Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 2895–2898

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Identification of a Novel Class of Orally Active Pyrimido[5,4-3][1,2,4]triazine-5,7-diamine-Based Hypoglycemic Agents with Protein Tyrosine Phosphatase Inhibitory Activity

Kevin R. Guertin,* Lina Setti, Lida Qi, Rachel M. Dunsdon, Brian W. Dymock, Philip S. Jones, Hilary Overton, Mathew Taylor, Glyn Williams, Joseph A. Sergi, Karen Wang, Ying Peng, Marcia Renzetti, Rogely Boyce, Fiorenza Falcioni, Ralph Garippa and Andrée R. Olivier

Roche Research Center, Hoffmann-LaRoche Inc., 340 Kingsland St., Nutley, NJ 07110, USA

Received 21 January 2003; accepted 14 May 2003

Abstract—A novel series of orally active pyrimido[5,4-3][1,2,4]triazine-5,7-diamine-based hypoglycemic agents have been identified. These compounds show non-selective inhibitory properties against a panel of protein tyrosine phosphatases including PTP1B. Compounds **12** and **13** display oral glucose lowering effects in *ob*/*ob* mice.

Insulin resistance in the liver and peripheral tissues coupled with elevated fasting plasma glucose and impaired glucose tolerance are the hallmarks of Type 2 diabetes.[1] The inability of endogenous insulin to suppress postprandial hepatic glucose output and stimulate glucose uptake and disposal by the peripheral tissues results in elevated plasma glucose levels. A sustained elevation in plasma glucose eventually culminates in an number of debilitating diabetic complications such as atherosclerosis, coronary artery disease, nephropathy, neuropathy and retinopathy.[2] Thus, tight control of blood glucose levels is essential in the early stages of the disease.

Protein tyrosine phosphatases (PTPases) are enzymes that regulate signal transduction pathways via dephosphorylation of biologically important proteins.[3] In the case of the insulin receptor (IR), PTPases believed to play a role in its negative regulation include PTP1B, leukocyte antigen receptor phosphatase (LAR), and PTPα.[4] These enzymes are believed to either directly or indirectly (via downstream signaling) dephosphorylate the active form (triphosphorylated in the regulatory domain) of the IR, resulting in attenuation of the intrisic tyrosine kinase activity of the receptor. The intracellular PTPase PTP1B has been demonstrated to play a significant role in the dephosphorylation of the IR.[5] Furthermore, PTP1B gene knockout mice have shown improved insulin sensitivity and resistance to weight gain.[6] Thus, small molecule PTP1B inhibitors have considerable therapeutic potential for the treatment of Type 2 diabetes and obesity.

Non-selective vanadium based PTPase inhibitors have been found to increase IR tyrosine phosphorylation levels in vitro and sensitize and mimic the actions of insulin in vivo, effectively lowering plasma glucose levels in diabetic animal models.[7] The clinical utility of vanadium based antidiabetic agents is limited by their cumulative toxicity and poor oral absorption and thus a small molecule equivalent may have considerable therapeutic advantage. Recently, Just and co-workers[8] reported a series of periodinate based oxidants with in vitro PTP1B inhibitory activity. The substituted pyrimido[5,4-3][1,2,4]triazine-5,7-diamine **1** was identified through high throughput screening against LAR. This compound was subsequently found to have residual PTP1B activity as well, and served as a starting point for the development of novel PTPase inhibitors. Herein, we describe the initial SAR surrounding a novel series

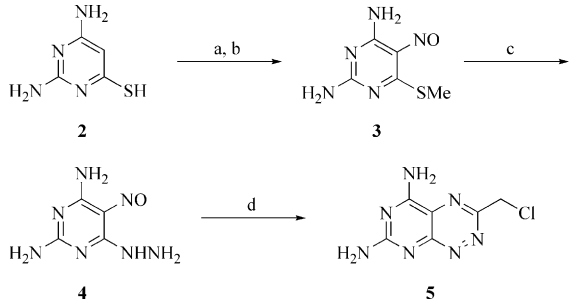
*Corresponding author. Tel.: +1-973-235-3635; fax: +1-973-235-7122; e-mail: kevin.guertin@roche.com

0960-894X/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0960-894X(03)00623-1

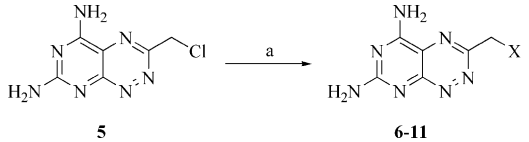
of orally active, non-selective 'vanadate like' pyrimido[5,4-3][1,2,4]triazine-5,7-diamine based-hypoglycemic agents with PTPase inhibitory activity.

