insulin secretion and evoke hypoglycemia. Furthermore, metformin may have special benefits in overweight patients with type 2 diabetes, since it does not promote weight gain.[6−8] In addition, metformin has favorable effects on dyslipidemia,[12] hypertension,[13] vascular function,[14] and fibrinolytic activity,[15] which are very beneficial to patients with NIDDM, the major risk factor group of atherosclerosis or cardiovascular disease. Unfortunately, this effective but highly basic antihyperglycemic agent is fully protonated under physiological conditions and therefore slowly and incompletely absorbed from the upper intestine after oral administration. Together with a rapid kidney excretion,[16] metformin suffers from variable bioavailability and causes uncomfortable gastrointestinal adverse effects at effective doses (0.5−2 g per day), such as abdominal discomfort and pain, nausea, vomiting, diarrhea, anorexia, and metallic taste, for nearly 30% of patients.[17−20] Previously, various formulation strategies, for example, extended-release formulations, have been developed attempting to ameliorate the malabsorption of metformin with trivial results.[21,22]

Prodrugs are pharmacologically inactive or less active bioreversible derivatives of drug molecules utilized to improve the unfavorable physicochemical, pharmaceutical, or biopharmaceutical properties of a parent drug.[23−25] Probably one of the most commonly introduced prodrug strategies is masking hydrogen bonding groups of an active compound to

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[a] Abbreviations: AUC, area under the plasma concentration−time curve; *F*, bioavailability; Cl, clearance; EDTA, ethylenediaminetetraacetic acid; IDDM, insulin-dependent diabetes mellitus; HP-β-CD, 2-hydroxypropyl-β-cyclodextrin; MW, microwave; NIDDM, non-insulin-dependent diabetes mellitus; OCT1, organic cation transporter 1; ROS, reactive oxygen species.

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achieve good membrane permeability for high passive transcellular absorption after oral administration. To date, the study of prodrugs of guanidines is confined,[26−30] despite the commonness and importance of guanidines and biguanidines in medical applications.[31,32] Using typical biodegradable prodrugs, such as amides and carbamates, is not viable strategy to prepare more lipophilic and membrane permeable prodrugs because of the powerful cyclization behavior of metformin.[33] In the present study, a novel prodrug approach of sulfenamides,[34,35] which has been previously developed to NH-acidic compounds, was applied to metformin. Consequently, the prodrugs **2a** and **2b** (Figure 1) were designed and synthesized, and the potential of this prodrug approach was evaluated in vitro and in vivo. As a result, we significantly improved the lipophilicity and the oral drug bioavailability of metformin by this prodrug approach, which is anticipated to endow clinical benefits, as the daily dosages could be decreased.

Results and Discussion

Design and Syntheses of Metformin Prodrugs. Two prodrugs of metformin were designed on the basis of sulfenamide prodrugs of NH-acidic compounds (Figure 1).[24,34,35] Sulfenamides have a covalent bond between the nitrogen atom and bivalent sulfur atom. Since the sulfur atom is susceptible to nucleophilic attack and subsequent displacement reaction, the N−S bond can act as a bioreversible bond between the parent drug and the prodrug promoiety. It has been proposed that free endogenous thiols, like glutathione and cysteine, participate in the bioactivation of sulfenamide prodrugs.[24,34] The advantage of this prodrug approach is that the R-group in the promoiety is not explicitly defined, which allows introduction of either hydrophilicity or lipophilicity to the prodrug. Since the more lipophilic prodrugs release metformin shortly after absorption, the risk of lactatic acidosis should not be relevant with these novel prodrugs of metformin. In the present study, two lipophilic prodrugs of metformin, **2a** and **2b** (cyclohexyl and phenyl derivatives, respectively), were synthesized in a straightforward manner by a substitution reaction with adequate yields (Scheme 1). Thiophthalimides were found to be the most efficient starting compounds to prepare the prodrugs of metformin, compared to sulfuryl chlorides or bromides. As far as we are aware, this novel prodrug structure is applied for the first time to a basic compound (the pK_a value of metformin hydrochloride is 12.4) in the present study.

