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Structure−Activity Relationships of Flavonoids as Potential Inhibitors of Glycogen Phosphorylase

ATSUSHI KATO,*,† NORIO NASU,† KENJI TAKEBAYASHI,† ISAO ADACHI,† YASUHIRO MINAMI,‡ FUJIKO SANAE,‡ NAOKI ASANO,‡ ALISON A. WATSON,⊥ AND ROBERT J. NASH⊥

Department of Hospital Pharmacy, University of Toyama, Toyama 930-0194, Japan, Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa 920-1181, Japan, and Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Ceredigion SY23 3EB, United Kingdom

Flavonoids are ubiquitous components in vegetables, fruits, tea, and wine. Therefore, they are often consumed in large quantities in our daily diet. Several flavonoids have been shown to have potential as antidiabetic agents. In the present study, we focused on inhibition of glycogen phosphorylase (GP) by flavonoids. 6-Hydroxyluteolin, hypolaetin, and quercetagetin were identified as good inhibitors of dephosphorylated GP (GPb), with IC_{50} values of 11.6, 15.7, and 9.7 μM, respectively. Furthermore, a structure−activity relationship study revealed that the presence of the 3′ and 4′ OH groups in the B-ring and double bonds between C2 and C3 in flavones and flavonols are important factors for enzyme recognition and binding. Quercetagetin inhibited GPb in a noncompetitive manner, with a K_i value of 3.5 μM. Multiple inhibition studies by Dixon plots suggested that quercetagetin binds to the allosteric site. In primary cultured rat hepatocytes, quercetagetin and quercetin suppressed glucagon-stimulated glycogenolysis, with IC_{50} values of 66.2 and 68.7 μM, respectively. These results suggested that as a group of novel GP inhibitors, flavonoids have potential to contribute to the protection or improvement of control of diabetes type II.

KEYWORDS: Glycogen phosphorylase; enzyme−inhibitor; flavonoids; 1,4-dideoxy-1,4-imino-D-arabinitol; multiple inhibition study

INTRODUCTION

The flavonoids are widely distributed in plants. Plants probably produce these flavonoids for the purpose of protection against pathogens and predators (*1*) and perhaps against damage by UV. They are important in food as flavors and pigments and also as components of herbal medicines around the world. In recent years, scientific studies were undertaken to test the validity of the medicinal claims made for flavonoids and gave some interesting results. The effect of flavonoids in diabetic animal models is one interesting example. The polyphenolic extract of red wine reduces glycemia and decreases food intake and body growth in diabetic and nondiabetic animals (*2*). Green and black teas also inhibit diabetic cataracts in a streptozotocin-induced rat diabetes model (*3*).

Noninsulin-dependent diabetes mellitus (type II diabetes) is a heterogeneous disease characterized by hyperglycemia, which is caused by a disorder of insulin secretion, insulin resistance in target tissues, and activation of the hepatic glucose production pathway in the liver (*4, 5*). The hepatic glucose level is affected by glycogenolysis (the breakdown of glycogen) and the gluconeogenesis pathway (the synthesis from three-carbon precursors). A number of studies have suggested that more than 70% of total hepatic glucose production is due to the breakdown of glycogen in type II diabetes patients (*6*). Furthermore, hepatic insulin resistance is also affected by a lack of suppression of glycogenolysis (*7*). Glycogen phosphorylase (GP, EC 2.4.1.1) is a key enzyme in the regulation of glycogen metabolism and catalyzes a degradative phosphorylysis of glycogen to glucose 1-phosphate (glucose-1-P). Thus, it is considered that inhibition of glycogenolysis by GP inhibitors may be beneficial in the treatment of type II diabetes.

GP exists in two interconvertible forms: a dephosphorylated form (GP b) and a Ser14-phosphorylated form (GP a). The active form of GP is a homodimer of 97 kDa subunits and has two conformational states, an inactive T state and an active R state. Allosteric effectors such as AMP are also important factors for the GP b activity. At present, the GP inhibitors are divided into five different groups. The first class of GP inhibitors described are the glucose analogues such as 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride and *N*-acetyl-β-D-glucopyranosylamine that bind to the catalytic site (*8, 9*). Spironohydantoin is one of the most potent inhibitors of this class and inhibits

* Author to whom correspondence should be addressed. Fax: +81 76 434 5155. E-mail: kato@med.u-toyama.ac.jp.
† University of Toyama.
‡ Hokuriku University.
⊥ Institute of Grassland and Environmental Research.

