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Study of the inhibition of two human maltase-glucoamylases catalytic domains by different α-glucosidase inhibitors

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ABSTRACT

In humans, both the N-terminal catalytic domain (NtMGAM) and the C-terminal catalytic domain (CtMGAM) of small intestinal maltase glucoamylase (MGAM) are α-glycosidases that catalyze the hydrolysis of α-(1→4) glycosidic linkages in the process of starch digestion, and are considered to be the main therapeutic targets for type 2 diabetes. In this work, recombinant human CtMGAM has been cloned for the first time, and this, combined with the expression of NtMGAM in *Pichia pastoris*, made it possible for us to study the catalytic mechanism of MGAM in a well-defined system. The enzymatic kinetic assays of the two catalytic domains suggest that CtMGAM has the higher affinity for longer maltose oligosaccharides. Kinetic studies of commercially-available drugs such as 1-deoxynojirimycin (DNJ), miglitol, voglibose, and acarbose along with a series of acarviosine-containing oligosaccharides we isolated from *Streptomyces coelicoflavus* against NtMGAM, CtMGAM, and human pancreatic α-amylase (HPA) provide us an overall profile of the inhibitory ability of these inhibitors. Of all the inhibitors used in this paper, DNJ was the most effective inhibitor against MGAM; the K_i values for the two catalytic domains were 1.41 and 2.04 μM for NtMGAM and CtMGAM, respectively. Acarviostatins 2-03 and 3-03 were the best inhibitors against HPA with relatively high inhibitory activity against CtMGAM. The acarviostatins 2-03 and 3-03 inhibition constants, K_i, for HPA were 15 and 14.3 nM, and those for CtMGAM were 6.02 and 6.08 μM, respectively. These results suggest that NtMGAM and CtMGAM differ in their substrate specificities and inhibitor tolerance despite their structural relationship.

1. Introduction

As the main dietary carbohydrate source for human consumption, starch plays a very important role in human life. However, excessive starch consumption or other carbohydrate-rich diets can cause high glucose levels in blood and result in chronic diseases like type 2 diabetes and obesity.[1,2] In humans, four enzymes are involved in the complete digestion of starch into glucose. Salivary and pancreatic α-amylases (EC 3.2.1.1) are endohydrolases that cleave the internal α-(1→4) bonds of starch into shorter linear and branched dextrin chains. The resultant mixture of dextrin is then further hydrolyzed at the nonreducing ends into glucose by two small-intestinal brush-border exohydrolases: maltase glucoamylase (MGAM; EC 3.2.1.20 and 3.2.1.3) and sucrose isomaltase (SI; EC 3.2.148 and 3.2.10).[3–6] MGAM is membrane-bound enzyme, each containing two independent catalytic domains: an N-terminal domain (NtMGAM) that is proximal to the membrane-bound end and a C-terminal luminal domain (CtMGAM) (Fig. 1).[7,8] These two catalytic domains are 40% identical in amino acid sequence, and all classified under glycosyl hydrolase family 31 (GH31).

In previous studies, the inhibition of MGAM and human pancreatic α-amylase (HPA) activity showed a significant effect in controlling blood glucose levels making them prefect targets for the treatment of type 2 diabetes and obesity.[1,4,9] Acarbose, miglitol, voglibose and 1-deoxynojirimycin (DNJ) are commercially-available glucosidase inhibitors with carbohydrate structures widely used in the treatment of type 2 diabetes (Fig. 2). There are many acarviosine-containing α-amylase inhibitors that have been studied, including acarbose, which display strong inhibitory potencies.[10] Acarbose is a pseudo-tetrasaccharide composed of an acarviosine group α-(1→4)-linked to a maltose (Fig. 2). Our group has isolated several aminooligosaccharide natural metabolic products of *Streptomyces coelicoflavus* ZG0656[11] named as acarviostatins 1-03, 2-03, 3-03, and 4-03 (referred to as A1-03, A2-03, A3-03, A4-03), which displayed much higher inhibitory activity against porcine α-amylase.[12] They have a repeating pseudotrisaccharide unit that consists of an acarviosine unit α-(1→4) linked to a glucose (Fig. 2). Especially, A3-03 is the most potent inhibitor against PPA known to date.[12] Moreover, they showed a significant effect in depressing blood glucose levels in mammalian systems and could be developed to possible therapeutic agents in the future..[13]