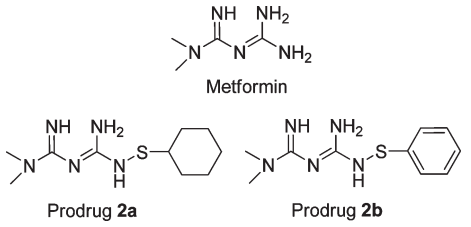


Figure 1. Structures of metformin and two novel prodrugs of metformin.

Stability of Metformin Prodrugs in Buffer and H_2O_2 Solutions. The chemical stabilities of the prodrugs **2a** and **2b** were studied in aqueous buffer solutions at pH values ranging from 1.0 to 9.0 and in hydrogen peroxide (H_2O_2) solution at 37 °C (Table 1). The hydrolysis of the prodrugs followed pseudo-first-order kinetics and released metformin. The prodrugs were reasonably stable in aqueous buffer solutions ($t_{1/2} \gtrsim 80$ h) at pH 4.0 and 7.4. In the acidic as well as oxidative conditions, these prodrugs showed lower stability ($t_{1/2}$ = 12−478 min). Since the releasing aromatic promoiety of the prodrug **2b** is highly resonance stabilized in the transition state, it is more susceptible to the cleavage from the prodrug than that of the prodrug **2a**. Therefore, it is possible that the conversion of the prodrug **2b** in vitro may be mediated by a free radical mechanism.

Distribution Coefficients of Metformin and Metformin Prodrugs in Octanol/Water. The octanol−water distribution coefficients (log D) of the prodrugs **2a** and **2b**, as well as metformin, were determined at pH values of 4.0 and 7.4 (Table 2). The log D values of the prodrugs were −1.10 to 0.49 and thus significantly higher and closer to the ideal log P than those of metformin (−3.37 and −3.41 at pH 4.0 and 7.4, respectively).[36] According to these data, it could be assumed that these more lipophilic prodrugs could greatly enhance the absorption of metformin, which consequently could yield higher bioavailability of metformin.

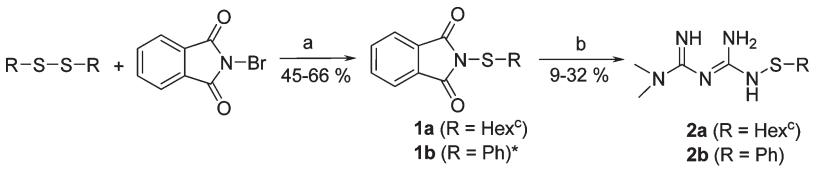
In Vitro Bioconversion of Metformin Prodrugs. The bioactivation of the prodrugs **2a** and **2b** was evaluated in vitro by using human serum, rat serum, and 20% rat liver homogenate at 37 °C (Table 1). The prodrug **2b** released metformin

Table 1. Half-Lives of the Metformin Prodrugs **2a** and **2b** in 50 mM Buffer Solutions, 15 mM Hydrogen Peroxide (H_2O_2) Solution, Human Serum, Rat Serum, and 20% Rat Liver Homogenate at 37 °C (Mean ± SD, n = 3)

solution	$t_{1/2}$ of **2a** (min)	$t_{1/2}$ of **2b**
buffer, pH 1.0	477.74 ± 41.37 min	12.13 ± 2.82 min
buffer, pH 4.0	[a]	78.60 ± 3.10 h
buffer, pH 7.4	[a]	78.56 ± 2.41 h
buffer, pH 9.0	[b]	18.14 ± 1.11 min
H_2O_2	35.77 ± 13.76 min	14.22 ± 1.01 min
human serum	[c]	3.74 ± 0.90 s
rat serum	[c]	36.81 ± 2.45 min
20% rat liver homogenate	[c]	14.98 ± 3.18 s

[a] 10−20% of the prodrug was degraded to metformin during the 4 weeks of incubation. [b] 40−50% of the prodrug was degraded to metformin during the 4 weeks of incubation. [c] 10−20% of the prodrug was degraded to metformin during the 24 h of incubation.