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GP b from rabbit muscle, with a K_i value of 3 μM (*10*). Caffeine and xanthine analogues bind to the nucleoside-inhibitor site (*11*). BAY R3401 and its active metabolite BAY W1807 bind to the allosteric site (AMP-activation site) (*12*). A new allosteric site, where inhibitors such as CP320626 and indole 2-carboxamides bind, is also reported (*13, 14*). Inhibitors binding to an allosteric site (AMP-activation site) can alter the equilibrium between the T and R state. Fosgerau et al. (*15*) have reported that 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) is a potent inhibitor of GP with antihyperglycemic effect in *ob*/*ob* mice and the mode of inhibition is uncompetitive or noncompetitive, with respect to glycogen and phosphate, respectively. However, Oikonomacos et al. (*16*) recently reported in an X-ray crystallographic investigation of a GP-D-AB1 complex that, in the presence of phosphate, D-AB1 binds at the catalytic site of GP b, with the three hydroxyl groups mimicking the hydroxymethyl and the hydroxyl groups in the C-6-, C-3-, and C-4-positions in a glucopyranose moiety.

In this paper, we investigated the structure−activity relationships of flavonoids against a glycogen phosphorylase. Although there are several reports that flavonoids and fractions containing them improve hyperglycemia (*17, 18*), the structural basis of glycogenolysis inhibition for the treatment of type II diabetes has never been reported. It was not possible to predict the structural requirements of flavonoids in these studies, because naturally occurring flavonoids usually exist as complex mixtures and consist of many different classes having the many hydroxyl and/or methoxy groups. Thus, to clarify the structure−activity relationships, we focused on five major classes of flavonoids (flavones, flavonols, flavanones, isoflavones, and catechins), which have OH groups at 5- and 7-positions on the A-ring. In addition, we investigated the effects of these compounds on the inhibition of glucagon-stimulated glucose production in rat hepatocytes.

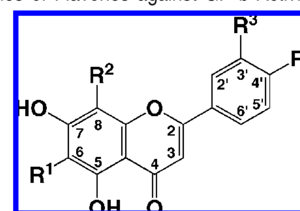
MATERIALS AND METHODS

Materials. Male Wistar rats with body weight of 130 g were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Phosphoglucomutase, glucose-6-phosphate dehydrogenase, glucose-1,6-bisphosphate, GP b, and α-D-glucose 1-phosphate dipotassium salt hydrate were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO). Glycogen and caffeine were purchased from Nacalai Tesque (Kyoto, Japan). Eagle MEM was purchased from Nissui Pharmaceutical (Tokyo, Japan). A protein assay kit was purchased from Nippon Bio-Rad Laboratories, Inc. (Tokyo, Japan). All standard flavonoids samples were purchased from Funakoshi Co. (Tokyo, Japan). Other chemicals were from Wako Pure Chemical Industries (Osaka, Japan).

Measurement of GP b Activity. GP b activity was measured in the direction of glycogenolysis using 2 mg/mL glycogen as the substrate in 45 mM phosphate buffer at pH 6.8, containing 0.1 mM EDTA, 0.34 mM $NADP^+$, 4 mM glucose-1,6-bisphosphate, 15 mM magnesium chloride, 1 mM AMP, phosphoglucomutase (0.8 units/mL), glucose 6-phosphate (glucose-6-P) dehydrogenase (3 units/mL), and GP b. The rate of enzyme-catalyzed reaction in the medium can be followed as the increase in absorbance at 340 nm and 25 °C due to the formation of NADPH. GP b activity was also measured in the direction of glycogen synthesis using 25 mM glucose-1-P as substrate in a 250 mM Tris-malate buffer (pH 6.8), containing 5 mg/mL glycogen, 1 mM AMP, 15 mM cysteine, and GP b. The assay mixture was incubated at 25 °C for 15 min and the reaction was stopped by adding 250 mM sulfuric acid. P_i was measured by the Fiske-Subbarow method (*19*). The rate of enzyme-catalyzed reaction in the medium was followed as the increase in absorbance at 660 nm. Flavonoids and caffeine had no effect on the coupling enzyme.