In this work, we first report the high quality and quantity cloning, expression and purification of human CtMGAM as well as

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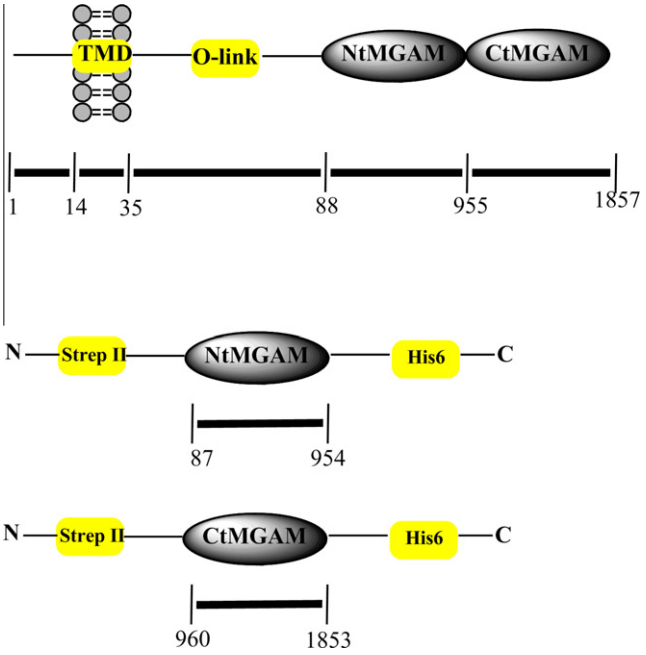

Figure 1. The schematic structure of MGAM and the construction of recombinant NtMGAM and CtMGAM protein inserted into pPIC9k expression vector. Features of MGAM include the following: a transmembrane domain (TMD; ~20 amino acids), an O-glycosylated linker (O-link; ~55 amino acids), and two homologous catalytic domains (NtMGAM and CtMGAM; each ~900 amino acids). Strep II and His6 indicate strep II tag and hexahistidine tag, respectively.

NtMGAM in the *Pichia pastoris* expression system for kinetic and inhibition studies. Kinetic studies of DNJ, miglitol, voglibose, and acarbose along with a series of acarviostatins against NtMGAM, CtMGAM, and HPA were developed. The results provide us valuable insight into the inhibitory mechanism of MGAM and HPA and will benefit future anti-diabetic drug design.

2. Results and discussion

MGAM is an important inhibition target in the treatment of type 2 diabetes and accounts for both glucoamylase activity and maltase activity.[2,7] Previous research has focused mainly on the N-terminal domain of MGAM after the successful expression of human NtMGAM in *Drosophila* cells.[7] The mouse CtMGAM has recently been cloned and expressed in Sf9 insect cells,[1] CtMGAM was considered to have greater catalytic efficiency than NtMGAM and to be responsible for the 'glucoamylse' activity of MGAM due to its high affinity for larger oligoglucans.[2] However, studies of CtMGAM, the most important catalytic domain of MGAM are far behind NtMGAM and HPA. In this paper, we report the first expression of human CtMGAM in the *Pichia pastoris* expression system. This allowed us to be the first to study human NtMGAM and CtMGAM independently at the same time.

2.1. Expression and purification of NtMGAM and CtMGAM in *Pichia pastoris*

The recombinant NtMGAM and CtMGAM proteins we constructed cover amino acids from 87 to 954 and from 960 to 1853 of full length human MGAM. They were fused to an N-terminal strep II tag and a C-terminal hexahistidine tag (Fig. 1). The expected size of both proteins was around 110 KD. The recombinant proteins were secreted to BMMY medium while being induced by methanol. The concentration of methanol was up to 1%. The proteins were harvested after three days and the secreted proteins were purified using Ni Sepharose resin from the BMMY media followed by anion exchange chromatography and size-exclusion column. Notable here was the high glycosylation degree of recombinant proteins due to the expression system. Deglycosylation was carried out by PNGase F using a size-exclusion column. The purity of both CtMGAM and NtMGAM proteins was up to 99% as analyzed by SDS–PAGE (Fig. 3). The total yield of pure recombinant NtMGAM and CtAMGM proteins were about 2–3 mg/L.