The chloromethylpyrimido[5,4-3][1,2,4]triazine-5,7-diamine compound **5** served as a convenient synthetic intermediate for the preparation of PTPase inhibitors **6–15** and was prepared as outlined in Scheme 1. *S*-Methylation of commercially available arylthiol **2** followed by nitrosylation afforded the nitroso-thiomethyl derivative **3**. Nucleophilic displacement of the thiomethyl moiety with hydrazine provided cyclization precursor **4** in good yield. Reaction of **4** with chloroacetaldehyde diethyl acetal under acidic conditions gave, upon dilution of the reaction mixture with water, compound **5** as a stable light brown solid. Upon heating in ethanol, intermediate **5** readily underwent reaction with a variety of secondary amines affording PTPase inhibitors **6–11** as outlined in Scheme 2. In particular, the piperazine derivative **9** could be further functionalized via alkylation with a variety of benzylic halides to provide PTPase inhibitors **12–15** as described in Scheme 3.[9]

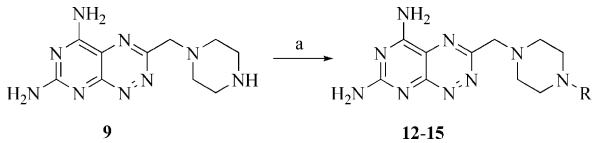
Initially, PTPase inhibitors **6–15** were evaluated for their PTP1B inhibitory activity and the results are outlined in Table 1. Compounds were assayed against PTP1B using a fluorescence based assay[10] and the IC_{50}'s were measured in the presence of 300 nM and 2 mM dithiothreitol (DTT). Clearly, hydrophobic benzylic sidechains (e.g., **12–15**) are favored on the piperazine ring with the 4-biphenylmethyl **13** showing the best PTP1B inhibitory potency (Table 1). In comparing the initial screening hit **1** and compound **13** the in vitro potency is significantly improved (ca. 33-fold) under the low DTT conditions. The precise role of the diaminopyrimidine moiety was not investigated in this study.



Scheme 1. Reagents and conditions: (a) KOH, MeI, H_2O, 71%; (b) $NaNO_2$, 2 N AcOH, 50 °C, 86%; (c) hydrazine hydrate, DMF, 50 °C, 86%; (d) chloroacetaldehyde diethyl acetal, concd HCl, DMF, rt to 80 °C then NH_4OH, 38%.



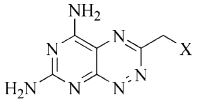
Scheme 2. Reagents and conditions: (a) secondary amine, EtOH, 80–100 °C.

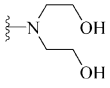
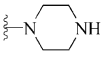
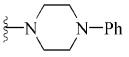
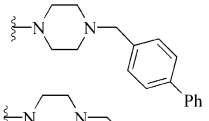
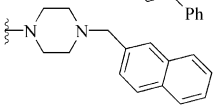
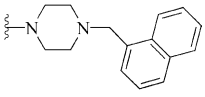


Scheme 3. Reagents and conditions: (a) RI or RBr, K_2CO_3, DMF, rt.

While the enzyme is active at both DTT concentrations, the in vitro inhibitory activity of the PTPase inhibitors appears to be sensitive to the concentration of DTT used in the assay. The sensitivity of the PTP1B inhibitory potency of inhibitors **6–15** to DTT concentration would suggest a possible redox reaction between the inhibitors and DTT. It is also possible that the compounds undergo redox chemistry with the PTP1B active site cysteine residue thus inactivating the enzyme. Shaver and co-workers have speculated that the mode of PTPase inhibition by peroxovanadium compounds may involve irreversible oxidation of the catalytic cysteine

