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Green tea flavonols inhibit glucosidase II

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Abbreviations:
EGC, epigallocatechin
EGCG, epigallocatechin gallate
NBDJ, *N*-butyldeoxynojirimycin
MUG, 4-methylumbelliferyl α-D-glucopyranoside
NPG, 4-nitrophenyl α-D-glucopyranoside
GC, gallocatechin
GCG, gallocatechin gallate
ECG, epicatechin gallate
PG, propyl gallate
MOPS, 4-morpholinepropanesulfonic acid
TFA, trifluoroacetic acid
MUGase, methylumbelliferyl glucosidase
NPGase, 4-nitrophenyl glucosidase

ABSTRACT

Green tea is getting into the focus of scientific interest due to its beneficial health effects, most of which are attributed to its catechin content. Polyphenolic tea catechins have antioxidant, antiproliferative, antiangiogenic and proapoptotic effects, which makes them promising anticancer compounds. Other poly-hydroxy molecules have similar antitumor potentials through the inhibition of glucosidase II, which affects the glycoprotein maturation and quality control in the endoplasmic reticulum. We investigated the effect of tea catechins on glucosidase II activity in rat liver microsomes using 4-methylumbelliferyl glucoside and 4-nitrophenyl glucoside as substrates. A concentration-dependent inhibition with non-competitive kinetics was found. The IC_{50} and K_i values for certain tea catechins were comparable with those of *N*-butyldeoxynojirimycin, the widely used glucosidase inhibitor. The possible interference of tea catechins with the glycoprotein processing in the endoplasmic reticulum should be considered as a potential mechanism of their dietary or pharmacological effects.

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1. Introduction

Tea is one of the most consumed beverages in the world. It is getting into the focus of scientific interest due to the wide range of its beneficial health effects and the apparent lack of toxicity. When it is prepared by steeping the green (dried and unfermented) leaves of the tea-plant (*Camellia sinensis*) it is especially rich in tea flavonols, which are polyphenolic catechins regarded as the biologically active constituents of tea. Epigallocatechin (EGC) and epigallocatechin gallate (EGCG) are the most abundant tea polyphenols. The beneficial effects of green tea are largely attributed to EGCG, and it is the most frequently studied flavonol. Tea catechins are very efficient antioxidants, their radical scavenging activity compares with that of ascorbate. This feature alone would make them good candidates for dietary cancer prevention. In addition, it has been proven in cell cultures and animal models that tea catechins have remarkable antiproliferative, proapoptotic and antiangiogenic effects. Despite the large number of elucidated mechanisms, the anti-carcinogenic effect of EGCG and related polyphenols is still the subject of extensive research (for review, see Refs. [1–3]).

Some cyclic poly-hydroxy compounds, such as castanospermine or *N*-butyldeoxynojirimycin (NBDJ) are potent inhibitors of microsomal glucosidase II [4]. This enzyme participates in the trimming of the N-linked oligosaccharide moiety of certain glycoproteins in the endoplasmic reticulum. After the transfer of an oligosaccharide precursor to the asparagine side chain of the nascent protein, glucosidase I removes the peripheral α 1,2-linked glucose residue. Subsequently, the removal of the α 1,3-linked glucose residues is catalyzed by glucosidase II. Therefore, this hydrolytic activity is necessary for the maturation of glycoproteins synthesized in the endoplasmic reticulum [5]. In addition, it has been found to play an important role in the quality control mechanism of glycoprotein folding. Immature or misfolded glycoproteins are labeled by monoglucosylated core glycan and sequestered by chaperones (calnexin or calreticulin) in the endoplasmic reticulum. The glycoprotein dissociates from the chaperones once the glucose residue has been removed by glucosidase II [6]. The inhibitors of certain glycosidase enzymes, such as glucosidase I and II, are regarded as promising antiviral and anticancer compounds [4,7].