Scheme 1. Synthetic Pathway to the Prodrugs of Metformin[a]

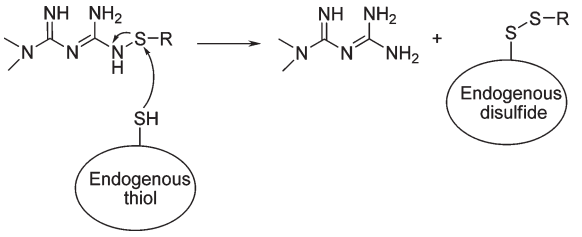


[a] Reaction conditions: (a) ACN, MW 100 °C, 10 min; (b) metformin (*N*,*N*-dimethylimidodicarbonimidicdiamide hydrochloride), DMF, room temp, 24 h.

Table 2. Octanol/Water Distribution Coefficients (log *D*) of Metfomin and the Metfomin Prodrugs **2a** and **2b** at pH Values of 4.0 and 7.4 (Mean ± SD, $n = 3$ unless Otherwise Mentioned)

	log *D*	
	pH 4.0	pH 7.4
metformin	−3.41 ± 0.71 ($n = 2$)	−3.37 ± 0.39
prodrug **2a**	−1.10 ± 0.02	0.49 ± 0.01
prodrug **2b**	−0.70 ± 0.01	−0.76 ± 0.02

Scheme 2. Proposed Bioactivation Mechanism of Metformin Prodrugs in Vivo



extremely quickly in human serum having a half-life of 4 s. Moreover, it underwent a reasonably fast bioconversion in rat serum and rat liver homogenate. On the other hand, the prodrug **2a** released hardly any metformin in vitro during 24 h. Furthermore, the prodrug **2b** was incubated with bovine serum albumin (BSA, Sigma-Aldrich Co.), which is known to have at least one free thiol group, but did not release any metformin during 2 h of incubation (data not shown). As speculated from the stability data of the prodrugs **2a** and **2b** in buffer and H_2O_2 solutions, the free radical mechanism was probably one reason for the rapid breakdown of the prodrug **2b**. Reactive oxygen species (ROS), such as hydroxyl radical (·OH), superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and nitric oxide (NO), are produced during normal cellular function and are present in in vitro samples regardless of their short half-lives.[37] Because of possible involvement of free radicals in the in vitro bioactivation, the prodrug **2b** was converted more rapidly compared to the prodrug **2a**. However, both prodrugs were converted readily to metformin in vivo in rats, which indicates that the bioconversion in vivo occurs by a different mechanism, this being most probably mediated by endogenous thiols (Scheme 2).

In Vivo Bioconversion of Metformin Prodrugs. The bioactivation of the prodrugs **2a** and **2b** was determined in vivo after both intravenous and oral administration in rats (Figure 2). Because of the decreased aqueous solubility, the prodrug **2a** was dissolved in 10% 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) in 0.6% NaCl solution, whereas metformin and the prodrug **2b** were dissolved in 0.9% NaCl solution. The plasma concentration of metformin was determined after immediate centrifugation and sample preparation of each blood sample. Since both prodrugs were rapidly converted to metformin in rats, only the concentrations of metformin, not the concentrations of the prodrugs, were quantified, although all compounds were detected in these samples. Unfortunately, the prodrug **2b** most probably liberated, in addition to metformin, thiophenol as a byproduct, which killed the rats shortly after the administration of the prodrug **2b**, and the in vivo studies with this prodrug were discontinued. Although thiophenols convert oxyhemoglobin to methemoglobin, which can induce hemolysis,[38] aliphatic thiols, such as those that exist intracellularly, do not interact with oxyhemoglobin like aromatic thiols and thus can serve as safer prodrug promoieties.