Table 1. PTP1B inhibitory activity of compounds **1** and **6–15**

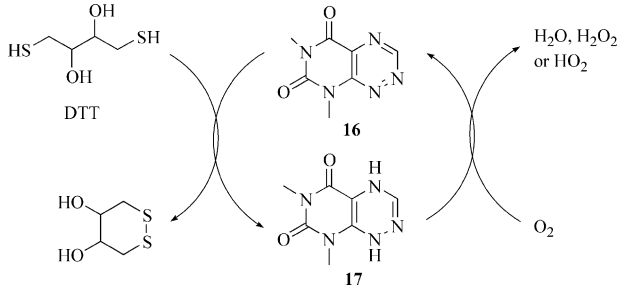


Compd	X	PTP1BIC (μM)[a]
1		97 (>100)
6		29 (>100)
7		24 (>100)
8		24 (>100)
9		29 (91)
10		21 (87)
11		>100 (>100)
12		6.2 (98)
13		2.9 (18)
14		3.5 (33)
15		4.5 (40)

[a]IC_{50} determined in the presence of 300 nM dithiothreitol (DTT). IC_{50} as measured in the presence of 2 mM DTT in parentheses. All measurements were carried out using a common MES buffer system.

residue.[11a] Under high DTT conditions, one may expect to observe less inhibition of PTP1B with inhibitors **6–15** as competing redox chemistry with DTT would become prominent. Indeed, Ramachandran and co-workers[11b] have demonstrated that increasing concentrations of DTT (as well as β-mercaptoethanol and reduced glutathione) effectively decrease the PTP1B inhibitory activity of pervanadate in vitro and similar inhibition kinetics were observed for pervanadate with PTP CD45.

It is likely that the triazine ring of inhibitors **6–15** is responsible for the redox activity of these PTPase inhibitors as we have observed using ^{1}H NMR and MS, that in the presence of DTT and absence of oxygen, the structurally related pyrimidotriazine **16** rapidly and quantitatively undergoes reduction to the corresponding dihydro derivative **17** which is reversible in the presence of atmospheric oxygen (Scheme 4).[12] A simple starch/iodide test indicates that ca. 1% of the reduction product of oxygen is either hydrogen peroxide or superoxide. It is possible that PTPase inhibitors **6–15** catalyse the reversible oxidation of the active site cysteine of PTPases to the sulfenic acid via a similar mechanism involving the generation of peroxide or superoxide. In an in vivo setting, DTT would be replaced by high levels of reduced glutathione. Recently, Goldstein et al. have demonstrated in vivo that insulin stimulation generates a burst of intracellular hydrogen peroxide that reversibly inhibits PTP1B, thereby enhancing the early insulin cascade.[13] Kinetic and reversibility experiments performed using GST-PTP1B (1–321) construct indicated that compounds of the pyrimido[5,4-3][1,2,4]triazine-5,7-diamine class are reversible and competitive inhibitors of PTP1B in the presence of low (300 nM) DTT concentrations.[14] Furthermore, the PTP1B inhibitory potency of these compounds is completely abolished when the assay is conducted in the presence of catalase to sequester any generated hydrogen peroxide. This result clearly indicates that hydrogen peroxide plays a central role in the inhibition of PTP1B under the assay conditions and a redox cycle similar to that outlined in Scheme 4 is likely to be operative for compounds **1** and **6–15**.[14] Although generated hydrogen peroxide is apparently responsible for the PTP1B inhibitory potency of **1** and **6–15**, the substituted piperazine side chains of **12–15** appear to improve potency somewhat, presumably by altering the standard redox potential of the triazine moiety.



Scheme 4. Catalytic cycle for the reduction of triazine derivatives with DTT.

The redox hypothesis described herein for PTPase inhibitors **1** and **6–15** would suggest that these compounds should be rather non-selective in their redox actions, as all PTPases (cytosolic and transmembrane bound receptor like enzymes) contain the conserved 11 amino acid sequence motif (I/V)HcxAGxxR(S/T)G which specifies the active site of the phosphatase. The conserved cysteine and arginine residues within this motif are essential for catalytic activity.[15] The PTPase inhibitors **6**, **12**, and **13** were further evaluated against other PTPases PTPα, LAR and SHP-2 and the results are outlined in Table 2. Indeed, the inhibitors show little selectivity in their inhibitory activity across this panel of PTPases and are reminiscent of the vanadate based inhibitors previously reported in the literature.[7]