Incubation of Hepatocytes and in Vitro Glycogen Phosphorylase Activity. The protocol was approved by the Animal Experiments Committee of the University of Toyama. Hepatocytes were prepared from male Wistar rats fed ad libitum as described by Bissell et al. (*20*). Cell viability was assessed by trypan blue exclusion and it was consistently greater than 80%. Cells (8×10^5 cells/mL) were plated onto collagen-coated 35 mm dishes in Eagle MEM basal medium with 5% fetal calf serum. The medium was replaced 2 h after initial plating in order to remove dead cells, and cells were cultured for 24 h. Twenty-four hours later, the medium was replaced with MEM basal medium supplemented with 5.5 mM glucose and 50 nM insulin to build up glycogen reserves, and glycogenolysis experiments were performed 20 h later (*21*). After 20 h of glycogen synthesis, hepatocytes were washed twice with buffer A [117.6 mM NaCl/5.4 mM KCl/0.82 mM Mg_2SO_4/1.5 mM KH_2PO_4/20 mM Hepes/9 mM $NaHCO_3$/0.1% (w/w) human serum albumin/2.25 mM $CaCl_2$ (pH 7.4)] and incubated in buffer A containing increasing concentrations of inhibitors in the presence of 10 nM glucagons for 120 min. Glucagon-induced glycogenolysis was measured as glucose released into buffer A by using the Glucose CII-test Wako (Wako Pure Chemical Ind.). Protein content in the cells was determined after solubilization in 1 M NaOH using the Bio-Rad protein-assay kit and bovine serum albumin as standard.

Table 1. IC_{50} Values of Flavones against GP b Activity



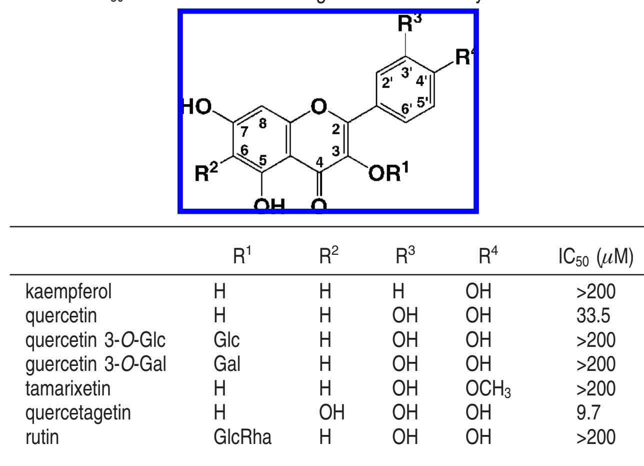
	R1	R2	R3	R4	IC_{50} (μM)
chrysin	H	H	H	H	>200
baicalein	OH	H	H	H	>200
norwogonin	H	OH	H	H	>200
apigenin	H	H	H	OH	>200
isoscullarein	H	OH	H	OH	46.1
luteolin	H	H	OH	OH	29.7
spiraeoside	H	H	OH	O-Glc	>200
6-hydroxyluteolin	OH	H	OH	OH	11.6
hypolaetin	H	OH	OH	OH	15.7
isoorientinglc	H	OH	OH	OH	>200
orientin	H	Glc	OH	OH	>200

RESULTS AND DISUSSION

Structure−Activity Relationships. Flavonoids basically consist of a fused aromatic ring (A-ring) and a heterocyclic ring (C-ring) connected through a carbon−carbon bridge to an aromatic B-ring. The two fused rings are generally planar and the B-ring can rotate (*22*). The biosynthesis of the flavonoids has been elucidated (*23*). The A-ring is mainly synthesized via the acetic acid−malonic acid pathway. Thus, the phenolic OH groups and methoxy groups conjugate similarly to phloroglucinol. On the other hand, the B-ring is synthesized via the shikimic acid pathway. Thus, the 3′-, 4′-, and 5′-positions are highly hydroxylated on the B-ring. Furthermore, flavonoids are often present in plants as glycosides. For the purpose of clarifying the structure requirements of flavonoids against GP b inhibitory activity, we selected the flavonoids that have OH groups at the 5- and 7-positions and investigated the structure−activity relationships.