The area under the plasma concentration−time curve (AUC) of metformin after intravenous administration of the prodrug **2a** (291 μg·min/mL ($AUC_{0-\infty}$)) was 1.7 times greater than that after administration of metformin (170 μg·min/mL ($AUC_{0-\infty}$)) (Table 3). However, the dose of the prodrug **2a** (69 μmol) was slightly greater (1.3 equiv) than that of metformin (53 μmol). Furthermore, as the detection of metformin after intravenous administration was closed after 30 min, a small proportion of metformin may be missing from the total AUC value. Nevertheless, it can be concluded that the prodrug **2a** was bioconverted in blood to metformin quantitatively when administered intravenously. The clearance (Cl) of metformin and the prodrug **2a** after intravenous administration was similar. The apparent maximum plasma concentration (C_{max}) of the prodrug **2a** (3.0 μg/mL) was lower, and the time to reach the maximum plasma concentration (t_{max}) of the prodrug **2a** (approximately 10 min (this was confirmed in the preliminary studies with time points of 1−30 min)) was higher compared to the C_{max} and t_{max} values of metformin (20.9 μg/mL and approximately 1 min, respectively) (Table 3). This may be attributable to the accumulation of the prodrug into the red blood cells. Glutathione activity is extremely high in red blood cells,[35] where the prodrug **2a** is readily bioconverted to metformin. The highly polar metformin was slowly released from the red blood cells, and since the concentration of metformin was determined from plasma and not from the whole blood, the concentrations of metformin were considerably lower than anticipated. Therefore, the biological half-life and distribution volume of the prodrug after intravenous administration cannot be accurately reported in the present study.

The dose of the prodrug **2a** (25 mg, 0.1 mmol) after oral administration was half of that of metformin (35 mg, 0.2 mmol), since we assumed that the prodrug would be absorbed more efficiently than metformin (Table 4). Furthermore, it has been reported that the extent of absolute oral bioavailability is dose-independent in rats after oral administration of 50−200 mg/kg of metformin.[39] Since the absorption profiles and the AUC and C_{max} values after oral administration of the prodrug **2a** (275 μg·min/mL ($AUC_{0-\infty}$) and 1.3 μg/mL, respectively) were almost equal to those of metformin after its oral administration (289 μg·min/mL ($AUC_{0-\infty}$) and 1.7 μg/mL, respectively), we can conclude that the prodrug **2a** was absorbed more efficiently from the gastrointestinal tract than metformin, although the t_{max} values were not significantly different from each other. Furthermore, the bioavailability (F) of the prodrug **2a** (64.5%) was significantly higher than that of metformin (43.0%) (Table 4). Since we could detect the prodrug **2a** from plasma after oral administration, although it was not quantified, it can be assumed that the prodrug **2a** is bioconverted at least partially if not completely after absorption. Metformin is known to be absorbed via saturable paracellular route,[40] and therefore, absorption of more lipophilic prodrug of metformin was improved most probably via increased passive transcellular diffusion. Moreover, more lipophilic prodrugs of metformin would also improve the hepatic uptake, which is known to be partly mediated by organic cation transporter 1 (OCT1).[41,42] Thus, this novel prodrug may improve the efficacy of metformin especially in the patients who have genetic polymorphism in the coding region of OCT1 and fail to reach the target glycemic control.