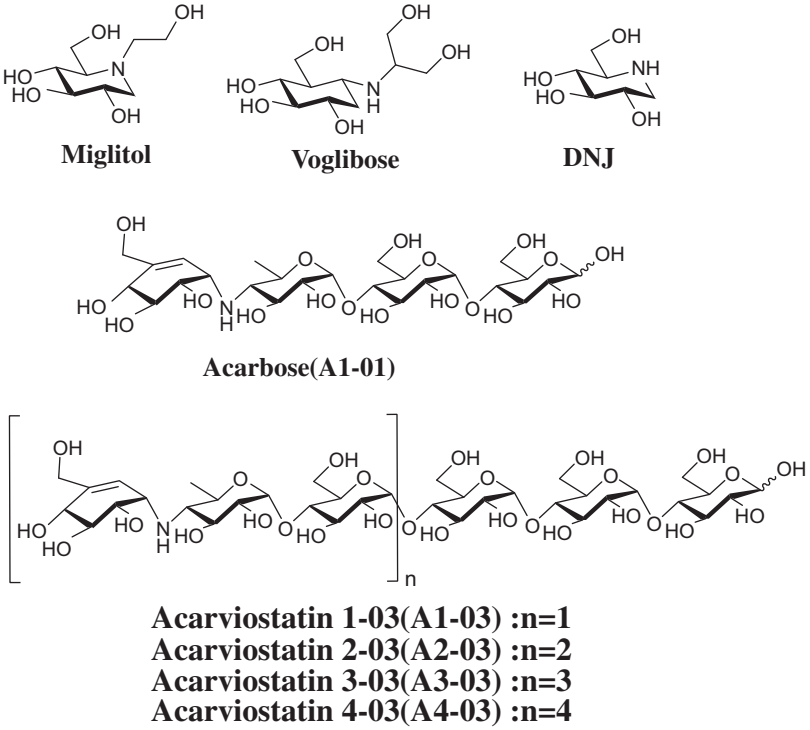

Figure 2. The structures of inhibitors mentioned in this paper: miglitol, voglibose, DNJ, acarbose, A1-03, A2-03, A3-03 and A4-03.

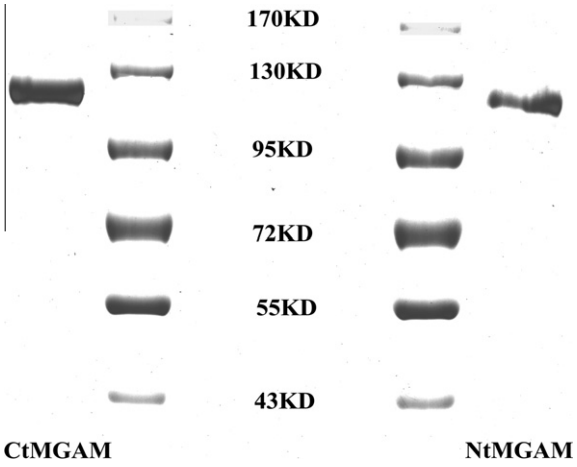

Figure 3. The image of SDS–PAGE showed the molecule weight and the purity of NtMGAN and CtMGAM proteins after S 200 column.

2.2. Catalytic studies of CtMGAM and NtMGAM

To compare the substrate specificities of NtMGAM and CtMGAM, we analyzed K_m and K_{cat} values with maltose and soluble starch. When maltose was used as the substrate, the K_m values of NtMGAM and CtMGAM were about 6.17 and 5.53 mM, respectively (Table 1, and no obvious affinity difference was observed between NtMGAM and CtMGAM. The K_{cat} of NtMGAM was 47.76 s^{-1}, which is about two times that of CtMGAM (21.99 s^{-1}). These results indicated that both catalytic domains of MGAM have maltose digestion activities; however, NtMGAM was more potent in degrading the shorter maltose substrate than CtMGAM.

Because of the uncertainty of the soluble starch molecular weight, we used the relation of its mass to its volume (percentage) to represent the concentration of the substrate. The K_m value of CtMGAM was about twice that of NtMGAM when soluble starch was used as substrate, and the K_{cat} was determined to be 213.84 s^{-1}, thirty times higher than that of NtMGAM (6.82 s^{-1}) (Table 1). Evidently CtMGAM has starch digestion activity and NtMGAM has little α-glucosidase activity against complex starch molecules. These results suggest that NtMGAM and CtMGAM differ in their substrate specificities and confirm the previous assumption that CtMGAM might have glucoamylase activities.[1,2]

2.3. Inhibition analysis

2.3.1. Inhibition against NtMGAM and CtMGAM

From the inhibition studies of NtMGAN, acarbose displayed poor inhibitory ability against NtMGAM with a K_i value of 51.88 μM (Table 2). This result was consistent with a previous result, which showed that acarbose inhibited NtMGAM with a K_i of 62 μM.[7] Furthermore, A1-03, A2-03, A3-03, A4-03 failed to show inhibitory activity against NtMGAM. However, we found DNJ, miglitol and voglibose had significant inhibitory activities against NtMGAM with K_i values of 1.14, 3.98, and 2.22 μM, respectively (Table 2).

