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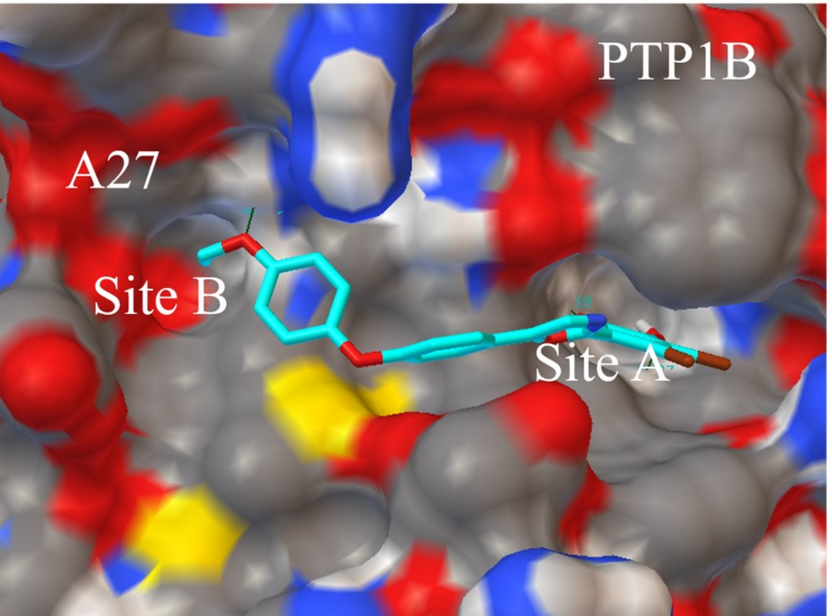
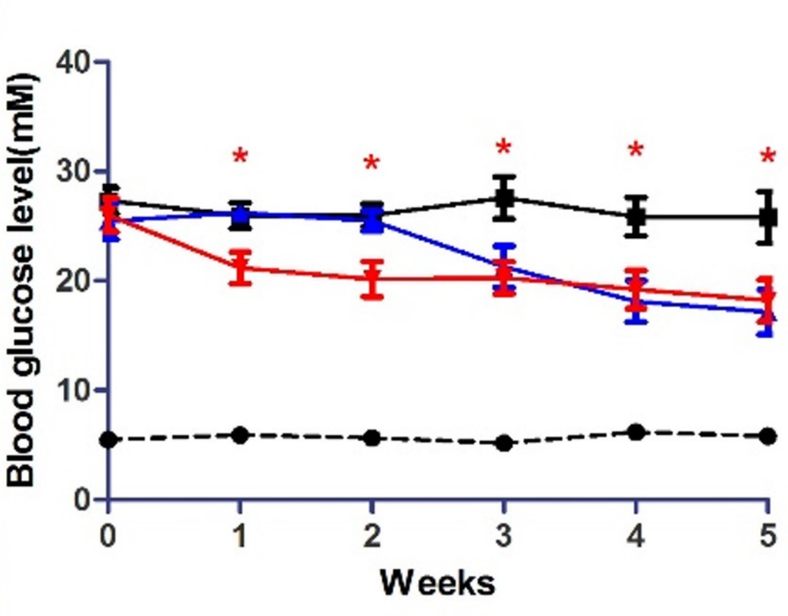

PTP1B
A27
Site B
Site A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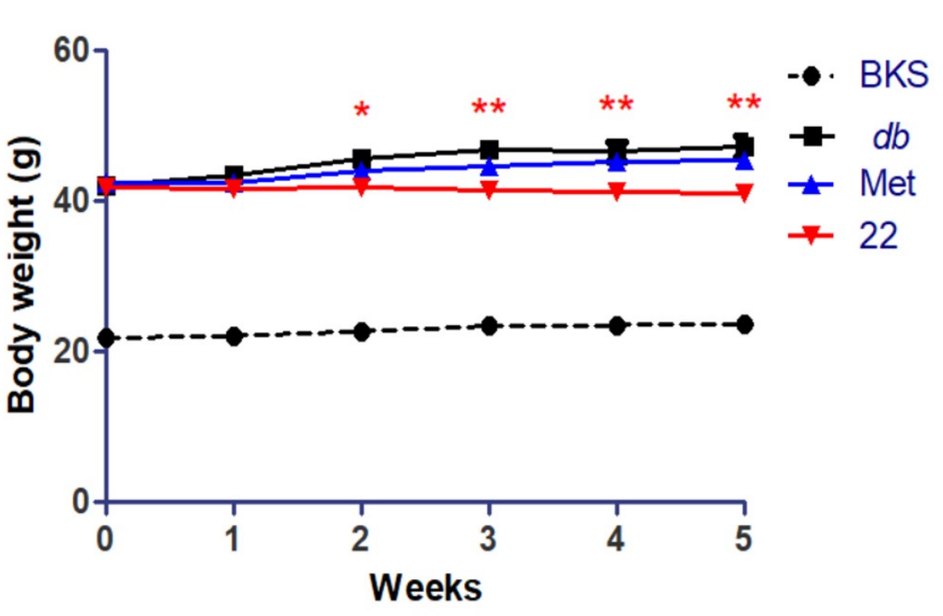
Body weight (g)
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BKS
db
Met
22