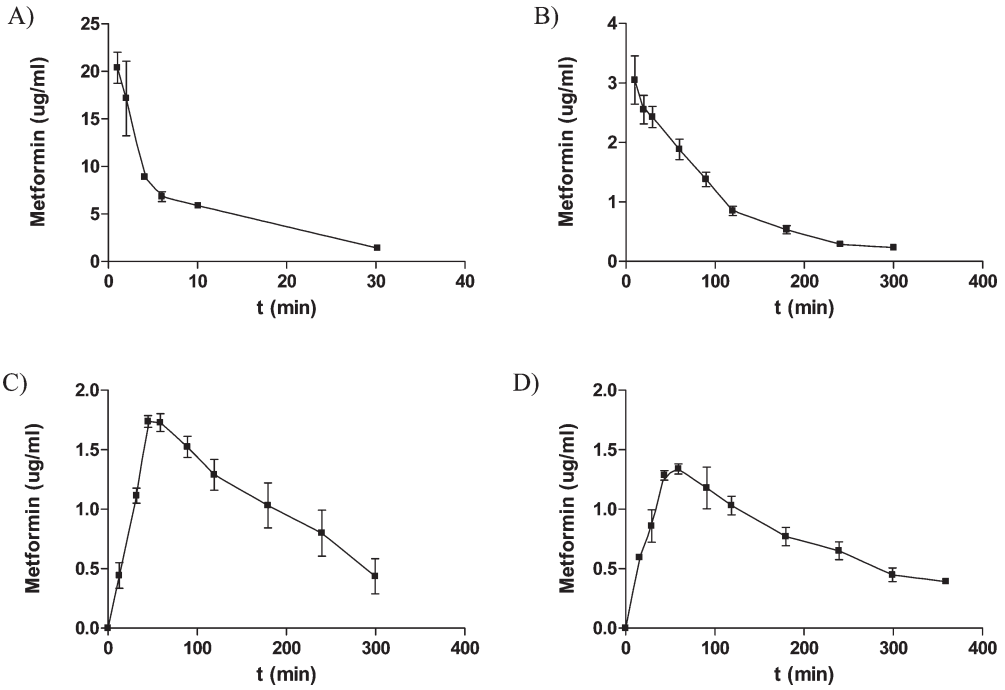


Figure 2. Mean plasma concentration−time profile of metformin (■) after (A) intravenous administration of metformin at a dose of 35 mg/kg, (B) intravenous administration of the metformin prodrug **2a** at a dose of 67 mg/kg, (C) oral administration of metformin at a dose of 140 mg/kg, and (D) oral administration of the metformin prodrug **2a** at a dose of 100 mg/kg to rats (mean ± SD, $n = 3$).

Table 3. Pharmacokinetic Parameters of Metformin and the Prodrug **2a** after Intravenous Administration into Rats (Mean ± SD, $n = 3$)

	metformin (iv)	prodrug **2a** (iv)
dose (mg/kg)	35.0 (53 μmol)	67.0 (69 μmol)
$AUC_{0-\infty}$ (μg·min/mL)	170 ± 20	291 ± 47
Cl ((mL/min)/kg)	52 ± 6	59 ± 11
t_{max} (min)	∼1	∼10
C_{max} (μg/mL)	20.9 ± 3.7	3.0 ± 0.7

Table 4. Pharmacokinetic Parameters of Metformin and the Prodrug **2a** after Oral Administration into Rats (Mean ± SD, $n = 3$)

	metformin (po)	prodrug **2a** (po)
dose (mg/kg)	140.0 (0.2 mmol)	100.0 (0.1 mmol)
$AUC_{0-\infty}$ (μg·min/mL)	289 ± 74	275 ± 29
t_{max} (min)	∼50	∼60
C_{max} (μg/mL)	1.7 ± 0.1	1.3 ± 0.1
F (%)	43.0	64.5[a]

[a] Statistically significantly different when compared with the respective control ($P = 0.001$, t-test).

In summary, these data show that this novel prodrug strategy can improve the oral absorption and bioavailability of metformin, which may be beneficial for current metformin therapy. Many of the unwanted gastrointestinal adverse effects could be ameliorated, since daily doses would be reduced. The results indicate that the daily doses could be reduced by approximately 30% assuming that clearances in rats will scale similarly to the human situation. However, the effectiveness of this prodrug will need to be evaluated in other animals and humans to estimate the true potential of this prodrug structure.

Conclusions

These results indicate that the novel and more lipophilic metformin prodrug **2a** can significantly enhance the oral absorption and bioavailability of metformin. This kind of prodrug should be a safer option for more lipophilic analogs of metformin, since they release the active drug molecule, metformin, shortly after absorption. Moreover, these novel prodrugs of metformin may improve the clinical usefulness of this widely used but very hydrophilic antidiabetic agent, as the required daily doses could be reduced, which may also decrease the unwanted adverse effects associated with metformin therapy.

Experimental Section

General Synthetic Procedures. All the reactions were performed with reagents of commercial high purity quality without further purification unless otherwise mentioned. Reactions were monitored by thin-layer chromatography using aluminum sheets coated with silica gel 60 F_{245} (0.24 mm) with suitable visualization. Microwave irradiation experiment was carried out in a Biotage Initiator microwave reactor (Biotage, Uppsala, Sweden) in a pressure-rated glass tube. Purifications by flash chromatography were performed on silica gel 60 (0.063−0.200 mm mesh). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin, Fallanden, Switzerland) operating at 500.13 and 125.75 MHz, respectively, using tetramethylsilane (TMS) as an internal standard. Furthermore, the products were characterized by mass spectrometry with a Finnigan LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ionization source, and the purity (≥95%) was determined by elemental analysis (C, H, N) with a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer (CE Instruments, Milan, Italy).

