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Tea contains potent inhibitors of tyrosine phosphatase PTP1B

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ABSTRACT

Tea is widely consumed all over the world. Studies have demonstrated the role of tea in prevention and treatment of various chronic diseases including diabetes and obesity, but the underlying mechanism is unclear. PTP1B is a widely expressed tyrosine phosphatase which has been defined as a target for therapeutic drug development to treat diabetes and obesity. In screening for inhibitors of PTP1B, we found that aqueous extracts of teas exhibited potent PTP1B inhibitory effects with an IC50 value of 0.4–4 g dry tea leaves per liter of water. Black tea shows the strongest inhibition activities, followed by oolong and then by green tea. Biochemical fractionations demonstrated that the major effective components in tea corresponded to oxidized polyphenolic compounds. This was further verified by the fact that tea catechins became potent inhibitors of PTP1B upon oxidation catalyzed by tyrosinases. When applied to cultured cells, tea extracts induced tyrosine phosphorylation of cellular proteins. Our study suggests that some beneficial effects of tea may be attributed to the inhibition of PTP1B.

1. Introduction

Protein tyrosine phosphatases (PTPs) constitute a large, structurally diverse family of tightly regulated enzymes [1,2]. As important signal transducers, deregulation of PTPs has major pathological implications [3–5]. Many PTPs have been identified as targets for therapeutic drug development, among which PTP1B is one of the best-validated [6–8]. Since its isolation as the first member of the PTP superfamily [9], mounting evidence has linked PTP1B to insulin resistance, obesity, and type 2 diabetes mellitus. Extensive studies have demonstrated that PTP1B is a negative regulator in both insulin and leptin signaling pathways. In the insulin signaling pathway, PTP1B dephosphorylates the insulin receptor and the insulin receptor substrate IRS-1 [10,11]. In the leptin pathway, PTP1B binds and dephosphorylates JAK2, a tyrosine kinase downstream of the leptin receptor [12,13]. Overexpression of PTP1B in cell cultures decreases insulin-stimulated phosphorylation of IR and IRS-1, whereas reduction in the level of PTP1B augments insulin-initiated signaling [14,15]. Analyses of quantitative trait loci and mutations in the human PTP1B gene support the notion that aberrant expression of PTP1B can contribute to diabetes and obesity [16–18]. More convincingly, PTP1B knockout mice display enhanced sensitivity to insulin and resistance to high-fat diet-induced obesity [19,20]. Further studies with tissue-specific PTP1B knockout mice demonstrated that body weight, adiposity, and leptin action are regulated by neuronal PTP1B [21]. In all, overwhelming evidence suggests that inhibiting PTP1B represents a highly promising approach to treat diabetes and obesity.

Tea is arguably the most popular beverage in the world. It is derived from leaves of the plant *Camellia sinensis*. Depending on the level of fermentation, tea can be categorized into mainly three types: green (unfermented), oolong (partially fermented), and black (fermented) tea. Although tea has been consumed for hundreds of years, it is not until recently that tea has been studied as a health-promoting beverage that can potentially prevent and treat a number of chronic diseases and cancers [22–24]. The beneficial role of tea in preventing obesity and diabetes has received greater attention in recent years [25,26]. Tea and tea catechins, especially (-)-epigallocatechin-3-gallate (EGCG), have been shown to display anti-obesity and anti-diabetic effects in various mouse and rat models [27–32]. Epidemiological and clinical studies demonstrated the potential role of tea and tea components in preventing obesity and diabetes in humans [33–36]. The mechanism underlying the functions of tea is not known. It is thought to be related to modulations of energy balance, endocrine systems, food intake, basic metabolisms, and the redox status. Needless to say, identifying molecular targets and biomarkers for tea polyphenols and other components is essential to understand the mechanism underlying the health-promoting functions of tea and to develop better drugs and food supplements.

In light of PTP1B as a target for anti-diabetes and anti-obesity drugs, we screened various herb products for their abilities to

Abbreviations: EGCG, (-)-epigallocatechin gallate; GCG, (-)-gallocatechin gallate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; *p*-NPP, para-nitrophenylphosphate; PTP, protein tyrosine phosphatase.

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inhibit PTP1B. Our study revealed that tea, particularly, black tea, contains potent PTP1B inhibition activities.