ACCEPTED MANUSCRIPT

Toward a treatment of diabesity: *in vitro* and *in vivo* evaluation of uncharged bromophenol derivatives as a new series of PTP1B inhibitors

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Abstract

Protein tyrosine phosphatase 1B (PTP1B) has been considered as a validated biological target for type 2 diabetes treatment, but past endeavors to develop inhibitors of PTP1B into drugs have been unsuccessful. Two challenging aspects are selective inhibition and cell permeability. A structure-based strategy was employed to develop uncharged bromophenols as a new series of PTP1B inhibitors. The most potent compound **22** (LXQ46) inhibited PTP1B with an IC_{50} value of 0.190 μM, and showed remarkable selectivity over other protein tyrosine phosphatases (PTPs, 20-200 folds). In the SPR study, increasing concentrations of compound **22** led to concentration-dependent increases in binding responses, indicating that compound **22** could bind to the surface of PTP1B via noncovalent means. By treating insulin-resistant C2C12 myotubes with compound **22**, enhanced insulin and leptin signaling pathways were observed. Long-term oral administration of compound **22** reduced the blood glucose level of diabetic BKS *db* mice. The glucose tolerance tests (OGTT) and insulin tolerance tests (ITT) in BKS *db* mice showed that oral administration of compound **22** could increase insulin sensitivity. In addition, long-term oral administration of compound **22** could protect mice from

obesity, which was not the result of toxicity. Our pharmacokinetics results from the rat-based assays showed that orally administered compound **22** was absorbed rapidly from the gastrointestinal tract, extensively distributed to the tissues, and rapidly eliminated from the body. All these results indicate that compound **22** could serve as a qualified agent to treat type II diabetes.

Keywords

Anti-diabetic; PTP1B inhibitor; Selectivity; BKS *db* mice; Toxicity.

1. Introduction

Protein tyrosine phosphatases (PTPs) are a superfamily of receptor-like, non-transmembrane proteins, whose members are crucial modulators of tyrosine phosphorylation-dependent cellular events [1, 2]. Among the PTPS, Protein tyrosine phosphatase 1B (PTP1B) is considered as a validated biological target to treat type 2 diabetes and obesity, owing to its regulatory role in insulin and leptin signaling [3-5]. In addition to normal development and longevity, PTP1B-deficient mice also display improved glycemic control and increased insulin sensitivity[6, 7]. The silencing of the PTP1B gene results in increased insulin sensitivity and resistance to weight gain on a high-fat diet without causing any abnormality in the animals [8, 9].

Extensive efforts have successfully identified potent PTP1B inhibitors, but endeavors to develop compounds into drugs have been largely unsuccessful [10, 11]. This situation was probably due to the highly polar nature of the PTP1B catalytic site, which has evolved to accommodate pTyr containing two negative charges at physiological pH[10]. Consequently, most active-site-directed PTP1B inhibitors possess a high charge density with limited cell membrane permeability and bioavailability [12]. Numerous nanomolar nonhydrolyzable phosphonate [13-15] and carboxylic acid [16-18] pTyr mimetics were suspended presumably due to their poor cell permeability.

Therefore, the identification of uncharged compounds has been conducted develop new small molecule PTP1B inhibitors. Previously, our group isolated and identified bromophenol compounds as PTP1B inhibitors [19-21] (Figure 1). Herein, we wish to report our structure-based design and antidiabetic study of potent uncharged PTP1B inhibitors, using these natural bromophenols as the initial lead compounds.

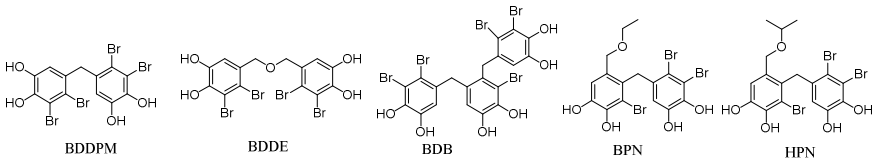


Figure 1. Representative bromophenol PTP1B inhibitors in our laboratory.