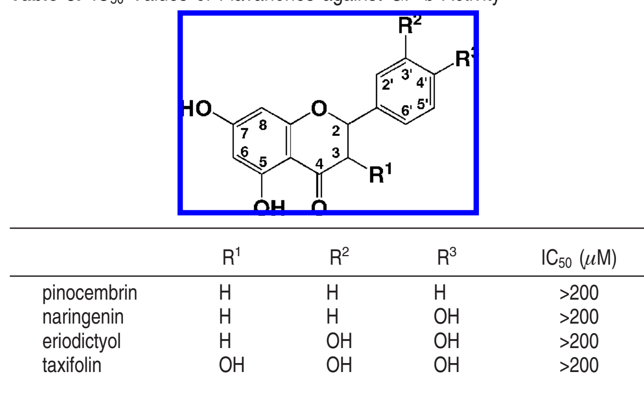
The IC_{50} values of flavones against GP b are shown in **Table 1**. Chrysin (5,7-dihydroxyflavone), which is the simplest flavone in this study, exhibited less than 50% inhibition, even at concentrations as high as 200 μM. Luteolin (3′,4′-dihydroxychrysin) is a good inhibitor of this enzyme with an IC_{50} value of 29.7 μM. An increase of hydroxylation of the A-ring results in enhanced potency of inhibition against GP b, as seen in IC_{50} values of 6-hydroxyluteolin (11.6 μM) and hypolaetin (8-

Table 2. IC_{50} Values of Flavonols against GP b Activity



	R^1	R^2	R^3	R^4	IC_{50} (μM)
kaempferol	H	H	H	OH	>200
quercetin	H	H	OH	OH	33.5
quercetin 3-*O*-Glc	Glc	H	OH	OH	>200
quercetin 3-*O*-Gal	Gal	H	OH	OH	>200
tamarixetin	H	H	OH	OCH_3	>200
quercetagetin	H	OH	OH	OH	9.7
rutin	GlcRha	H	OH	OH	>200

Table 3. IC_{50} Values of Flavanones against GP b Activity



	R^1	R^2	R^3	IC_{50} (μM)
pinocembrin	H	H	H	>200
naringenin	H	H	OH	>200
eriodictyol	H	OH	OH	>200
taxifolin	OH	OH	OH	>200

hydroxyluteolin, 15.7 μM). In contrast, the deoxygenation at C3′ of luteolin and hypolaetin to give apigenin and isoscullarein decreased their inhibition toward this enzyme, with an IC_{50} value of >200 and 46.1 μM, respectively. Furthermore, baicalein and norwogonin, which do not have the 3′- and 4′-OH groups, also did not show inhibitory activity. These results suggested that the presence of the 3′- and 4′-OH groups in the B-ring are an essential feature for recognition and strong binding.

The IC_{50} values of flavonols against GP b are shown in **Table 2**. Quercetin, which has a catechol moiety, is a moderate inhibitor of GP b (IC_{50} = 33.5 μM). The introduction of the OH group at C6 to give quercetagetin enhanced its inhibitory activity (IC_{50} = 9.7 μM), but deoxygenation at C3′ of quercetin to give kaempferol reduced its inhibitory potential. These results support that an OH at C3′ is an essential feature for the inhibitory activity of flavones. Furthermore, from a comparison of the potency of luteolin and quercetin (3-hydroxyluteolin), the presence of the C3 OH group does not appear to be essential for binding to this enzyme. Flavonoids are often present in plants as glycoside conjugates. The present work revealed that every glycoside lost inhibitory activity (**Tables 1** and **2**). It seems to be independent on position and sugar type. It may well be that on ingestion, however, sugars are removed.

The IC_{50} values of flavanones against GP b are shown in **Table 3**. Eriodictyol and taxifolin also have a catechol moiety but did not show inhibitory activity. These results suggested that the 2−3 double bond of flavones and flavonols is also an important factor for the inhibitory activity. We furthermore tested the inhibitory activities of two isoflavones (genistein and biochanin A) and three catechins [(+)-catechin, (−)-epicatechin, and (−)-epigallocatechin] against GP b. Although all these compounds have hydroxyl groups at positions 5 and 7 on the A-ring and a catechol-type moiety (3′,4′-dihydroxyl group) on the B-ring, these compounds showed no significant inhibitory activity.