2. Materials and methods

2.1. Materials

ΔPTP1B and ΔTC-PTP, which represent recombinant proteins containing the catalytic domains of PTP1B and TC-PTP, respectively, were purified from recombinant *Escherichia coli* cells as previously described for the catalytic domain of other PTPs [37,38]. PTP substrate para-nitrophenylphosphate (*p*-NPP), (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin gallate (GCG), mushroom tyrosinase, bovine serum albumin, buffers, and salts were obtained from Sigma–Aldrich. Reverse-phase C18 column was purchased from Waters Corporation. All the teas were purchased from local markets.

2.2. Preparation of tea extracts

Except for the use deionic water, tea extracts (infusions) were prepared the way normal drinking tea is made. In brief, 0.2 g dry tea leaves were added to 5 ml boiling water in a glass tube and kept at 95 °C on a heating block for 20 min. After cooling down to room temperature, the supernatant were collected, and any debris was removed by centrifugation. The concentration of the crude extracts was defined as 40 g/L. Note that the true concentration of solutes in the extract is substantially less because a significant portion of tea leaves is insoluble. This concentration of tea extracts is equivalent to that of regular drinking teas.

2.3. PTP activity assays

The PTP activity assay system contained 25 mM MOPS–NaOH (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, 0.1 M NaCl, 10 mM *p*-NPP, and 40 nM recombinant ΔPTP1B or ΔTC-PTP. The assays were performed in a 100 μl total volume with no more than 10 μl of tea extracts at room temperature for 10 min. The reactions were terminated by addition of 800 μl 0.1 M sodium bicarbonate, and absorbance at 410 nm was measured to determine the product, para-nitrophenol. A blank was run without enzymes. One unit of PTP activity is defined as 1 nmol of phosphate released per minute. The IC50 value refers to the concentration of inhibitors at which PTPs are inhibited by 50%. One PTP inhibition unit is defined as the amount of inhibitors required to cause 50% reduction of PTP1B activity in our assays system.

2.4. Fractionation of tea extracts

A reverse-phase C18 column was used to fractionate tea extracts to enrich PTP inhibition activity. Tea extracts were loaded onto the column equilibrated with water and then eluted with a 0–100% methanol gradient. Each fraction was monitored for absorbance at 450 nm and analyzed for PTP1B inhibitory activity as described above.

2.5. Tyrosinase-catalyzed oxidation of tea extracts and tea catechins

We employed tyrosinase to oxidize tea extracts and isolated tea catechins. This essentially mimics the process that occurs during tea fermentation. In brief, tea extracts, EGCG, and GCG were treated with mushroom tyrosinase in the aforementioned PTP assay buffer at room temperature for different periods of time. The products were directly used for PTP inhibition assays as described above.

2.6. Cell culture and stimulation with tea extracts

NIH-3T3 cells were cultured to near confluency in DMEM medium supplemented with 10% fatal bovine serum. The cells were then treated with various tea extracts. After 30 min incubation, cells were extracted in a whole cell extraction buffer containing 25 mM β-glycerophosphate (pH 7.3), 5 mM EDTA, 2 mM EGTA, 5 mM β-mercaptoethanol, 1% Triton X-100, 0.1 M NaCl, and a protease inhibitor mixture (Roche Applied Science). After centrifugation at 14,000*g* for 10 min, the supernatants were resolved on a 10% SDS polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membrane for Western blot analysis with anti-phosphotyrosine antibody. Detection was made by using the enhanced chemiluminescence (ECL) method.

3. Results

3.1. Tea extracts contain potent PTP1B inhibition activity

We purified ΔPTP1B, namely, the catalytic domain of PTP1B, from recombinant *E. coli* cells. The purified enzyme was essentially homogenous and had a specific activity of 17,000 units/mg under our assay condition at pH 7.0. Our PTP assay buffer contained 50 mM MOPS–NaOH plus 0.1 M NaCl to mimic the physiological ionic strength. With the recombinant ΔPTP1B, we screened many different teas for inhibitory effects. These teas were prepared with water similar to the way drinking teas are brewed. They were slightly acidic (pH 6–7). When added to the assay system, they did not cause changes of pH. Fig. 1A shows the results of PTP activity assays obtained with 12 teas. They all exhibited PTP1B inhibition activity, with black showing the strongest activity followed by oolong tea and then green teas. A comparison of IC50 values for different types of teas is illustrated in Fig. 1B. The average IC50 values for five black teas were around 0.4 g/L, corresponding to a dilution of the original tea extracts by 100-fold. Oolong and green teas displayed average IC50 values of 2 and 4 g/L, respectively.