2. Results and Discussions

2.1 Design of selective PTP1B inhibitors

Our previous analysis indicated that the bromophenol moiety is essential for PTP1B inhibitory activity. Two hydroxyls of the bromophenol moiety (Figure 2a, light blue) are involved in hydrogen bonding interactions with Ser216, Ile219, Gly220 and Arg221 in the catalytic site (site A). This group in part mimics the action of the phosphoryl group (Figure 2a, pink) in pTyr1162 (insulin receptor kinase). Hence, natural bromophenols were simplified to two active fragments, 3-bromo-4,5-dihydroxybenzaldehyde (**1**) and 2,3-dibromo-4,5-dihydroxybenzaldehyde (**2**). PTP1B inhibition studies identified that compound **2** exhibited inhibitory activity with an IC_{50} value of 128 μM, while compound **1** inhibited PTP1B weakly (354 μM, Figure S1). Based on these two weak but well characterized fragments, we sought to design a series of PTP1B inhibitors to interact with Ala27 in site B, which is a specific recognition site of PTP1B over other PTPs.

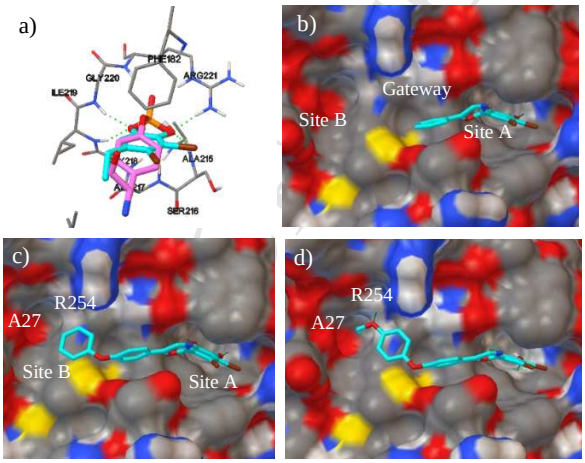
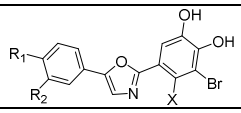


Figure 2. Predicted binding models of PTP1B (1G1H) with (a) compound **2** (light blue) and overlay with pTyr1162 (pink), (b) compound **4**, (c) compound **11**, and (d) compound **22**.

Our procedure was to construct compounds from site A toward B in a stepwise manner, in which inhibitors could be optimized with more efficiently. An oxazole group was introduced to pass through the narrow area between site A and B (Figure 2b). To effect the extension of the molecule, a phenyl group was introduced as a linker to offer a favorable trajectory toward site B. Compounds **3** and **4** were synthesized and determined to inhibit PTP1B with IC_{50} values of 6.68 and 4.27 μM, respectively (Table 1). Modeling suggests that the additional 5-phenyloxazole group lies along the tunnel wall and offers an appropriate direction toward site B (Figure 2b, Figure S1).

Table 1. Inhibitory activity of the compounds.

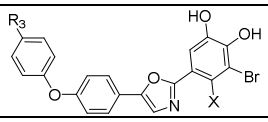


Comp	X	R_1	R_2	IC_{50} (μM)
3	H	H	H	6.68±0.5
4	Br	H	H	4.27±0.2
5	H	OMe	H	9.13±1.6
6	H	OMe	Br	18.47±2.3
7	Br	OMe	Br	10.26±1.2
8	H	OBn	H	4.00±0.6
9	Br	OBn	H	3.75±0.2
10	H	OPh	H	3.34±0.1
11	Br	OPh	H	1.99±0.2
	Na_3VO_4			3.09±0.13

The small hydrophobic cavity adjacent to Ala27 was next targeted; its occupation with a proper hydrophobic group could increase inhibitor potency and selectivity. This strategy led to the design of **12**-**17**, which contain multiple hydrophobic groups of different sizes extending out from the para-position of the terminal phenyl ring. The methyl group did endow compound **12** with improved inhibitory activity (IC_{50}=0.87 μM, Table 2). When

the large groups, such as *i*-propyl, *t*-butyl, and phenyl were incorporated, inhibitory activity showed no improvement (or decreased efficacy). The molecular modeling predicted that the methyl group does not fully fill this hydrophobic "hole", but instead points to the side chain of Arg254, which could stabilize the interactions between PTP1B and inhibitors.

Table 2. Inhibitory activity of the compounds.