In contrast to NtMGAM, all these α-glucosidase inhibitors used in this paper showed inhibitory activities against CtMGAM with K_i values in range from 1.72 to 171.57 μM (Table 2). The inhibitory ability of acarbose, DNJ, and voglibose were at almost the same level, with K_i values of 1.72, 2.04, and 3.31 μM, respectively. In addition, A1-03, A2-03, A3-03, A4-03 have shown high inhibitory ability against CtMGAM with K_i values in the range of 6.02–19.18 μM. However, miglitol was a very poor inhibitor against CtMGAM. These inhibitors were all competitive inhibitors against NtMGAM or CtMGAM.

The current antidiabetic compound, acarbose, was found to be a micromolar inhibitor of the human NtMGAM (K_i = 51.88 μM). In contrast, acarbose is a 100-fold better inhibitor of human CtMGAM (K_i = 1.72 μM) and 1000-fold better for mouse CtMGAM-N2 (K_i = 0.009 μM), CtMGAM-N20 (K_i = 0.028 μM).[1] This supports the notion that the tetrasaccharide analog occupies extended binding subsites in the C-terminal catalytic subunits as compared to the N-terminal domains.

2.3.2. Inhibition against HPA

The catalytic and inhibitory mechanism of HPA, an endoglycosidase, has been studied in a multifaceted way.[14–19] In our previous work, acarviosine-containing α-amylase inhibitors have shown tremendous inhibitory effect on HPA. Compounds A2-03 and A3-03 have displayed 170–180 times higher inhibitory activity than acarbose.[2] Their inhibition types were mixed. These results show HPA has a very clear preference for inhibitors with longer sugar chains than acarbose (AI-01).

In this research, DNJ and miglitol were found to be non-competitive inhibitors against HPA, indicating the molecules might be too small to have enough affinity to bind in the HPA active site alone. As expected, their inhibitory ability against HPA was very low, which was only 2% and 8% compared to acarbose. Additionally, as we observed, voglibose was unable to inhibit HPA.

We were interested in finding the best inhibitors against MGAM and HPA in this research. In the inhibition analysis, aside from acarbose, DNJ and miglitol were proven to have a broad spectrum of inhibition effect on these glycosidases (NtMGAM, CtMGAM and HPA), which catalyze the hydrolysis of α-(1→4)-glycosidic linkages in starch digestion. No particular inhibitor was found to be a very good inhibitor of all three targets. Among all inhibitors tested in this study, the K_i of DNJ for NtMGAM and CtMGAM were small, suggesting that DNJ had the strong ability to 'brake' the entire catalytic ability of MGAM. In addition, A2-03 and A3-03 were not only two of the strongest inhibitors against HPA, but also effective inhibitors against CtMGAM, which will make them very valuable in future drug design. In summary, to effectively block the pathway of starch digestion, it is important to restrain both the beginning and the end process of starch digestion to which the main targets are referred to HPA and MGAM. According to our results, at least two α-glucosidase inhibitors were required to efficiently inhibit the activities of MGAM and HPA. The combination of A2-03 (or A3-03) and DNJ may potentially have stronger inhibitory effects than a single inhibitor.