The well characterized *ob*/*ob* mouse model was chosen for in vivo evaluation of selected inhibitors.[16] The glucose lowering properties of inhibitors **12** and **13** in male *ob*/*ob* mice are summarized in Table 3. Compounds were dosed po q.d. for 5 days at 50 mg/kg and blood glucose was measured 2 h post dose on day 5. Inhibitors **12** and **13** display significant glucose lowering effect in mice with an ED_{25} (parent drug) around 30 mg/kg po for **12**.[17,18] The pharmacokinetic properties of **13** are summarized in Table 4. The compound is rapidly and extensively distributed in mice. The achieved systemic exposure (C_{max}) of **13** approximates the measured IC_{50}

Table 2. Selectivity of inhibitors **6**, **12**, and **13** against relevant PTPases

Compd	IC_{50} (μM)[a]			
	PTP1B	LAR	PTPα	SHP-2
6	12	28	28	14
12	8	12	24	9
13	8	16	15	5

[a]IC_{50}'s were determined in a common glutarate buffer system in the presence of 300 nM DTT.

Table 3. Glucose lowering properties of **12** and **13** in male *ob*/*ob* mice

Compd	% change in blood glucose[a]
Vehicle	−0.5
12	−23[b]
13	−19[b]

[a]Compounds were dosed at 50 mg/kg orally for a 5-day period and blood glucose was measured 2 h post dose on day 5; values are percent change relative to pretreatment group with 10 mice per group.
[b]$p < 0.05$ when compared to vehicle treated mice.

Table 4. Pharmacokinetic (PK) properties of **13**

Compd	PK Properties[a]				
	$t_{1/2}$ h	CL (mL/kg/min)	V_{ss} (L/kg)	C_{max} (μM)	F (%)
13	1.0	104	3.1	4.0	97

[a]iv and po pharmacokinetic parameters were determined post 10 mg/kg bolus and 50 mg/kg doses, respectively, in male C57BL/6J mice.

against PTP1B in vitro under the low DTT conditions (Table 1). The compound has a favorable $t_{1/2}$ and excellent oral bioavailability. The large steady state volume of distribution (V_{ss}) of **13** is approximately 6 times the total body water volume of the animal suggesting deep tissue and cell penetration. On the other hand, the compound suffers from high systemic clearance which exceeds hepatic blood flow of the mouse.

In conclusion, a novel series of pyrimido[5,4-3]-[1,2,4]triazine-5,7-diamine-based hypoglycemic agents have been discovered. These compounds display non-selective or 'vanadate like' PTPase inhibitory properties in vitro and **12** and **13** were found to effectively lower blood glucose levels in *ob*/*ob* mice upon oral administration. From a toxicological standpoint, the lack of a metallic component suggests that compounds of this class may offer an advantage over the vanadium-based anti-diabetic agents currently in clinical development.

Acknowledgements

We thank Drs. Jeff Tilley and Nader Fotouhi for helpful discussions and Dr. Hans Kuehnle for in vivo evaluation of compounds **12** and **13**.