Comp	X	R_3	IC_{50} (μM)
12	Br	Me	0.87±0.3
13	H	*i*-Pr	7.44±0.4
14	Br	*i*-Pr	3.09±1.2
15	H	*t*-Bu	2.29±0.8
16	H	Ph	2.45±0.4
17	Br	Ph	3.55±0.6
18	H	OMe	1.07±0.3
19	Br	OMe	2.14±0.2
20	H	EtOMe	3.81±0.3
21	H	OEt	0.285±0.1
22	Br	OEt	0.190±0.05
Na_3VO_4			3.09±0.13

Three groups, methoxyl, ethoxyl, and methoxyethyl, were selected to optimize these interactions, leading to the design of **18**-**22**. These compounds contain (a) a hydrogen bond receptor that was designed to interact with Arg254, providing an anchor site, and (b) a small flexible hydrophobic group that was expected to occupy the hydrophobic cavity adjacent to Ala27, which is related to selectivity. Consistent with our prediction,

compounds with the ethoxyl group at the para-position of the phenyl ring, **21** and **22**, showed inhibitory activity against PTP1B with IC_{50} values of 0.285 and 0.190 μM, respectively (Table 2). Modeling suggests that the additional ethoxyl group not only optimally fills the hydrophobic hole, but also forms a hydrogen bond with Arg254 (Figure 2d).

2.2 Compound 22 is a selective PTP1B inhibitor

Selectivity study. Selective inhibition of PTP1B over other PTPs represents another challenging aspect, because of the high homology combined with different biological functions [22]. *In vitro* selectivity over PTPs was evaluated for the most potent compounds (**11**, **12**, **14**, **19** and **22**) in Table 3. Compounds **19** and **22** showed 17 to 20-fold selectivity over TCPTP, the phosphatase with the highest homolog to PTP1B. The methoxyl and ethoxyl substituents in **19** and **22** interacted effectively with Ala27 of PTP1B, but they were less tolerated by the Ser29 in TCPTP. Furthermore, compounds **19** and **22** also showed good selectivity over other PTPs, such as SHP-1, SHP-2 and LAR (approximately 20-200-fold).

Table 3. Inhibition of phosphatases by selected compounds.

Comp	IC_{50} (μM)					
	PTP1B	TCPTP	PTP1B (A27S)	SHP-1	SHP-2	LAR
11	1.99±0.20	5.49±1.2	3.98±0.63	5.16±2.1	7.78±0.32	>40
12	0.87±0.28	2.55±0.46	1.38±0.27	29.9±6.3	>38.9	>38.9
14	3.09±1.23	3.02±0.28	1.03±0.38	2.54±1.8	8.11±0.06	>36.9
19	2.14±0.16	36.42±5.3	12.9±1.52	27.1±0.51	>37.7	>37.7
22	0.190±0.05	3.81±0.64	1.78±0.21	>36.8	>36.8	>36.8
Na_3VO_4	3.09±0.13	2.38±0.10	2.01±0.2	2.63±0.14	10.68±2.08	32.62±0.44

A27S mutant. To study the function of Ala27 in the selectivity between PTP1B and TCPTP, we applied a site-directed A27S mutant of PTP1B to test the inhibitory activity of compounds **11**, **12**, **14**, **19** and **22**. As expected, the substitution of Ala27 to Ser (as in the TCPTP sequence) elicited selectivity between the derivative and the wild-type proteins. Compounds **19** and **22** exhibited lower inhibitory activity against A27S mutant than wild-type PTP1B (6-9-fold, Table 3), while the control Na_3VO_4 showed no selectivity. This result further confirmed that the capture of Ala27 is a selectivity determinant and is sufficient to confer selectivity.

Kinetics study. Inhibition kinetics studies were carried out in the absence or presence of inhibitors with different concentrations of pNPP. The experimental data were analyzed by the double reciprocal plot method in order to determine the type of inhibition. As shown in Figure 3, with the increasing concentrations of compounds **11** and **22**, the K_m values increased accordingly, while the V_{max} values remained almost constant. The graphs exhibited straight lines which intersect each other on the vertical axis, indicating that compounds **11** and **22** acted as competitive inhibitors.

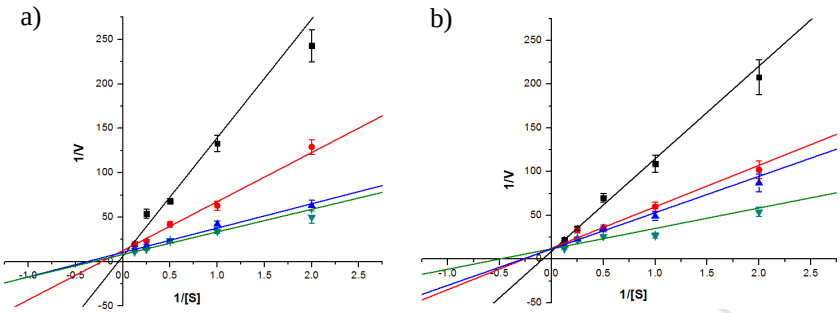


Figure 3. Inhibition kinetics studies of compounds **11** and **22**. (a) concentrations of compound **11** were 1 (green), 2 (blue), 4 (red), and 8 µM (black). (b) concentrations of compound **22** were 0 (green), 0.5 (blue), 1 (red), and 2 µM (black).