2-(Cyclohexylthio)isoindoline-1,3-dione (1a). A mixture of dicyclohexyl disulfide (0.51 g, 2.21 mmol) and *N*-bromophthalimide (0.50 g, 2.21 mmol) in anhydrous ACN (20 mL) was irradiated at 100 °C (1 bar) in a microwave reactor for 10 min. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography, eluting with hexane/ethyl acetate (2:1) solution to obtain compound **1a** as

a light-yellow solid (0.29 g, 52%). ^{1}H NMR ($CDCl_3$): δ 7.95−7.91 (2H, dd, $^3J_{HH}$ = 5.46 Hz, $^4J_{HH}$ = 3.07 Hz), 7.81−7.76 (2H, dd, $^3J_{HH}$ = 5.46 Hz, $^4J_{HH}$ = 3.07 Hz), 3.19 (1H, tt, $^3J_{HH(eq)}$ = 3.63 Hz, $^3J_{HH(ax)}$ = 10.88 Hz), 1.95−1.88 (2H, m), 1.84−1.75 (2H, m), 1.65−1.57 (1H, m), 1.43−1.33 (2H, m), 1.32−1.18 (3H, m). ^{13}C NMR: δ 168.82 s, 134.53 s, 132.03 d, 123.86 d, 49.39 d, 31.11 t, 25.54 t, 25.46 t.

N^1,N^1-Dimethyl-*S*-cyclohexyl-N^4-thiohydroxylbiguanidine (2a). Metformin (*N*,*N*-dimethylimidodicarbonimidicdiamide hydrochloride) (1.0 g, 6.0 mmol) in 10 mL of 1 M NaOH was stirred at room temperature for 30 min. Water was evaporated in vacuo, and the residue was dissolved in 30 mL of MeOH. The solvent was evaporated, and the residue was redissolved in 20 mL of MeOH. NaCl was filtered out of the solution and the filtrate was evaporated to yield basic metformin as a white solid (0.77 g, 99%).

The basic metformin (0.15 g, 1.15 mmol) and 2-(cyclohexylthio)isoindoline-1,3-dione (0.29 g, 1.15 mmol) in anhydrous DMF (10 mL) was stirred overnight. The solvent was evaporated in vacuo and the residue was purified by flash chromatography, eluting with MeOH/DCM (0.5:10) solution to obtain the white solid compound **2a** (88 mg, 33%). ^{1}H NMR (DMSO): δ 8.33 (1H, s), 7.50 (2H, s), 7.18 (1H, s), 2.95 (6H, s), 2.93−2.87 (1H, m), 1.91−1.84 (2H, m), 1.76−1.69 (2H, m), 1.61−1.55 (1H, m), 1.33−1.15 (5H, m). ^{13}C NMR: δ 160.39 s, 159.01 s, 49.11 d, 38.03 q, 30.71 t, 25.75 t, 25.59 t. ESI-MS: m/z = 244.2 $(M+H)^+$. Anal. Calcd for ($C_{10}H_{21}N_5S$): C, 49.35; H, 8.70; N, 28.78; S, 13.17. Found: C, 49.25; H, 8.94; N, 28.45; S, 13.54.

N^1,N^1-Dimethyl-*S*-phenyl-N^4-thiohydroxylbiguanidine (2b). ^{1}H NMR (DMSO): δ 8.97 (1H, s), 7.65 (2H, s), 7.40−7.34 (2H, m), 7.30−7.25 (3H, m), 7.23−7.18 (1H, m), 2.90 (6H, s). ^{13}C NMR: δ 160.73 s, 157.49 s, 140.28 s, 129.45 d, 126.50 d, 123.68 d, 38.03 q. ESI-MS: m/z = 238.1 $(M + H)^+$. Anal. Calcd for ($C_{10}H_{15}N_5S \cdot 0.1H_2O$): C, 50.23; H, 6.41; N, 29.29; S, 13.41. Found: C, 50.09; H, 6.80; N, 29.56; S, 13.67.