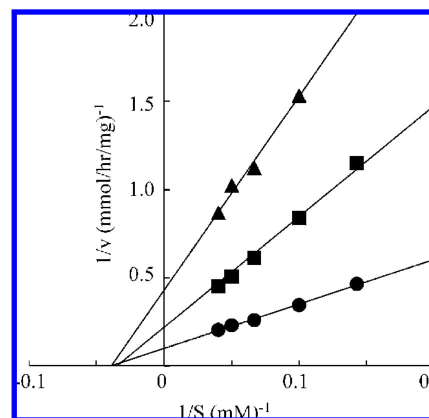


Figure 1. Lineweaver−Burk plots of quercetagetin inhibition of glycogen phosphorylase b. The increasing concentrations of glucose-1-phosphate were used to determine the K_m and K_i values. Concentrations of quercetagetin were 0 μM (●), 5 μM (■), and 10 μM (▲). Glycogen phosphorylase activities were measured in the direction of glycogen synthesis and the data were plotted as $1/v$ against $1/S$. The calculated K_m of glycogen phosphorylase was 22.8 mM.

Multiple Inhibition Study. GP is an allosteric enzyme and contains at least four potential regulatory sites: the catalytic site, the allosteric site, the nucleoside-inhibitor site, and the new allosteric inhibitor site (*24*). In order to investigate the interaction of flavonoids with the catalytic site of GP, we measured the GP b activity in the direction of glycogen synthesis using glucose-1-phosphate as the substrate, and the enzyme inhibition mode was determined from the slope of Lineweaver−Burk plots. As shown in **Figure 1**, quercetagetin inhibited GP b in a noncompetitive manner, with a K_i value of 3.5 μM. We also examined the interaction of quercetagetin with other regulatory sites. The Dixon plot is a useful method for analysis of the interaction of inhibitors and distinguishes whether the two inhibitors interact with same site or different sites of the enzyme (*25, 26*). Dixon plots run parallel to the line obtained with one inhibitor alone if the two inhibitors compete with each other at the same site, whereas they cross at the left side of the $1/v$ axis if they do not compete with each other. Caffeine and AMP are known to bind to the nucleoside-inhibitor site and the allosteric site, respectively. The effect of quercetagetin on GP activity measured by Dixon plots in the presence of caffeine (0.5 and 2 mM) or AMP (0.1, 0.2, 1.0 mM) is shown in **Figure 2**A,**B**. Quercetagetin and caffeine do not compete with each other, since the lines describing the inhibition by quercetagetin in the presence and absence of caffeine cross at the left side of the $1/v$ axis (**Figure 2**A). In contrast, quercetagetin and AMP compete with each other, since the line obtained with quercetagetin in the presence of AMP is parallel to that obtain with quercetagetin alone (**Figure 2**B). These results suggested that quercetagetin binds to the allosteric site and not to the nucleoside-inhibitor site.

Inhibition on Glycogenolysis in Primary Cultured Rat Hepatocytes. More than 70% of the total hepatic glucose production is due to the breakdown of glycogen in type II diabetes patients (*6*). Thus, we next investigated the inhibition of glucose production from glycogenolysis by quercetagetin and quercetin (**Figure 3**A,**B**). Primary cultured rat hepatocytes were preincubated in the presence of 5.5 mM glucose and 50 nM