SPR study. A novel screening technique [23, 24] was employed to monitor the binding of PTP1B and compounds. Using a surface plasmon resonance (SPR) biosensor, we evaluated the dynamic interaction of small molecules with PTP1B immobilized on the sensor surface of a single chip. Association and dissociation of inhibitors could be exhibited and analyzed with continuous response values. Compounds **11**, **12**, **14**, **19** and **22** displayed binding affinities with the PTP1B surface, which were characterized by slow on-rates and off-rates (Figure 4a). To determine the kinetic rate constants, increasing concentrations of compound **22** were injected over the immobilized PTP1B surface. As shown in Figure 4b, compound **22** showed concentration-dependent increases in binding responses to PTP1B with a K_D value of 2.238×10^{-6} M. Increasing concentrations of compound **22** led to concentration-dependent increases in binding responses. The SPR studies indicated that these compounds could bind to the surface of the catalytic site in PTP1B via noncovalent means.

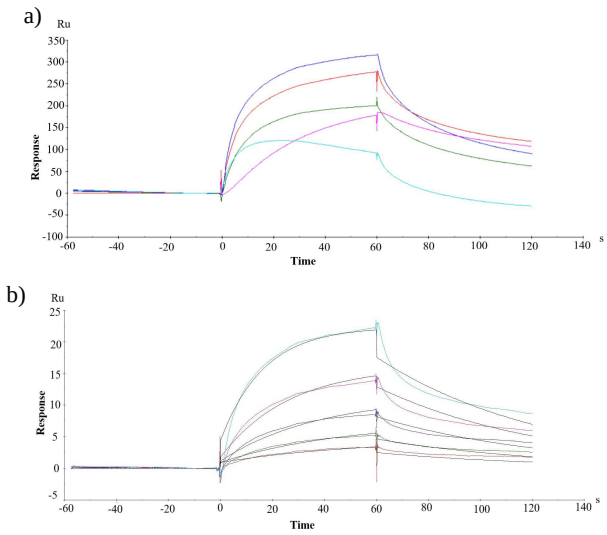


Figure 4. Surface plasmon resonance studies of inhibitor binding. (a) Representative sensorgrams for the interactions of compounds **11**, **12**, **14**, **19** and **22** (50 μM) with the PTP1B surface. (b) Sensorgrams and curve fits (smooth lines) for the interactions of increasing concentrations of inhibitor **22** (6.25, 3.125, 1.562, 0.781, and 0.390 μM) with the PTP1B surface, providing $k_a = 6.124\times10^{3}$ M^{-1} s^{-1}, $k_d = 1.371\times10^{-2}$ s^{-1}, and leading to $K_D = 2.238\times10^{-6}$ M.

2.3 Compound 22 activates insulin and leptin signaling pathway in cells

As a critical regulator of insulin and leptin receptors, PTP1B is an ideal therapeutic target for type II diabetes and obesity [25-27]. The activation of insulin[6, 7] and leptin signaling (JAK2-STAT3) [28, 29] is the indirect reflection of PTP1B inhibition. Stabilization of PTP1B in an inactive, oxidized conformation by small molecules can promote insulin and leptin signaling [30]. The role of compound **22** was also investigated in C2C12 myotubes. As shown in Figure 5, the phosphorylation levels of IRS-1, Akt, JAK2 and STAT3 were significantly increased in a dose-dependent manner. These results indicated that compound **22** could activate the insulin and leptin signaling pathways in cells.