To see if the inhibition activity of teas has any selectivity towards PTP1B, we analyzed the activity of TC-PTP in parallel. Among all the classic PTPs, PTP1B shares the highest homology with TC-PTP exhibiting 54% identity and 70% similarity in amino acid sequences. Following a similar strategy used for ΔPTP1B, we isolated ΔTC-PTP, the catalytic domain of TC-PTP, from recombinant *E. coli* cells. The purified ΔTC-PTP displayed a specific catalytic activity comparable to that of ΔPTP1B. Interestingly, ΔTC-PTP appeared to be resistant to the inhibition by both black and green extracts with IC50 values at least 20-fold higher than those seen with ΔPTP1B (Fig. 1C). These data demonstrated that tea extracts selectively inhibit PTP1B.

3.2. Pre-incubation of tea extracts with PTPs increases inhibitory effects

The kinetic mechanism of PTP1B inhibition by tea extracts appeared to be complex. On one hand, it exhibits certain features of competiveness since changing of substrate concentration altered the degrees of inhibition, but this does not follow the typical competitive inhibition pattern (data not shown). On the other hand, pre-incubation of the enzyme with the tea extracts significantly enhanced the inhibitory effects (Fig. 2), suggesting involvement of slow binding to the enzyme or chemical modifications. We also noticed that decreasing the concentration of reducing reagent dithiothreitol enhanced the sensitivity to tea extracts, suggesting involvement of possible oxidation reactions.

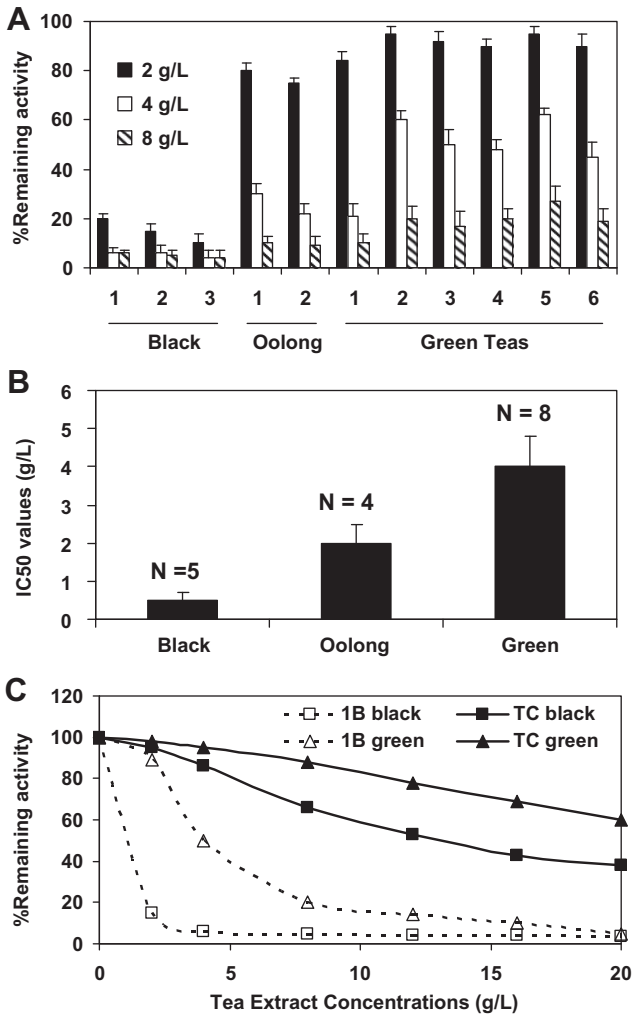

Fig. 1. Inhibitory effects of different teas on ΔPTP1B. (A) The activity of ΔPTP1B was analyzed in the presence of the indicated concentrations of 11 different teas (three red, two oolong, and six green). (B) IC50 values were determined by analyzing the activity of ΔPTP1B at various concentrations of teas. Data represent average IC50 values for each type of tea. (C) The activity of ΔPTP1B and ΔTC-PTP was analyzed in the presence of indicated concentrations of a black and a green tea. PTP activity assays were started by addition of enzymes. Error bars denote standard deviation ($n \geqslant 3$ unless indicated otherwise).