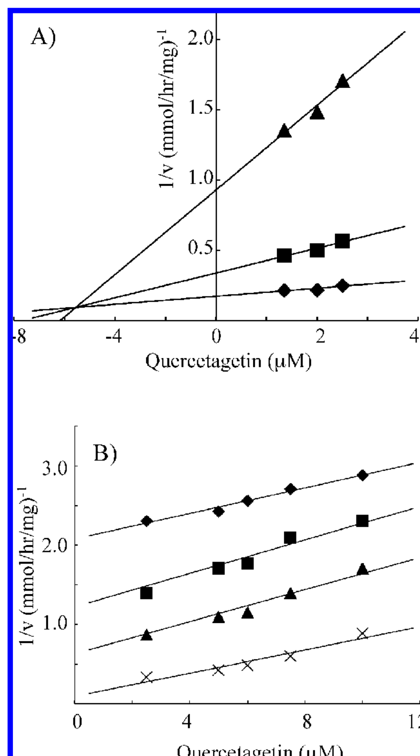


Figure 2. Multiple inhibition kinetics in the presence of two inhibitors. (**A**) The lack of competition between quercetagetin and caffeine. Inhibition of glycogen phosphorylase activity by quercetagetin was measured in the absence (◆) and the presence of 0.5 mM (■) and 2 mM (▲) caffeine. (**B**) The mutual competition between quercetagetin and AMP. Inhibition of glycogen phosphorylase activity by quercetagetin was measured in the absence (◆) and the presence of 0.1 mM (■), 0.2 mM (▲), 1 mM (×) AMP.

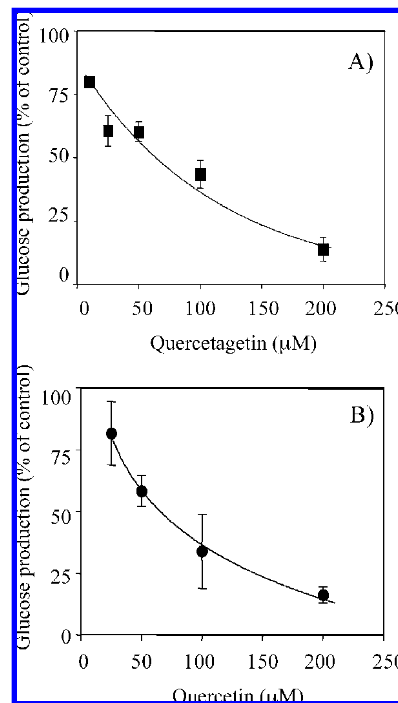


Figure 3. Concentration-dependent inhibition of glycogenolysis in rat hepatocytes by quercetagetin (**A**) and quercetin (**B**). Rat hepatocytes were incubated in the presence of 5.5 mM glucose and 50 nM insulin for 20 h. Subsequently, glucose productions from 10 nM glucagons-stimulated cells were measured over 120 min. Results are expressed as a percentage of control without inhibitors. Each value represents the mean ± SEM (n = 3).

insulin for 20 h. From a comparison of untreated and 10 nM glucagon-stimulated cells, the level of glucose released was 3 times higher than that in untreated cells (data not shown). Quercetagetin and quercetin inhibited glucose production from the 10 nM glucagon-stimulated hepatocytes in a dose-dependent manner (**Figure 3A**,**B**). The IC_{50} values of quercetagetin and quercetin were calculated as 66.2 and 68.7 μM, respectively. These results suggested that these flavonoids could inhibit the glucose production in hepatocytes.

In this study, our purpose was to reveal structural requirements of flavonoids that had GP inhibitory activity and also to investigate whether inhibition of glycogenolysis could be useful in the treatment of type II diabetes. We screened 11 flavones, 7 flavonols, 4 flavanones, 2 isoflavones, and 3 catechins against GP b, and selected quercetagetin (3,3′,4′,5,6,7-hexahydroxyflavone) as the most effective one of all the compounds tested. The structure−activity relationships revealed that the presence of the 3′- and 4′-OH groups in the B-ring and the 2−3 double bond are important factors for inhibition. Quercetagetin did not compete with caffeine and the nucleoside-inhibitor site but did bind to the allosteric site. However, more crystal structural analysis of enzyme-flavonoid complexes may indicate that quercetagetin can alter the equilibrium between the T state and R state because the known allosteric site inhibitor BAY R3401 and its active metabolite Bay U6751 can alter this equilibrium (*12*). Furthermore, we demonstrated that quercetagetin and quercetin inhibited hepatic glucose production from glycogenolysis in rat hepatocytes. Quercetin is one of the major components of apple, onion, and red wine, and quercetagetin is also distributed widely in fruits and herbs. Thus, as dietary supplements, the flavonoids would be potentially useful in the prevention and self-medication of diabetes.

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