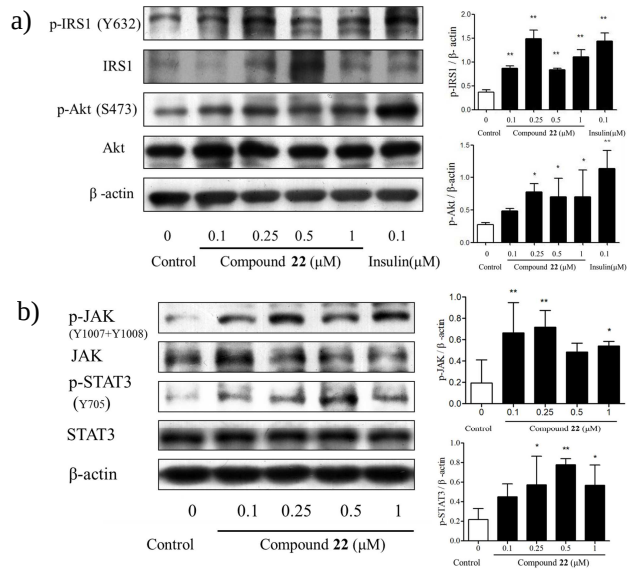


Figure 5. Compound **22** activates (a) insulin and (b) JAK2-STAT3 signaling pathway. C2C12 myotubes were treated with compound **22** for 8 h in serum-free DMEM. Then, phosphorylation levels were determined by immunoblotting. Band density was quantified and normalized with β-actin. The results shown are means ± SD (n = 3). *p < 0.05 versus vehicle treated control.

2.4 Oral administration of compound 22 controls body weight of BKS *db* mice

Since the typical symptoms of T2DM are polydipsia and polyphagia, we studied the dynamic effects of compound **22** on food intake, water intake and body weight of BKS *db* mice during a treatment of five weeks. Oral administration of compound **22** (100 mg/kg·day^{-1}) initiated a trend toward decreasing food intake, but no suppressive effect on the water intake (Figure 6a, b). In detail, food intake of **22**-treated mice was significantly reduced at the fourth week compared to the administered vehicle. As shown in Figure 6c, mice treated with compound **22** maintained a steady weight during five weeks, while mice of other groups continued to increase in weight. By the fifth week, **22**-treated mice had gained approximately 15% less weight (average 7.18 g) than the controls. The weight of the abdominal fat pad in **22**-treated mice was also reduced compared with the mice that were administered vehicle and metformin (Figure 6d). These results indicate that long-term oral administration of **22** could protect mice from obesity.

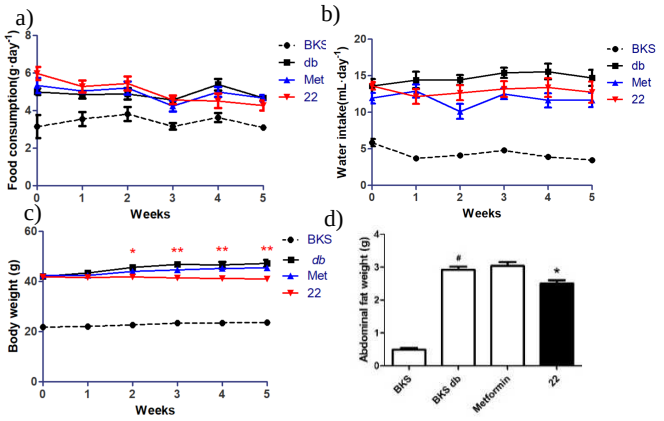


Figure 6. Effects of 5 weeks treatment with compound **22** (100 mg/kg·day^{-1}) on mean (a) food intake, (b) water intake (data are expressed as the mean ± SEM (g·day^{-1}, n = 8)); (c) body weight and (d) abdominal fat weight (data are expressed as the mean ± SEM (n = 8)). BKS *db* mice were treated with vehicle, compound **22** and metformin, and BKS mice were treated with vehicle. *P < 0.05 versus BKS *db* group; $^{\#}$P < 0.05 versus BKS group.

2.5 Oral administration of compound 22 ameliorates insulin sensitivity of BKS *db* mice

Blood glucose levels of BKS *db* mice were measured during the 5 weeks of treatment to further investigate the anti-diabetic effect. Compared with BKS mice, diabetic BKS *db* mice exhibited hyperglycemia with blood glucose levels of approximately 28 mM (Figure 7a). A five week treatment with compound **22** (100 mg·kg^{-1} body wt·day^{-1}) showed a trend toward decreased blood glucose levels compared with vehicle. In the **22**-treated group, the blood glucose levels of BKS *db* mice were significantly decreased from the first week and stable within the following four weeks. Compared to **22**'s rapid effect, metformin was slow-acting, there was not a visible downregulation of glucose levels until the third week. By the fifth week, the blood glucose levels of **22**-treated mice were similar with those of metformin-treated mice (approximately 18 mM), suggesting that compound **22** could serve as a qualified agent to treat type II diabetes.