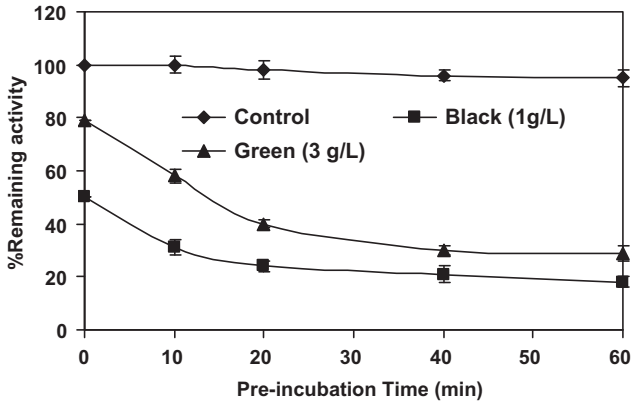

Fig. 2. Enhancement of inhibitory effects by pre-incubation of tea extracts with the enzyme. ΔPTP1B were pre-incubated with a black tea extract at 1 g/L or a green tea extract at 3 g/L for indicated periods of time. PTP activity assays were started by addition of substrate pNPP. Data represent relative remaining PTP activity, and error bars denote standard deviation ($n = 3$).

3.3. PTP1B inhibition activity of tea extracts co-migrates with intensity of tea colors on reverse-phase chromatography

To separate the effective components in tea extracts, we fractionated them on C18 reverse-phase chromatographic columns. Upon elution of the loaded columns with methanol, we were able to enrich the inhibition activity. When the most effective fractions were dried, weighted, and re-dissolved in water, we were able to see an IC50 value of 50 mg solute per liter of water in comparison with the 4 g dry tea/L in original green tea. Interestingly, the PTP inhibition activity co-migrated with yellow/red color of tea fractions as indicated by absorbance at 450 nm (Fig. 3A). For simplicity, Fig. 3A only illustrated data obtained with green tea extracts. Similar results were seen with black and oolong teas. Together, the data essentially explain why black tea extracts displayed a deeper color and thus possessed higher PTP1B inhibition activity than green tea extracts.

3.4. Tyrosinase-catalyzed oxidation of tea extracts and catechins generates PTP1B inhibition activity

The color of tea comes from theaflavins and thearubigins, oxidation products of tea catechins [22]. Black tea has a deeper color because it contains a higher content of theaflavins and thearubigins, which result from oxidation of catechins during the tea fermentation process. Together with chromatographic separation results from Fig. 3A, the data suggest that the oxidized catechins are responsible for the PTP1B inhibition activity. To verify this, we oxidized the tea extracts and isolated catechins with tyrosinase to mimic the tea fermentation process. In the presence of tyrosinase, the color of green tea became more like that of black tea, and the colorless catechins solution turned red–orange. The data shown in Fig. 3B demonstrate that tyrosinase-catalyzed oxidation of green

