Pyrrolopyrazinedione-Based Inhibitors of Human Hormone-Sensitive Lipase

Deborah H. Slee,* Abhijit S. Bhat, Truc N. Nguyen, Mary Kish, Katy Lundeen, Michael J. Newman, and Stephen J. McConnell

Ontogen Corporation, 6451 El Camino Real, Carlsbad, California 92009

Received October 17, 2002

Abstract: The regulation of lipid metabolism and it's effect on glucose control and diabetes has received intense interest. Hormone-sensitive lipase (HSL) is a vital enzyme in lipid metabolism. A series of novel pyrrolopyrazinediones has been discovered that demonstrate submicromolar activity both in the enzyme assay and in a ^{14}C-emulsion assay employing cholesteryl oleate as a substrate as a secondary measure of HSL activity. These compounds represent novel inhibitors of the human HSL enzyme.

Introduction. In recent years interest has grown in the area of lipid metabolism and its effect on the regulation of glucose control and, in turn, diabetes. Hormone-sensitive lipase (HSL) is a vital enzyme in lipid metabolism and general energy homeostasis in mammals. HSL catalyzes the rate-limiting step in the hydrolysis of stored triglycerides in adipose tissue. The hormonal and neuronal control and regulation of HSL activity is mediated through reversible cyclic adenosine monophosphate-dependent serine phosphorylations mediated by protein kinase A.[1] Elevated levels of free fatty acids have been shown to be associated with increased insulin resistance and increase risk for type 2 diabetes.[2,3] The two products of HSL-mediated hydrolysis are glycerol and free fatty acids, thus HSL inhibition could potentially decrease plasma levels of free fatty acids, implicated in a variety of pathological conditions.[4] There is an interest in finding compounds that modulate the activity of HSL, as this may shed light on the pathogenesis and potential treatment of human diseases such as diabetes and obesity. Schoenafinger et al. have recently described a series of 3-phenyl-5-alkoxy-1,3,4-oxadiazol-2-ones (general formula **1**) as HSL inhibitors with IC_{50} values ranging from 0.02 to 60 μM,[5] and Beltrandelrio et. al. have reported that certain (3,4-dihydro-1*H*-isoquinolin-2-yl) carbamates (general formula **2**) inhibit HSL with the most potent compound having an IC_{50} of 0.003 μM[6] (Figure 1). Herein we report a novel series of pyrrolopyrazinediones (general formula **3**) that demonstrate submicromolar activity against HSL.

The HSL enzyme shares a very minimal homology with other mammalian proteins. Although the enzyme shares a *Gly-Xaa-Ser-Xaa-Gly* motif in the active site, with other mammalian lipases, primary sequence alignments have failed to reveal any significant sequence similarity between HSL and other mammalian lipases.[7]

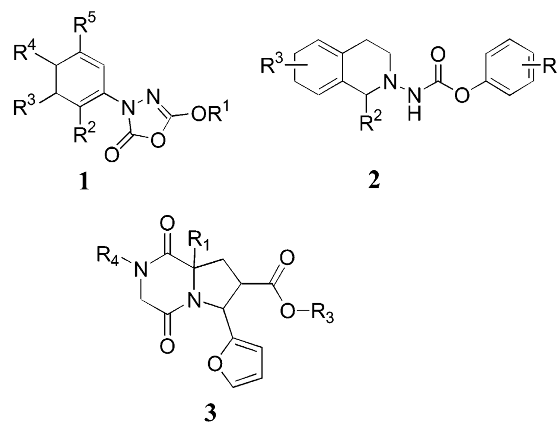
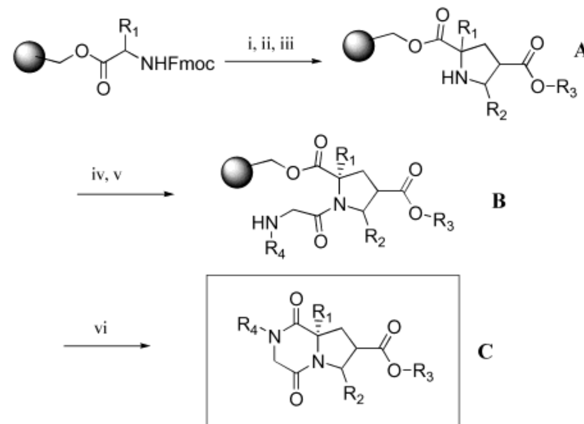


Figure 1. Known Inhibitors of HSL

Contreras et al. recently reported a secondary structure homology between HSL and a super family of esterases and lipases that include acetyl cholinesterase, bile salt stimulated lipase, and several fungal lipases.[8] However, there is little known about the catalytic mechanism of HSL, and there is no crystal structure currently available for HSL. Owing to the lack of relevant structural information about HSL enzyme, we exclusively relied on high throughput screening (HTS) of our compound collection for the identification of the initial lead inhibitors. A high throughput screening assay was developed and used for the screening of over 100 000 compounds from our compound collection. Several novel pyrrolopyrazinediones were identified as submicromolar inhibitors of HSL.