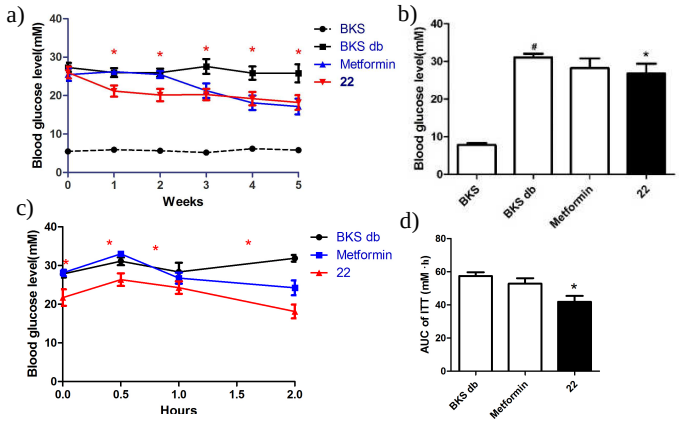


Figure 7. Effects of compound **22** in BKS *db* mice. (a) Fasting blood glucose levels were measured at week 0, 1, 2, 3, 4 and 5 during the five-week period. (b) Blood glucose levels 120 min after a dose of oral glucose (2 g/kg). (c) ITT in the 2nd week (insulin dose, 0.75 U/kg). (d) The area under the curve (AUC) of ITT. Data are expressed as the mean ± SEM (n = 8). *P < 0.05 versus BKS *db* group, $^{\#}$P < 0.05 versus BKS group.

We next performed oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT) in BKS *db* mice treated with vehicle, metformin or **22**. BKS *db* mice treated with compound **22** showed decreased blood glucose levels at 120 min after a dose of oral glucose (2 g/kg) compared with the control BKS *db* mice (Figure 7b). Increased insulin sensitivity was observed in BKS *db* mice after **22** administration (Figure 7c). The area under the curve (AUC) values of ITT in the compound **22** group were significantly lower than those in the control group (Figure 7d).

2.6 Oral administration of compound 22 increases phosphorylation level of Akt in muscle tissues of BKS *db* mice

To determine whether compound **22** improves glucose homeostasis and insulin resistance through inhibition of PTP1B, the phosphorylated and total levels of Akt were evaluated in the muscle tissue of BKS *db* mice with or without compound **22**. As shown in Figure 8, the phosphorylation level of Akt in muscle of BKS *db* mice were decreased, compared with the normal BKS mice. After oral administration of compound **22**, phosphorylation level of Akt was elevated compared with the control group, indicating that compound **22** administration enhanced insulin signaling in muscle of BKS *db* mice.

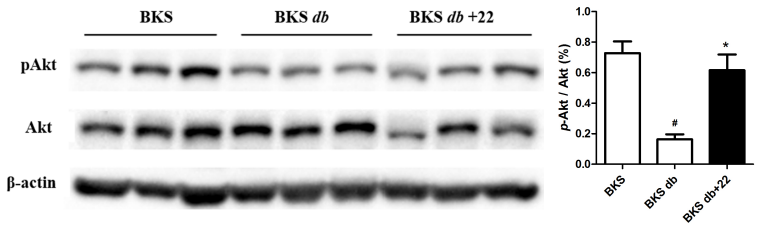


Figure 8. Phosphorylated and total Akt in muscle tissues of BKS *db* mice. Data are expressed as the mean ± SEM (n = 3). *P < 0.05 versus BKS *db* group, $^{\#}$P < 0.05 versus BKS group.

2.7 Toxicological safety evaluation of compound 22 *in vivo*

To assess the safety of compound **22** for oral administration, acute and subacute toxicological evaluation were conducted. Single doses (500, 1000 and 2000 mg/kg body wt.) elicited no significant changes in food intake, water intake or body weight (not shown). Repetitive doses (1000 mg/kg body wt·day^{-}) for 14 days initiated a trend toward decreased food intake and body weight compared with vehicle, but no suppressive effect on the water intake (Figure 9). In addition, none of the mice showed visible toxic effects, behavioral changes or mortality during acute and subacute toxicity studies. Histological analysis by gomori staining of **22**-treated mice livers, hearts, kidneys and lungs showed normal histological structure and normal sized cells (Figure 10). Long-term oral administration of compound **22** could protect mice from obesity, which was not the result of toxicity.

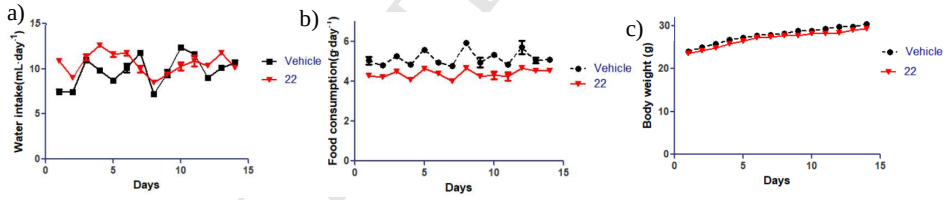


Figure 9. Effects of 14 days treatment with compound **22** (1000 mg/kg·day^{-1}) on mean (a) water intake, (b) food intake, and (c) body weight (data are expressed as mean ± SEM (n = 10)).