References and Notes

1. Defronzo, R. A. *Diabet. Rev.* **1997**, *5*, 177.
2. Klein, R. *Diabetes Care* **1995**, *18*, 258.
3. Neel, B. G.; Tonks, N. K. *Curr. Opin. Cell Biol.* **1997**, *9*, 93.
4. (a) Goldstein, B. J. *J. Cell. Biochem.* **1992**, *31*, 33. (b) Goldstein, B. J. *Receptor* **1993**, *3*, 1. (c) Ahmad, F.; Goldstein, B. J. *Biochim. Biophys. Acta* **1995**, *1248*, 57.
5. Byon, J. C. H.; Kusari, A. B.; Kusari, J. *Mol. Cell. Biochem.* **1998**, *182*, 101.
6. Elchebly, M.; Payette, P.; Michaliszyn, E.; Cromlish, W.; Collins, S.; Loy, A. L.; Normandin, D.; Cheng, A.; Himms-Hagen, J.; Chan, C. C.; Ramachandran, C.; Gresser, M. J.; Tremblay, M.; Kennedy, B. P. *Science* **1999**, *283*, 1544.
7. (a) Huyer, G.; Liu, S.; Kelly, J. *J. Biol. Chem.* **1997**, *272*, 843. (b) Tsiani, E.; Fantus, G. *Trends Endocrinol. Metab.* **1997**, *8*, 51. (c) Posner, B. I.; Faure, R.; Burgess, J. W.; Bevan, A. P.; Lachance, D.; Zhang-Sun, G.; Fantus, I. G.; Ng, J. B.; Hall, D. A.; Lum, B. S. *J. Biol. Chem.* **1994**, *269*, 4596. (d) Yale, J. F.; Lachance, D.; Bevan, A. P.; Vigeant, C.; Shaver, A.; Posner, B. I. *Diabetes* **1995**, *44*, 1274. (e) Halberstan, M.; Cohen, N.; Shlimovich, P.; Rosetti, L.; Shamoon, H. *Diabetes* **1996**, *45*, 659. (f) Fantus, I. G.; Tsiani, E. *Mol. Cell. Biochem.* **1998**, *182*, 109.
8. Leung, K. W. K.; Posner, B. I.; Just, G. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 353.
9. Satisfactory spectral data was obtained for all compounds. Final products **6**–**15** were purified by C18 reversed-phase HPLC, eluting with 0.1% TFA, H_2O/CH_3CN gradient, lyophilized and isolated as their trifluoroacetate salts.
10. Assay conditions: PTP1B (0.5–2 nM) was incubated with compound for 15 min in buffer containing 37.5 mM MES pH 6.2, 140 mM NaCl, 2 mM EDTA, 0.05% BSA and either 300 nM or 2 mM DTT. The reaction was initiated by the addition of 50 μM substrate [phosphorylated peptide 1146(TRDI-(pY)E]. After 20 min at room temperature (22–25 °C), the reaction was stopped with KOH and the amount of free phosphate measured using malachite green as previously described (Harder, K. W.; Owen, P.; Wang, L. K.; Asbersold, R.; Clark-Lewis, I.; Jirik, F. R. *Biochem J.* **1994**, *298*, 395). In order to measure the PTPase activity across a panel of phosphatases, similar assay conditions were used as described except 6,8-difluoro-4-methylumbelliferylphosphate (DiFMUP, Molecular Probes) was used as substrate at the K_m for each enzyme and 37.5 mM diethylglutarate pH 6.2 was used in place of MES. The reaction was stopped with KOH and the fluorescence of the dephosphorylated substrate measured (excitation: 360 nM/emission: 460 nM).
11. (a) Shaver, A.; Ng, J. B.; Hall, D. A.; Soo Lum, B.; Posner, B. I. *Inorg. Chem.* **1993**, *32*, 3109. (b) Huyer, G.; Liu, S.; Kelly, J.; Moffat, J.; Payette, P.; Kennedy, B.; Tsaprailis, G.; Gresser, M. J.; Ramachandran, C. *J. Biol. Chem.* **1997**, *272*, 843.
12. Upon reaction of **16** with DTT, the CH proton of the triazine ring of product **17** is shifted upfield by approximately 3 ppm compared to **16**, indicating a loss in aromaticity of this ring. In DMSO-d_6, two NH protons could be detected at 6.93 and 8.73 ppm. Mass spectrometry revealed a species whose mass is 2 units higher than parent **16,** corresponding to the addition of hydrogen and consistent with the structure **17**.
13. Mahadev, K.; Zilbering, A.; Zhu, L.; Goldstein, B. *J. J. Biol. Chem.* **2001**, *276*, 21938.
14. Olivier, A. R. Unpublished results. A detailed account of this work will be published separately.
15. (a) Fischer, E. H.; Charbonneau, H.; Tonks, N. K. *Science* **1991**, *253*, 401. (b) Pot, D. A.; Dixon, J. E. *Biochim. Biophys. Acta* **1992**, *1136*, 35.
16. Coleman, D. L. *Diabetologia* **1978**, *14*, 141.
17. As determined by C, H, N, O, F combustion analysis, the trifluoroacetate salt of compounds **12** and **13** contains 3 mol of TFA per mol of parent **12** and **13**. Thus a 50-mg/kg dose of **12** corresponds to 28 mg/kg of parent drug.
18. Preliminary toxicological data was obtained following the 5 day dosing study in *ob*/*ob* mice (Table 3). No significant adverse treatment related effects were observed on organ weights, hematological parameters or serum chemistries. A histopathological evaluation of selected tissues was performed and no treatment related microscopic changes were observed.