Chemistry. The pyrrolopyrazinediones were synthesized as shown in Scheme 1. The chemistry was performed using the OntoBLOCK system to generate the screening libraries.[9] Highly functionalized pyrrolidines (**A**) were synthesized on Wang resin, using the 1,3-dipolar cycloaddition reaction of acrylates with resin-bound azomethine ylides as described by Murphy et al.[10] The pyrrolidines were acylated with bromoacetic acid in the presence of DIC and then treated with various

Scheme 1[a]



[a] (i) Piperidine/THF. (ii) R_2CHO, $CH(OCH_3)_3$, rt, 15 h. (iii) $CH_2{=}CH{-}C({=}O)OR_3$, $AgNO_3$, Et_3N, CH_3CN, rt, 15 h. (iv) $BrCH_2COOH$, DIC, DMF, rt, 16 h. (v) 2M R_4NH_2/DMSO, rt, 3 h. (vi) Toluene, 110 °C, 3 d.

* To whom correspondence should be addressed. Tel: 760-930-0100 x3034 Fax: 760-930-0955. E-mail: Debbie.slee@ontogen.com or debbieslee@yahoo.com.

10.1021/jm020460y CCC: $25.00 © 2003 American Chemical Society
Published on Web 02/28/2003

primary amines to give the resin-bound intermediate (**B**). Upon refluxing in toluene or *n*-butanol, these intermediates underwent a cyclodehydration reaction to liberate the desired pyrrolopyrazinediones (**C**) in solution. The yields for the final cyclization and release were moderate (12−40%), but provided the compound in high purity (>80% in most cases) and predominantly as a single diastereomer (The development and validation of the chemistry and the stereochemistry issues are discussed in detail in a separate manuscript under preparation (Short, K. et al., 2003)). The major reason for the low yields was incomplete acylation of the pyrrolidine nitrogen with bromoacetic acid; however, this nonacylated pyrrolidine stays bound to the resin during the final cyclization step and does not appear as an impurity. This procedure was employed for the library synthesis, as it provided the target compounds in adequate amounts and high purity for high throughput screening. The hits from the library were analyzed by LC-MS to confirm purity and identity of the compounds. This analysis, along with the extensive studies that were done during the chemistry validation stage for this library, gave us strong confidence regarding the identity of the compounds. A subset of pyrrolopyrazinediones (including all compounds reported herein) was resynthesized on a larger scale with the aid of the OntoBLOCK system to confirm activity against HSL. TLC analysis revealed pyrrolopyrazinediones as the major product along with minor baseline impurities. The baseline impurities were removed via purification using silica gel chromatography, and purified samples were characterized by ^{1}H, ^{13}C NMR and/or LC-MS ELSD. The purified pyrrolopyrazinediones (purity >95%) appear to be single diastereomers based on NMR analysis; however, additional studies were not performed to characterize the diastereomers. The IC_{50} values from the assay of the purified samples correlated quite well to the data from the unpurified compounds from the initial library (within 2 -fold).

Biology. The human HSL gene was cloned and expressed in the baculovirus/insect cell system.[11] Protein purification and specific activity measurements were carried out as described.[12] Specific activity of the enzyme was measured using both PNPB and cholesteryl [^{3}H]-oleate as substrates for HSL and has been previously described.[13] A high throughput screen using *p*-nitrophenyl butyrate (PNPB) to measure the activity of human HSL was used to screen compounds from the Ontogen combinatorial chemistry library. PNPB has been routinely used to measure HSL activity.[14] This substrate has the advantage of being both nonradioactive and water-soluble. All compounds were initially tested at 50 μM final assay concentration. Compounds showing initial activity were retested to confirm activity. Full 8-point IC_{50} determinations were performed in triplicate, in both PNPB and [14C] cholesteryl oleate assays on the confirmed hits.