Interestingly, out results reveal both NtMGAM and CtMGAM have the ability of digesting soluble starch to produce glucose and that CtMGAM has more significant efficiency than NtMGAM. In comparison to acarbose, A2-03 and A3-03 display much higher inhibitory activities against HPA, lower inhibitory activity against CtMGAM, and failed to inhibit NtMGAM. The selectivity of the two human MGAM catalytic domains toward different inhibitors was also reflected in the report as mouse CtMGAM-N2 and N20 were more sensitive to the longer inhibitors than human ntMGAM.[1] We propose that the explanation of these phenomena

Table 1
Substrate kinetics of NtMGAM and CtMGAM

Enzyme	Maltose		Soluble starch	
	K_m (mM)	K_{cat} (S^{-1})	K_m (mass/volume)	K_{cat} (S^{-1})
NtMGAM	6.17 ± 0.66	47.76 ± 10.34	1.18% ± 0.42%	6.82 ± 1.35
CtMGAM	5.53 ± 0.32	21.99 ± 1.25	2.72% ± 1.45%	213.84 ± 43.42

Table 2
Inhibition of acarbose, A1-03, A2-03, A3-03, A4-03, DNJ, miglitol, voglibose against HPA, NtMGAM, and CtMGAM

Inhibitor	HPA		NtMGAM		CtMGAM	
	Inhibition type	K_i (μM)	Inhibition type	K_i (μM)	Inhibition type	K_i (μM)
Acarbose	Mixed[a]	2.59 ± 0.010[a]	Competitive	51.88 ± 3.06	Competitive	1.72 ± 0.05
A1-03	Mixed[a]	1.25 ± 0.030[a]	—	—	Competitive	12.20 ± 2.05
A2-03	Mixed[a]	0.015 ± 0.0012[a]	—	—	Competitive	6.02 ± 0.68
A3-03	Mixed[a]	0.014 ± 0.00020[a]	—	—	Competitive	6.08 ± 0.18
A4-03	Mixed[a]	0.47 ± 0.0096[a]	—	—	Competitive	19.18 ± 2.43
DNJ	Non-competitive	122.36 ± 10.18	Competitive	1.41 ± 0.16	Competitive	2.04 ± 0.19
Miglitol	Non-competitive	31.50 ± 6.50	Competitive	3.98 ± 0.04	Competitive	171.57 ± 13.39
Voglibose	—	—	Competitive	2.22 ± 0.26	Competitive	3.31 ± 0.21

K_i is the inhibition constant, defined as [E][I]/[EI].
—: no significant inhibition was observed.
[a] Data from Qin and Ren.[3]

may lie in the structure of the active cleft of these glucosidases. Relatively small inhibitors such as DNJ, miglitol, voglibose displayed tremendous inhibitory effect on NtMGAM suggesting a relatively small catalytic cleft, which is consistent with the observation of the active cleft in the crystal structure of NtMGAM.[4] On the other hand, acarviosine-containing α-amylase inhibitors including acarbose have shown strong inhibitory ability against CtMGAM and HPA indicating that they might have a larger catalytic cleft. This was confirmed by our previous studies on the crystal structure of HPA in complex with acarviostatins.[3] Additionally, all the inhibitors used in this paper had the inhibitory ability against CtMGAM, further indicating that it may have a flexible and adaptable active cleft.

3. Experimental

3.1. General methods

All chemicals and buffers salts used were from Sigma unless otherwise indicated. The expression and purification of recombinant HPA was performed as described previously.[2] HPA was dialyzed against 20 mM potassium phosphate buffer, with pH 6.9 and the protein concentration was 2 mg/mL. In the enzyme kinetic studies, reactions were performed in 96-well plates in triplicate. Due to the different optimal buffers for each enzyme, substrates and inhibitor were diluted in the buffer 100 mM sodium acetate trihydrate, pH 4.8 for NtMGAM or 20 mM Tris–HCl, pH 7.0 for CtMGAM.

3.2. Cloning, expression and purification of NtMGAM and CtMGAM

Recombinant NtMGAM and CtMGAM proteins were expressed in the *Pichia pastoris* protein expression system. The NtMGAM and CtMGAM cDNA were cloned from pReceiver-Y01 vector contain the cDNA of full length human MGAM (Genbank Accession NM_004668.1 from GeneCopoeia). NtMGAM and CtMGAM contain residues from 87 to 954 and from 960 to 1853 of full length human MGAM. NtMGAM was amplified by the upstream primer 5′-AAGGAAAAAAcctaggTGGAGCCACCCGCAGTTCGAAAAG tctgctgaatgtccag tgg-3′ and downstream primer 5′-AAGGAAAAAA cctaggATGATGG TGATGGTGATGccttatctttatgctccattcc-3′. CtMGAM were amplified by the upstream primer 5′-AAGGAAAAAAcctaggTGGAGCCACC CGCAGTTCGAAAAG gatgaagaaaaaatagactgttac-3′ and downstream primer 5′-AAGGAAAAAAcctaggATG ATGGTGATGGTGATGccac gtcaatgaagtaaaattatg-3′. The PCR products were cloned into pPIC9k expression vector by Avr2 site. After being linearized by *Sal*I and *Sac*I, respectively, recombinant pPic9k-NtMGAM and pPic9k-CtMGAM vectors were electroporated into *Pichia pastoris* (GS115). The positive strains were obtained though G418 screening in 96-well plates and confirmed by dot-blotting using the antibody of his6. Recombinant NtMGAM and CtMGAM proteins were secreted into BMMY medium while being induced with 1% methanol. The proteins were harvested between 72 and 96 h.