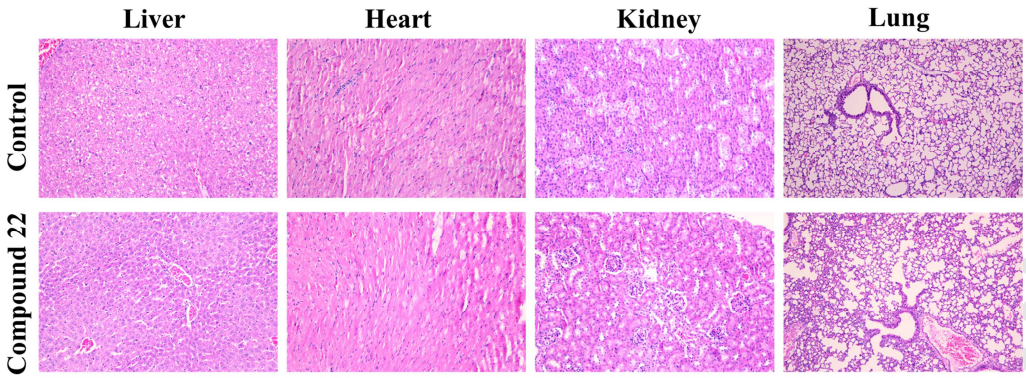


Figure 10. Histological analysis by gomori staining of livers, hearts, kidneys and lungs in the mice treated with compound **22** (1000 mg/kg body wt·day, 14 days) and vehicle .

2.8 Pharmacokinetics study of compound 22 in specific pathogen free rates.

The highest plasma concentration C_{max} of compound **22** had a mean value of 3350 ± 707 nmol/L after i.v. administration (5 min post dosing, 2 mg·kg^{-1}). The mean V_{ss} was 4.25 ± 0.47 L/kg, suggesting that compound **22** could be extensively distributed to the tissues. The plasma level of compound **22** declined rapidly during the first 2 h (MRT 0.58 ± 0.04 h), as indicated by a short mean $t_{1/2}$ of approximately 0.40 ± 0.03 h. After p.o. administration, compound **22** was absorbed rapidly from the gastrointestinal tract (T_{max} 0.5 h). The bioavailability F of compound **22** was low, with a mean value of 8.31 ± 2.96 %.

Our pharmacokinetics results from the rat-based assays indicated that orally administered compound **22** was absorbed rapidly from the gastrointestinal tract and extensively distributed to the tissues. In addition, compound **22** was rapidly eliminated from the body, resulting in poor availability to systemic circulation. The reason for this result might be that the phenolic hydroxyl groups were eliminated rapidly [31]. To circumvent the undesirable presystemic metabolism, a slow-release formulation or a biomimetic nanocarrier approach [32] might be employed as promising strategies to overcome this deficiency.

3. Conclusions

In summary, we have utilized a structure-based approach to design a series of non-phosphonate PTP1B inhibitors with good specificity. When an ethoxyl group was introduced to occupy the hydrophobic pocket containing Ala27 and Arg254, a marked inhibitory effect (**22**, IC_{50}=0.190 μM) and selectivity (20-200 folds

over other PTPs) were observed resulting from the competitive inhibition of PTP1B. Further studies on cellular activities revealed that compound **22** increased the phosphorylation levels of IRS-1, Akt, JAK2 and STAT3 in the C2C12 myotubes and the muscles of BKS *db* mice, indicating the activation of insulin and leptin signaling pathways. Long-term oral administration of compound **22** could ameliorate insulin sensitivity, reduced blood glucose levels (fast-acting) in BKS *db* mice, and protect mice from obesity, while no visible toxic effects were observed. Orally administered compound **22** was absorbed rapidly from the gastrointestinal tract, extensively distributed to the tissues and rapidly eliminated from the body of rat. These novel bromophenol derivatives reported in this study could provide a possible opportunity for the development of potent PTP1B inhibitors to treat type II diabetes.

Additional files

Additional figures, synthesis and full spectroscopic data for all new compounds can be found at Electronic Supplementary Information (ESI).

Declarations

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Highlights:

1. A complete process of PTP1B inhibitor identification is proposed, from enzymatic to mouse model.

2. The difficult points in PTP1B inhibitors, selective inhibition and cell permeability, were overcome.

3. Oral administration of **22** prevented weight gain, improved insulin sensitivity and decreased blood glucose level in BKS *db* mice.

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