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Notes

Isoxazolone Derivatives as Potent Inhibitors of PTP1B

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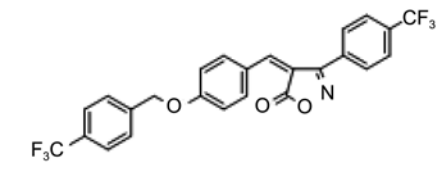
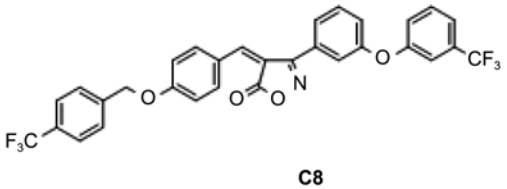
Metabolic syndromes such as obesity and diabetes pose serious health threats in modern society. These chronic ailments can cause secondary complications including the increase of cardiovascular risk, which is a leading cause of death.[1,2] Numerous researches have been conducted to develop novel therapeutics against obesity and diabetes with improved safety and efficacy.[3-6] One of the strategies is to develop chemical inhibitors targeting PTP1B, which was identified as a negative regulator of insulin and leptin signaling pathways.[7-10] Biochemical studies revealed that PTP1B inhibition prolongs the tyrosine phosphorylated states of insulin and leptin receptors, resulting in the augmentation of insulin sensitivity and the suppression of weight gain.[7-10] PTP1B knockout mice were resistant to weight gain and maintained insulin sensitivity upon feeding a high fat diet (HFD).[11] Researches during the last two decades produced numerous PTP1B inhibitors, none of them yet satisfied the criteria for therapeutic uses. As a result of our ongoing efforts toward the search for PTP1B inhibitors, we reported several chemical scaffolds that inhibited PTP1B effectively.[12-17] Among those, an isoxazol-5(4*H*)-one derivative **C0** inhibited PTP1B with an IC_{50} value of 2.3 μM and suppressed weight gain in mice upon feeding a HFD.[17] In the present study, further structural variation was made on the isoxazol-5(4*H*)-one moiety to improve the inhibitory potency of the compound **C0**.



C0



C8

Results and Discussion

The first set of compounds, **C1-C9**, was prepared by the condensation of benzaldehyde, **C**, with isoxazolone precursors, **1-9**. The general synthetic route for the preparation of isoxazol-5(4*H*)-one derivatives has been previously reported. Briefly, isopropanol solutions of an arylaldehyde (**A-J**) and an isoxazolone precursor (**1-9**) were mixed in a test tube and heated at 65 ºC for 4 h without additional reagents or catalysts. Upon cooling to room temperature, the condensation products were obtained as precipitates. Removal of the solvent and washing with isopropanol afforded reaction products which are pure in TLC analysis. Using this protocol, the condensation products could be harvested conveniently without aqueous workup and additional purification steps. This strategy saves time for synthetic efforts sacrificing a precise structural characterization, which can be accomplished only for the compounds progressed for further experiments.

The first set of compounds, **C1-C9**, was evaluated for their inhibitory activity against PTP1B. In initial experi-

Table 1. Inhibitory effect of the compounds **A0-J9** on PTP1B. Numbers are IC_{50} values or percent inhibition (in the parenthesis) at 10 μM concentrations of the compounds[a]

	0[b]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
A	2.5								1.1	
B	3.9								1.6	
C	2.3	3.2	2.7	4.0	2.3	6.8	13	1.5	0.7	19
D	(28)								(75)	
E	(57)								(88)	
F	(86)								(89)	
G	(35)								(70)	
H	(39)								(87)	
I	(18)								(63)	
J	(84)								1.3	

[a]IC_{50} values were determined only for the compounds with > 90% inhibition. Values are means of 2 or more experiments. Standard deviations are not shown. [b]Data for **A0-J0** were reproduced from our previous publication.[17]