HPLC Analysis of Metformin and Metformin Prodrugs. The analysis was performed on the HPLC system, which consisted of a Agilent 1100 binary pump, a 1100 micro vacuum degasser, a HP 1050 autosampler, and a HP 1050 variable wavelength detector (operated at 235 nm) (Agilent Technologies, Waldbronn, Germany). The chromatographic separations were achieved on a Supelco Supelcosil LC-Si analytical column (4.6 mm × 250 mm, 5 μm) (Supelco Inc. Bellefonte, PA) by using isocratic elution of acetonitrile and 10 mM ammonium acetate buffer (pH 5.0) with a ratio of 60:40 (v/v) at a flow rate 1.0 mL/min at room temperature.

Stability of Metformin Prodrugs in Buffer and H_2O_2 Solutions. The rates of chemical stability of the metformin prodrugs **2a** and **2b** were determined at 37 °C in 50 mM (ionic strength 0.15) HCl buffer at pH 1.0, acetate buffer at pH 4.0, phosphate buffer at pH 7.4, and borate buffer at pH 9.0. The incubation mixtures were prepared by dissolving 5−10 mM stock solutions of prodrugs in H_2O in preheated buffer solutions. The prodrug mixtures of about 200 μM were incubated in a water bath at 37 °C, and the samples were withdrawn at appropriate intervals. ACN was added to the samples (1:1, v/v) to quench the degradation reaction during the HPLC analyses. The concentration of the prodrugs **2a** and **2b** and of metformin were analyzed by HPLC as described in the previous section. The pseudo-first-order half-lives ($t_{1/2}$) for the chemical stability of the prodrugs were calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug concentration versus time.

The chemical stability of the metformin prodrugs **2a** and **2b** in 15 mM hydrogen peroxide (H_2O_2) solution was determined at 37 °C. The incubation mixture contained 30% H_2O_2 solution, 1 mM prodrug stock solution in phosphate buffer, and 50 mM phosphate buffer, pH 7.4. The experiment was carried out as described above. ACN was added to the samples (4:1, v/v) to quench the degradation reaction during the HPLC analyses.

Distribution Coefficients of Metformin and Metformin Prodrugs in Octanol/Water. The distribution coefficients (log *D*) of metformin and metformin prodrugs **2a** and **2b** were determined at room temperature from the distribution of the compounds between a mixture of 1-octanol and 50 mM acetate/phosphate buffer (pH 4.0 or 7.4) system. Then 1 mL of metformin or prodrug solution (150 μg/mL) in 50 mM buffer was added to 4 mL of 1-octanol saturated with desired buffer. The mixtures were shaken for 1 h, and the phases were separated. The concentration of metformin or the metformin prodrug **2a** or **2b** in the buffers before and after the shaking were analyzed by HPLC as described earlier.

Biological Material. The pooled human and rat sera were obtained from normal human donors or control rats, respectively, and collected aseptically from whole blood. The pooled human and rat sera were stored at −80 °C until used. The 20% liver homogenate was prepared by homogenizing rat livers with 4 equiv of isotonic phosphate buffer (pH 7.4). The homogenate was centrifuged for 90 min at 9000*g* at 4 °C, and the supernatant was stored at −80 °C until use.

In Vitro Bioconversion of Metformin Prodrugs. The bioconversion of the metformin prodrugs **2a** and **2b** were determined in human serum, rat serum, and 20% rat liver homogenate at 37 °C. The prodrugs were dissolved in 50 mM phosphate buffer, pH 7.4 (ionic strength 0.15), and the stock solutions and the biological material were preheated in a water bath. Then 2.5 mM stock solutions were mixed with serum (1:4; v/v) and 1 mM stock solutions were mixed with 20% liver homogenate (1:1, v/v). The mixtures were placed in a water bath at 37 °C, and the samples were withdrawn at appropriate intervals. Ice-cold ACN was added to the samples (4:1, v/v) to precipitate proteins and quench the degradation reaction. The samples were centrifuged for 10 min at 12 000 rpm and kept on ice until the HPLC analysis. The concentration of the prodrugs **2a** and **2b** and of metformin were analyzed from the supernatants by the HPLC as described above. The pseudo-first-order half-lives ($t_{1/2}$) for the bioconversion of the prodrugs were calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug concentration versus time.