Results and Discussion. Pyrrolopyrazinediones that inhibit HSL in low micromolar concentrations are summarized in Table 1. It appears that for HSL inhibition, R_1 and R_4 positions of the pyrrolopyrazinedione scaffold need flexible hydrophobic groups. It was found that other pyrrolopyrazinediones containing smaller, non-hydrophobic groups such as hydroxyl, amino alkyl, methyl, or bulky groups such as *tert*-butoxymethyl (compound **13**, 67 μM) in the R_1 position did not show any significant HSL inhibitory activity. One exception was compound **10** which showed moderate HSL inhibition with a hydroxymethyl group in the R_1 position. Two very closely related compounds **4** and **11** show dramatic differences in their HSL activity. While **4** is a potent inhibitor of HSL, **11** is inactive. Compound **4** has a trifluoroethyl ester at R_3 while **11** has an ethyl group in the same position, which indicates that the ester plays a key role in the enzyme inhibition. This hypothesis is also supported by the activity shown by the esters **5**, **6**, **7**, and **8** (0.21−1.3 μM) where the corresponding acid **12** shows only moderate HSL inhibition (48 μM). These compounds were also tested in the [^{14}C]cholesteryl oleate assay. This assay was chosen for two reasons. First, this assay has been used historically in the literature and is predictive of HSL-specific activity. Second, it was important to show that these compounds specifically inhibited the HSL enzyme and the activity would be independent of the substrate used. It is interesting to note that compounds give comparable activity in both PNPB and [^{14}C]cholesteryl oleate assays, which demonstrates that the activity of the compounds is specific against the enzyme and not the substrate used to measure HSL activity. As shown in Table 1, the IC_{50} measurements using the PNPB assay are comparable to IC_{50} values generated using the [^{14}C]cholesteryl oleate assay. Efforts are presently underway to understand and optimize the structural features essential for HSL inhibition on the pyrrolopyrazinediones. The discovery and characterization of compounds that modulate HSL activity may shed light into the mechanisms

Table 1

Compd	Structure			IC_{50}* (μM)	
	R_1	R_3	R_4	# HSL PNPB	⊥HSL [^{14}C] COA
4	$PhCH_2OCH_2$	CF_3CH_2		0.12	0.18
5	$PhCH_2OCH_2$	CH_3	$Ph(CH_2)_4$	0.21	0.26
6	$PhCH_2OCH_2$	^{t}Bu	$Ph(CH_2)_4$	0.21	0.20
7	$PhCH_2OCH_2$	$MeOCH_2CH_2$	$Ph(CH_2)_4$	0.76	0.77
8	$PhCH_2OCH_2$	$CNCH_2CH_2$	$Ph(CH_2)_4$	1.3	1.9
9	$PhCH_2OCH_2$	$PhCH_2$		8.5	6.2
10	CH_2OH	Ph	$Ph(CH_2)_4$	5.5	8.3
11	$PhCH_2OCH_2$	CH_3CH_2		>100	n.d.
12	$PhCH_2OCH_2$	H	$Ph(CH_2)_4$	48	n.d.
13	CH_2O^tBu	Ph	$Ph(CH_2)_4$	67	n.d.

HSL PNPB Assay ⊥, HSL [^{14}C] Cholesteryl Oleate Assay,* assays done in triplicate. S.D.<5%.

regulating lipolysis and energy utilization in humans. The control and regulation of HSL-specific pathways may have fundamental importance in the study and treatment of several pathophysiological conditions including diabetes and obesity.

Acknowledgment. The authors thank Dr. Henry Choy (Lipid Research Laboratory, UCLA Department of Medicine) for helpful discussions on PNPB assay development and the preparation of cholesteryl oleate emulsions. Dr. Eugene Coates (Ontogen Corporation) is gratefully acknowledged for his help in computational analysis of the HTS data.