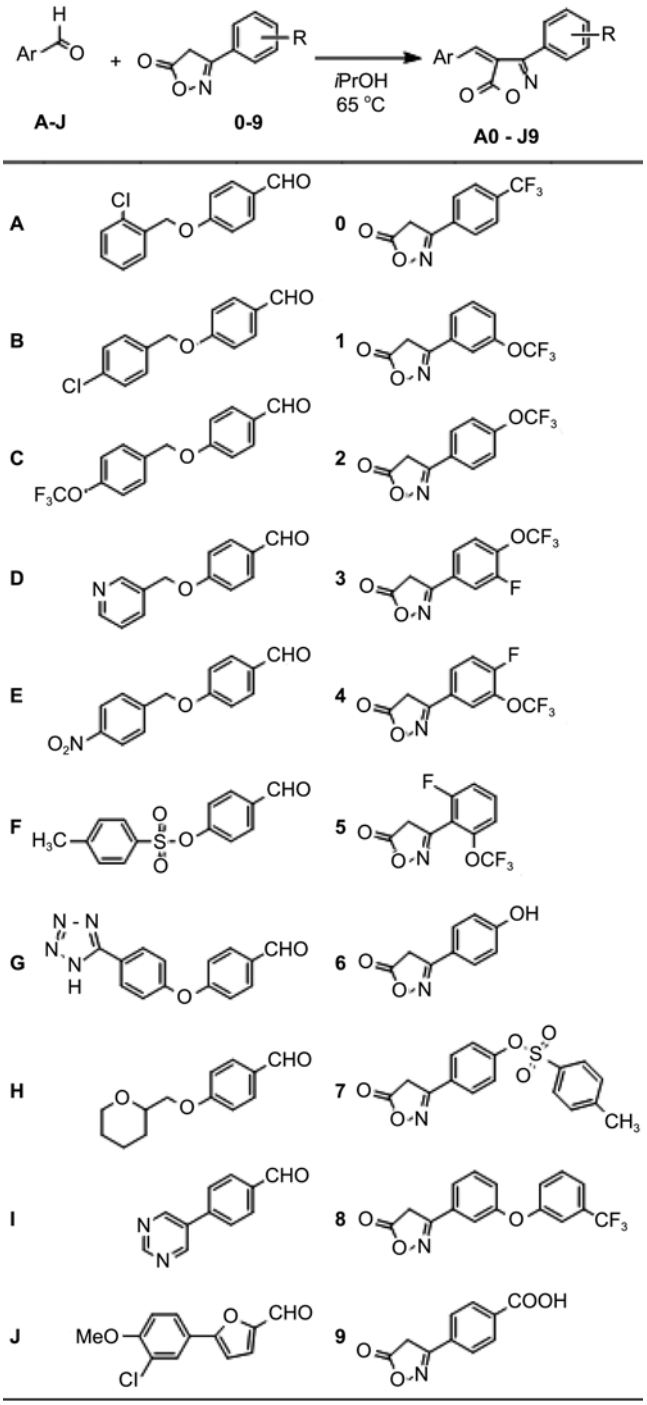


Figure 1. Preparation of the isoxazol-5(4*H*)-one chemical library.

ments, *p*-nitrophenyl phosphate (*p*NPP) hydrolase activity of PTP1B was measured in the presence of the compounds at a 10 μM concentration. All of the compounds inhibited > 90% of the enzyme activity. Determination of the IC_{50} values revealed **C8** the most potent among the series with an IC_{50} value of 0.7 μM (Table 1). Second set of compounds (**A8-J8**) were prepared with the isoxazolone precursor fixed to **8** and the arylaldehyde precursors varied to **A-J**. Four compounds in this series inhibited > 90% of the enzyme activity of PTP1B with the IC_{50} values in a 0.7-1.6 μM range

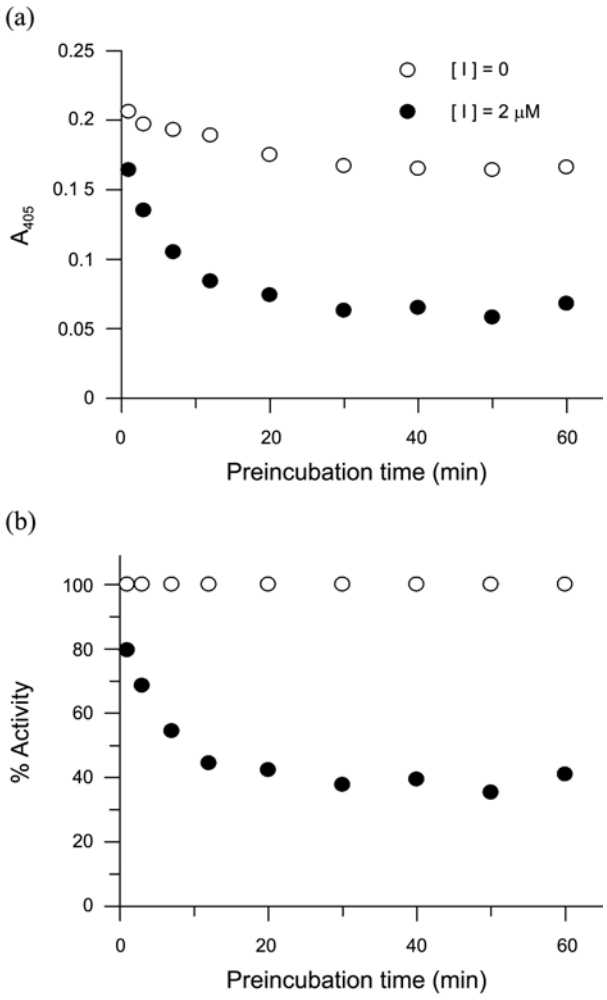


Figure 2. Time-dependent inhibition kinetics of PTP1B with **C8**. (a) PTP1B was incubated in the absence (○) and presence (●) of **C8** (2 μM) in the reaction buffer. At intervals, the enzyme reaction was initiated by addition of *p*NPP and the initial velocity was measured. (b) Percent remaining activity of the inhibited reaction was calculated at each time points.