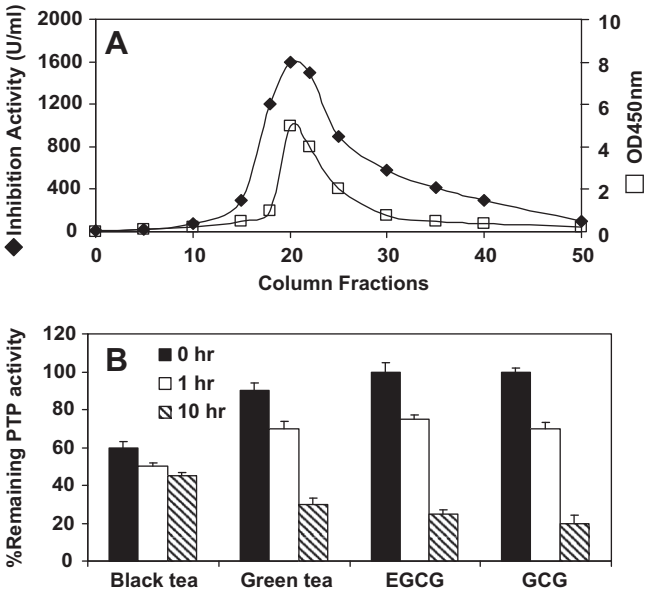

Fig. 3. Reverse-phase chromatographic separation of tea extracts and tyrosinase-catalyzed conversion of tea catechins to PTP1B inhibitors. (A) Green tea extracts were loaded onto a C18 column equilibrated with water. After washing with water, the column was eluted with a 0–100% methanol gradient. PTP1B inhibitory activity of selected fractions was measured and expressed as units/ml. Absorbance of each fraction at 450 nm which coincides with the red/yellow color was also measured. (B) Black tea extracts at 0.5 mg/ml, green tea extracts at 1 mg/ml, 50 μM EGCG, and 50 μM GCG were incubated with five unit of tyrosinase in the PTP assay buffer (25 mM MOPS–NaOH, pH 7.0, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, 0.1 M sodium chloride) for 0, 1, and 10 h. PTP assays were started by addition of pNPP and ΔPTP1B. Error bars denote standard deviation ($n = 3$).

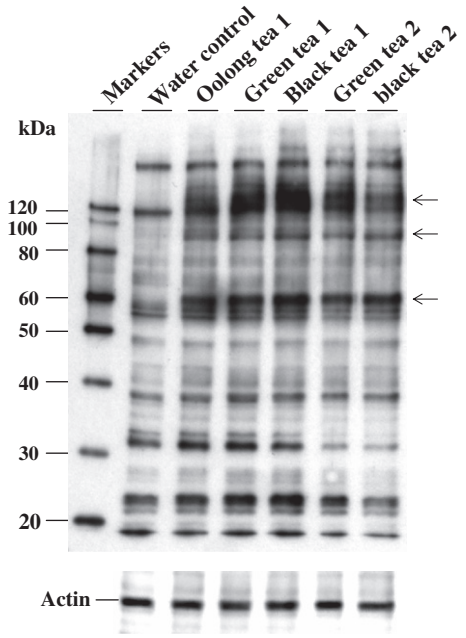

Fig. 4. Tyrosine phosphorylation of cellular proteins induced by tea extracts in cultured cells. NIH-3T3 cells were grown to near confluency in 2 ml complete culture medium, and then 0.1 ml of tea extracts (5 g/L) or water was added. Cells were further cultured for 30 min and then extracted in a whole cell extraction buffer. Equal amounts of total proteins were subjected to Western blot analysis with anti-phosphotyrosine (4G10) and anti-actin antibodies. Arrows indicate proteins whose phosphorylation is significantly induced by tea extracts.

tea extracts, EGCG, and GCG greatly increased their respective PTP1B inhibition activities. The oxidized green tea extracts were as potent as black extracts, while EGCG and GCG changed from non-effective to strongly inhibitory with IC50 values below 10 μM. These data indicate that oxidized catechins are responsible for the PTP1B inhibition activity in teas. Note that leaving the tea extracts or tea catechins for prolonged time without tyrosinase did not cause significant changes in inhibitory activity and that tyrosinase per se has no inhibitory effects on PTP1B (data not shown).

3.5. Tea extracts induce tyrosine phosphorylation of cellular proteins in cultured cells

By inhibiting PTP activities, tea extracts presumably increase protein tyrosine phosphorylation. To see if this is indeed the case, we treated NIH3T3 cells with tea extracts for 30 min. Western blotting analyses of cell extracts revealed all three types of teas can induce strong tyrosine phosphorylation of cellular proteins (Fig. 4). Identities of these proteins are to be determined. PTP1B has been known to represent a major PTP in NIH-3T3 cells [39]. It is conceivable the inhibition of PTP1B is at least partly responsible for the increased tyrosine phosphorylation. The concentrations of tea extracts used in these experiments were equivalent to 20-fold diluted drinking teas.