Animals. Adult male Wistar rats weighing 250 ± 5 g were supplied by the National Laboratory Animal Centre (Kuopio, Finland). Rats were housed in stainless steel cages on a 12 h light (07:00−19:00) and 12 h dark (19:00−07:00) cycle at an ambient temperature of 22 ± 1 °C with a relative humidity of 50−60%. All experiments were carried out during the light phase. Tap water and food pellets (Lactamin R36; Lactamin AB, Sodertalje, Sweden) were available ad libitum. All procedures were reviewed and approved by the Animal Ethics Committee at the University of Kuopio.

In Vivo Bioconversion of Metformin Prodrugs. Rats were anesthetized with ketamine (90 mg/kg, ip) and xylazine (8 mg/kg, ip). Small incisions were made in the neck to right side of midline and in the region of the left groin. The right jugular (and left femoral vein) were exposed aseptically[43] and cannulated with PE-50 catheters filled with 100 IE/mL heparin in order to collect blood samples and to inject drug solutions. The catheters were exteriorized through a small incision made in the back of the neck. The rats were allowed to recover until the following day.

Metformin hydrochloride and metformin prodrug **2b** were dissolved in 0.9% NaCl solution at a dose of 35 and 50 mg/kg, respectively, and infused via the jugular vein over 0.5 min (total injection volume of 0.3 mL) to the rats, and the catheter was rinsed with 0.9% NaCl. An approximately 200 μL aliquot of blood was collected via the jugular vein at 0, 1, 2, 4, 6, 10, and 30 min after the start of the intravenous administration to micro test tubes containing 20 μL of 3% ethylenediaminetetraacetic acid (EDTA) in 0.7% NaCl solution. Metformin prodrug **2a** was dissolved in 10% HP-β-CD in 0.6% NaCl solution at a dose of 67 mg/kg and administered via the femoral vein over 1 min

(total injection volume of 0.5 mL) to the rats, and the catheter was rinsed with 0.9% NaCl. An approximately 200 μL aliquot of blood was collected via the jugular vein at 0, 10, 20, 30, 60, 90, 120, 180, 240, and 300 min after the start of the intravenous administration to micro test tubes containing 20 μL of 3% EDTA in 0.7% NaCl solution. The drawn blood volume was substituted by 0.9% NaCl immediately after each blood sampling.

Metformin hydrochloride was dissolved in 0.9% NaCl solution at a dose of 140 mg/kg and administered orally (total injection volume of 1.0 mL) to the rats using a feeding tube. An approximately 200 μL aliquot of blood was collected via the jugular vein at 0, 15, 30, 45, 60, 90, 120, 180, 240, and 300 min after the start of the oral administration to micro test tubes containing 20 μL of 3% EDTA in 0.7% NaCl solution. Metformin prodrug **2a** was dissolved in 10% HP-β-CD in 0.6% NaCl solution at a dose of 100 mg/kg and administered orally (total injection volume of 1.0 mL) to the rats using a feeding tube. An approximately 200 μL aliquot of blood was collected via the jugular vein at 0, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min after the start of the oral administration to micro test tubes containing 20 μL of 3% EDTA in 0.7% NaCl solution. The drawn blood volume was substituted by 0.9% NaCl immediately after each blood sampling.

Blood samples were centrifuged 5 min at 14 000 rpm immediately after each blood sampling, and 100 μL aliquot of each plasma sample and 20 μL of 5.2 mg/mL 1,1,3,3-tetramethylguanidine as an internal standard were vortexed with ice-cold ACN (1:4, v/v) to precipitate proteins and quench the degradation reaction. The samples were centrifuged 5 min at 14 000 rpm and kept on ice until injected into the HPLC system. The pharmacokinetic analysis was performed with GraphPad Prism software, version 4.03 (GraphPad Software, Inc., San Diego, CA). The area under the plasma concentration−time curve (AUC) was calculated using the linear trapezoidal method. The maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (t_{max}) were read directly from the plasma concentration−time data. The clearance and the bioavailability were calculated from the following equations:

$$\mathrm{Cl} = \frac{\mathrm{dose}}{\mathrm{AUC}}; \quad F = \frac{\mathrm{AUC_{po}}}{\mathrm{AUC_{iv}}} \frac{\mathrm{dose_{iv}}}{\mathrm{dose_{po}}}$$

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