(Table 1). Taken together, **C8** was identified as the most potent PTP1B inhibitor among the compounds synthesized in the present study. The IC_{50} value was 0.7 μM and 3.3-fold lower compared to 2.3 μM of **C0**.

To determine the mode of PTP1B inhibition by **C8**, kinetic experiments were performed. A bulk solution of PTP1B was preincubated with **C8** and aliquots were taken at time intervals for activity measurements. Compound **C8** inhibited PTP1B in a time-dependent fashion suggesting irreversible or slow-binding inhibition (Figure 2). To distinguish the mode of inhibition between these possibilities, progress of the enzyme reaction was monitored continuously in the presence of 2 μM **C8** and, when the reaction rate reached steady-state, 10-fold excess *p*NPP was added to the mixture. The increase of reaction rate by addition of excess substrate indicates that binding of **C8** to PTP1B was reversible (Figure 3).

In the present study, significant improvement was achieved in the inhibitory potency against PTP1B. Compound **C8** was

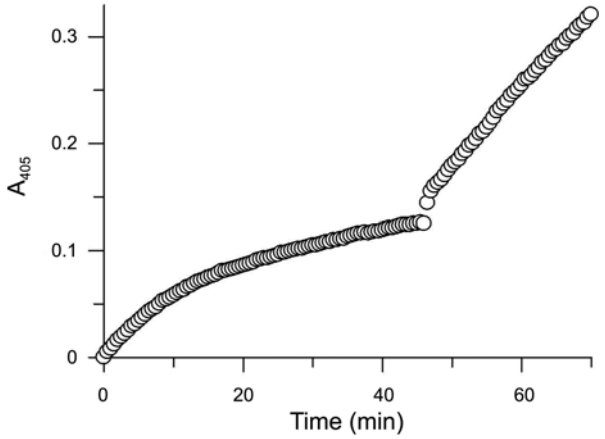


Figure 3. Slow-binding inhibition kinetics of PTP1B with **C8**. Enzyme reaction was initiated by addition of PTP1B to the reaction mixture containing 0.1 mM *p*NPP and 2 μM **C8**, and the absorbance at 405 nm was monitored continuously. After 46 min, additional *p*NPP was added to a final concentration of 1 mM.

found 3.3-fold more potent compared to **C0**, which exhibited anti-obesity effects in our previous mouse experiment. Strongly hydrophobic nature of the compound (cLogP value of 8.96), however, could be a hurdle for **C8** to be used for clinically relevant trials. Further studies are required to improve the physicochemical properties of **C8**.

Synthesis. The benzaldehyde and isoxazolone precursors were dissolved in minimum volumes of isopropanol at 65 ºC. Solutions of arylaldehyde derivatives **A-J** and isoxazolone precursors **1-9** were added into test tubes to provide appropriate combinations of the precursors (0.05 mmol each). The test tubes were then heated at 65 ºC in a dry heating block for 4 h. In all of the test tubes, precipitates formed during the reaction or on cooling to room temperature. The supernatant liquid was removed and the precipitate was washed with isopropanol (0.5 mL). Residual solvent was evaporated by heating in a dry heating block. The products exhibited essentially a single spot in TLC analysis. The compounds, thus obtained, were dissolved in dimethyl sulfoxide (DMSO) and used for enzyme assay to determine the inhibitory activity against PTP1B.

PTP1B Assay. Enzymes were diluted before the experiment to 400 nM in enzyme dilution buffer (25 mM HEPES, 5.0 mM EDTA, 1.0 mM DTT, 1.0 mg/mL bovine serum albumin, pH 7.3). Inhibitors were dissolved in DMSO. The enzyme reaction buffer was buffer A (50 mM HEPES, 5.0 mM EDTA, pH 7.0). For a typical 50 μL reaction, inhibitor (5.0 μL) was added to a reaction mixture containing enzyme (5.0 μL), 5× buffer A (10 μL) and H_2O (25 μL), and the resulting mixture was incubated at 37 °C for 10 min. The enzyme reaction was initiated by addition of *p*NPP (20 mM, 5.0 μL). After 3 min at 37 °C, the reaction was quenched by addition of 0.5 M NaOH (950 μL) and the absorbance at 405 nm was measured. IC_{50} values of the inhibitors were determined by measuring the *p*NPP hydrolase activity at a series of different inhibitor concentrations.

Enzyme Kinetics. To examine the time-dependent mode of inhibition, bulk solutions of PTP1B in buffer A were incubated in two Eppendorf tubes without and with **C8** (2 μM). Aliqouts (45 μL) of the mixture were taken at time intervals and the enzyme reaction was initiated by addition of *p*NPP (5 μL). After 3 min at 37 °C, the progress of the reaction was measured as described above. Progress curves for slow-binding inhibition analysis were obtained by performing an enzyme reaction in a cuvette and monitoring the absorbance continuously at 405 nm by a spectrophotometer. The reaction mixture contained 8 nM PTP1B, 2 μM **C8** and 0.1 mM *p*NPP in buffer A. When the reaction rate reached steady-state, additional *p*NPP was added to a final concentration of 1 mM and the absorbance was recorded for a further 30 min.

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