4. Discussion

The present study demonstrated that teas contain potent PTP1B inhibition activity. The active components correspond to oxidized catechins. This is supported by the fact that black tea displayed stronger inhibition activity than green tea, that chromatographically enriched fractionations exhibited properties of oxidized catechins, and that tyrosinase-catalyzed oxidation of tea catechins installed inhibition activity. Green, oolong, and black teas are produced by different manufacturing processes. To produce green tea, freshly harvested leaves are steamed and then dried, yielding a stable product preserving most of the catechins. In manufacture of black tea, tea leaves are crushed and then allowed to ferment, during which over 80% of catechins undergo enzymatic transformation consisting of oxidation and partial polymerization. The main products of catechins oxidation are theaflavins and thearubigins [22], which give black teas the strong red–orange color. In between green and black teas, oolong teas are made through partial fermentation and thus have a color stronger than green teas but lighter than black teas. Together, non-oxidized and oxidized catechins account for 25–30% of dry weight in teas [24]. Our biochemical assays demonstrated a good correlation between colors of the teas, representing the level of oxidized catechins, with PTP1B inhibition activity. This, however, does not necessarily indicate their abilities to inhibit PTP1B in cells. On one hand, highly oxidized and polymerized catechins found in black teas may be poorly permeable to cells. On the other hand, catechins found in green teas have a better permeability to cells and can be oxidized inside cells. Indeed, our cell-based assays demonstrated that green tea and black teas essentially work equally well to stimulate tyrosine phosphorylation of cellular proteins in NIH3T3 cells (Fig. 4).

Our study demonstrated that the tea extracts inhibit PTP1B with IC values of 0.4–4 g dry tea leaves per liter of water. These concentrations correspond to 10–100-fold dilutions of regular drinking teas which have a concentration of 4–40 g/L. Assuming all the active components can get into the circulation, the amount of teas required to reach an effective PTP1B inhibition concentration in the human body can be easily achieved. Thousands of different flavonoids have been identified in the tea [22]. Some of these are desirable and beneficial to health, and some may not be. To produce tea-based therapeutic drugs or food supplements for prevention or treatment of diabetes and obesity, some of the components such as caffeine need to be removed, while other components such as PTP1B-inhibitory catechins should be enriched, modified, and formulated to improve efficacy. With PTP1B as a target, our study provides both biochemical assays to isolate effective components for further therapeutic drug development.

By comparing the inhibition of PTP1B to that of TC-PTP, which is structurally the most similar to PTP1B in the PTP superfamily, our study demonstrated certain selectivity of the tea components as inhibitors of PTPs. Of course, there are over one hundred members in the tyrosine phosphatase family [1,2], and many of them may be effectively inhibited by tea extracts. However, by identifying the most effective species of oxidized catechins and analyzing the structures of individual PTPs we should be able to enhance such selectivity further. The fact that pre-incubation of tea extracts with PTP1B enhances inhibitory effects suggests the involvement of oxidation reactions in the inhibition process. All PTPs have a highly active cysteinyl residue in the catalytic center that is absolutely required for the de-phosphorylation reaction [1,2]. Oxidation of the SH group of this cysteinyl residue causes total inactivation of the enzymes. We believe oxidized catechins inhibit PTPs through two steps. First, as phenolic compounds, they mimic the structure of tyrosine and thus have an easy access to the catalytic center of PTPs. Second, once in the catalytic pocket, they cause oxidation of the key cysteinyl residue thereby inducing permanent inaction of the enzymes. This explains the mixed inhibition pattern observed in our enzyme kinetic analyses and also makes the inhibitors identified in our study highly unique.

Overwhelming evidence supports the beneficial role of teas in prevention and treatment of diabetes and obesity. This may be partly mediated by the inhibition of PTP1B, a well-established anti-diabetes and anti-obesity drug target. Interestingly, black teas appeared more effective than green teas according to several stud-

ies [35,36]. This agrees with our observation that black teas possess higher PTP1B inhibition activity.

The percentage of the US adult population affected by diabetes is projected to increase from 14% in 2010 to 21% by 2050 [40]. The obesity rate is expected to hit 42% by 2050 in USA [41]. Such huge public health problems must be combated from multiple fronts through managing drug intake, selecting nutritious food and beverages, and by maintaining healthy life styles. Tea, nature's gift to humanity, is represented in all these fronts. Our study of the inhibitory effects of tea on PTP1B provides a new arsenal to the battle against diabetes and obesity.

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