Ni Sepharose™ 6 Fast Flow resin (GE healthcare) was used to purify the secreted NtMGAM and CtMGAM proteins from BMMY medium (10–15 ml resin/L media). Resin was pre-equilibrated by 20 mM Tris–HCl, pH 7.0 containing 200 mM NaCl. The recombinant proteins bound to the resin overnight at 4 °C. After being washed by pre-equilibration buffer, the proteins were eluted by 20 mM Tris–HCl pH 7.0, contains 200 mM NaCl and 300 mM imidazole. The eluted fractions were concentrated and dialyzed against 20 mM Tris–HCl, pH 7.0, which is also the start and equilibration buffer of HiTrap Q HP anion exchange column (GE Healthcare). Then, the recombinant proteins were eluted with a linear gradient of 0–500 mM NaCl. The fractions were concentrated to 2 mL and deglycosylated by PNGase F overnight at room temperature. Finally, a Superdex S 200 size-exclusion column (GE Healthcare) was used to further purify NtMGAM and CtMGAM. The S 200 column was pre-equilibrated with the running buffer 20 mM Tris–HCl, pH 7.0, containing 200 mM NaCl. The proteins obtained were up to 99% pure as analyzed by SDS–PAGE.

3.3. Kinetic assays of CtMGAM, NtMGAM and HPA

K_m values of NtMGAM and CtMGAM were determined by measuring the production of glucose (Glu Kit, Biosino Bio-technology and Science Inc.) using maltose and soluble starch as substrates. The catalytic rate constant (K_{cat}) values of NtMGAM and CtMGAM were determined by the number of glucose that was converted from each NtMGAM or CtMGAM substrate per unit time, and in which the enzyme was at its maximum efficiency. The reaction mixture contained 10 μL NtMGAM (0.2 μM) or CtMGAM (0.2 μM) and 10 μL maltose (1–10 mM). The reactions were carried out at 37 °C for 30 min. 20 μL 2 M Tris–HCl, pH 7.0 was used to stop the reaction, then the reactions were developed with 150 μL Glu kit reagent at 37 °C for 15 min to determinate the amount of glucose produced. Absorbance was measured at 490 nm in a bio-rad microplate spectrophotometer. The volume of reaction mixture was 200 μL when the substrate was soluble starch. The reaction mixture contains 150 μL soluble starch (0.1–1%) and 50 μL NtMGAM (1 μM) or 50 μL CtMGAM (0.1 μM). After incubation at 37 °C for 30 min, the reactions were stopped by 200 μL 2 M Tris–HCl pH, 7.0. 40 μL samples were taken to determine the glucose level. The following procedures were the same as we mentioned above.

The inhibitory constants (K_i) values of miglitol, voglibose, DNJ, acarbose, A1-03, A2-03, A3-03, and A4-03 against NtMGAM and CtMGAM were determined by glucose kit using maltose as substrate. The reactions began when 10 μL CtMGAM (0.2 μM) or NtMGAM (0.2 μM) were added to 10 μL mixture of maltose (1–10 mM) with inhibitors of different concentrations (0.5–40 μM). The reactions were carried out at 37 °C for 30 min and 20 μL 2 M Tris–HCl, pH 7.0 was used to stop the reaction. The glucose detection method was the same as mentioned above. The change of the concentration of glucose in 30 min was a function of the reaction speed (rate). The inhibition mode, K_m and V_{max} were determined by generating a Lineweaver–Burk plot (1/rate vs 1/concentration of substrates), see Supplementary data. K_i values were determined from a Dixon plot (1/rate vs concentration of inhibitors), see Supplementary data. Inhibition of HPA by different inhibitors was carried using the DNS reagent, as reported previously.[3]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2011.09.012.

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