References

(1) Osterlund, T. Structure−function relationships of hormone-sensitive lipase. *Eur. J. Biochem.* **2001**, *268*, 1899−1907.
(2) Bergman, R. N.; Ader, M. Free fatty acids and pathogenesis of type 2 diabetes mellitus. *Trends Endocrinol. Metab.* **2000**, *11*, No 9, 351−356.
(3) Roden, M.; Stingl, H.; Chanramouli, V.; Schumann, W. C.; Hofer, A.; Landau, B. R.; Nowotny, P.; Waldhausl, W.; Shulman, G. I. Effects of free fatty acid elevation on postabsorptive endogenous glucose production and gluconeogenesis in humans. *Diabetes* **2000**, *49*, 701−707.
(4) Holm, C.; Osterlund, T.; Laurell, H.; Contreras, J. A. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Annu. Rev. Nutr.* **2000**, *20*, 365−393.
(5) Schoenafinger, K.; Petry, S.; Mueller, G.; Baringhaus, K.-H. Preparation of 3-phenyl-5-alkoxy-1,3,4-oxadiazol-2-ones as hormone-sensitive lipase inhibitors. PCT Int. Appl. WO 0166531, 2001.
(6) Beltrandelrio, H.; Jacobsen, P.; Cornelis De Jong, J. Preparation of (3,4-dihydro-1H-isoquinolin-2-yl)carbamates for treating disorders where a decreased level of plasma FFA is desired. PCT Int. Appl. WO 0187843, 2001.
(7) Li, Z.; Sumida, M.; Birchbauer, A.; Schotz, M. C.; Reue, K. Isolation and Characterization of the gene for mouse hormone-sensitive lipase. *Genomics* **1994**, *24* (2), 259−265.
(8) Contreras, J. A.; Karlsson, M.; Osterlund, T.; Laurel, H.; Svensson, A.; Holm, C. Hormone-sensitive Lipase is structurally related to acetylcholinesterase, biel salt-stimulated lipase, and several fungal lipases. *J. Biol. Chem.* **1996**, *49*, 31426−31430.
(9) Cargill, J.; Maiefski, R. Automated Combinatorial Chemistry on Solid Phase. *Lab. Rob. Autom.* **1996**, *3*, 139−148.
(10) Murphy, M. M.; Schullek, J. R.; Gordon, E. M.; Gallop, M. A. Combinatorial synthesis of highly functionalized pyrrolidines: Identification of a potent angiotensin converting enzyme inhibitor from a mercaptoacyl proline library. *J. Am. Chem. Soc.* **1995**, *117*, 7029−7030.
(11) Cloning and protein purification: The full-length human HSL gene was cloned by PCR from human adipocyte cDNA (Clontech). A PCR fragment containing the full-length human HSL gene was cut with the restriction enzymes Eco RI and Bgl II and ligated into the plasmid pAcHLT-A (PharMingen), a vector suitable for His6-tagged expression in the baculovirus/insect cell system. The sequence of the human HSL gene was confirmed to be 100% correct compared to the human HSL sequence from genbank (accession # 896474). This HSL cDNA/pAcHLT-A construct and BaculoGold DNA were cotransfected into Sf9 insect cells according to manufacturer instructions (PharMingen).
(12) For large-scale production of recombinant human HSL, Sf9 cells were grown in suspension culture in TNM-FH medium. Cells were infected with a recombinant high titer viral stock at a multiplicity of infection (MOI) between 5 and 10 and incubated at 27 °C for 60 h. Cells were harvested by centrifugation at 2500*g* for 5 min. Pellets were processed in 500 mL batches and lysed in 25 mL lysis buffer (0.25 M sucrose, 10 mM 2-mercaptoethanol + protease inhibitor cocktail (Sigma P-8849, used at 1: 100 dilution) using 10 strokes of a dounce homogenizer. 1% CHAPS was added followed by sonication on ice (8×, 30 s pulses, Branson 450 sonifier). Insoluble material was removed by centrifugation at 10 000*g* for 30 min at 4 °C. To this 25 mL of buffer A (100 mM sodium phosphate, pH 8.0, 600 mM NaCl, 20% glycerol, 10 mM 2-mercaptoethanol + protease inhibitor cocktail was added. This material was loaded onto a 2 mL Ni−NTA column and washed with 20 bed volumes of wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10% glycerol, 0.3% CHAPS + protease inhibitor cocktail). HSL protein was eluted with 8 mL of wash buffer + 60 mM imidizole. Protein was concentrated to 4 mL using a stirred-cell concentrator with 30 000 molecular weight cutoff (Amicon). Protein was then dialyzed against 3 × 1 L changes of 50 mM phosphate, pH 8.0, 0.03% CHAPS, 1 mM EDTA, 1 mM DTT, 10% glycerol. Protein determination was accomplished by BCA assay (Pierce).
(13) Osterlund, T.; Danielsson, B.; Degerman, E.; Contreras, J. A.; Edgren, G.; Davis, R. C.; Schotz, M. C.; Holm, C. Domain-structure analysis of recombinant rat hormone-sensitive lipase. *Biochem. J.* **1996**, *319*, 411−420.
(14) Each 100 uL reaction contained 10 ng of HSL protein, 20 mM KPO4, pH 7.4, 1 mM EDTA, 0.1% Triton X-100, 5 mM PNPB, 5% DMSO or test compound. Reactions were incubated for 1 h at 27 °C and immediately read at OD 400 nM.

JM020460Y