We investigated the effect of EGCG, other green tea flavonols and a non-catechin gallate (Fig. 1) on glucosidase II in rat liver microsomal vesicles, using two specific artificial substrates, 4-methylumbelliferyl α-D-glucopyranoside (MUG) and 4-nitrophenyl α-D-glucopyranoside (NPG) [8].

2. Materials and methods

2.1. Materials

4-Methylumbelliferyl α-D-glucopyranoside (MUG), 4-nitrophenyl α-D-glucopyranoside (NPG), 4-methylumbelliferone, 4-nitrophenol, gallocatechin (GC), gallocatechin gallate (GCG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), propyl gallate (PG), *N*-butyldeoxynojirimycin (NBDJ), alamethicin, 4-morpholinepropanesulfonic acid (MOPS), methanol and trifluoroacetic acid (TFA), were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

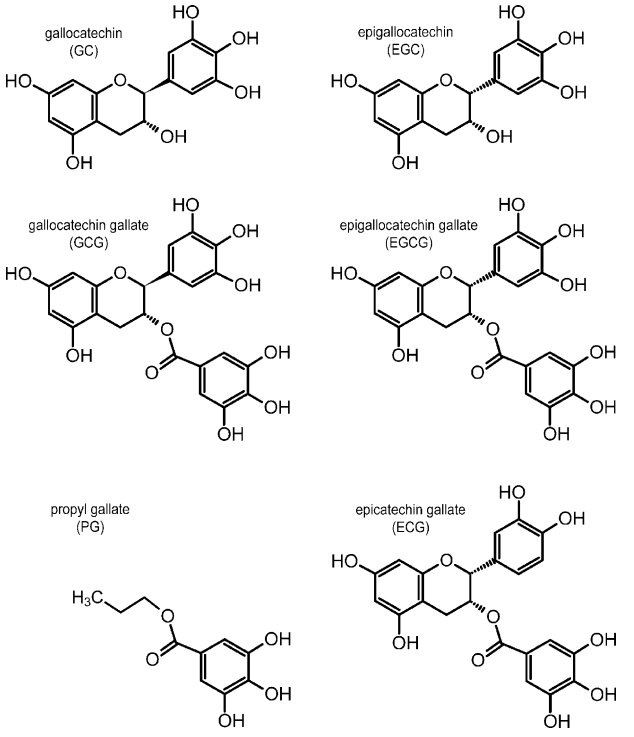

Fig. 1 – Structure of the investigated catechins and propyl gallate.

2.2. Preparation of rat liver microsomal vesicles

Microsomes were prepared from livers of overnight fasted male Wistar rats (180–230 g), using fractional centrifugation [9]. The vesicles were washed and re-suspended in MOPS–KCl buffer (100 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$, 20 mM MOPS; pH 7.2) then immediately frozen in liquid nitrogen and kept in liquid nitrogen until use (within 6 months). The protein concentration in microsomal samples was determined using the method of Lowry et al. [10] with bovine serum albumin as a standard.

Purity of the microsomes was assessed by a marker-enzyme analysis as described earlier [11]. The integrity of the microsomal membranes was assessed using the mannose-6-phosphatase assay [12], which showed a latency greater than 95%.

2.3. Glucosidase II activity measurements

Two specific artificial substrates of glucosidase II were used in the enzyme activity assays. Methylumbelliferyl glucosidase (MUGase) activity was measured by the fluorescent detection of methylumbelliferone, while 4-nitrophenyl glucosidase (NPGase) activity was quantified by the determination of 4-nitrophenol using HPLC.

The MUGase activity assay was carried out in a Cary-Varian Spectrofluorimeter equipped with temperature controller.

Microsomes (0.1 mg protein/ml in MOPS–KCl buffer) were incubated at 37 °C in the fluorimeter cuvette under continuous stirring. MUG was added to the sample and the linear fluorescence increase due to the formation of methylumbelliferone was recorded for 1 min (at 360 nm excitation and 440 nm emission wavelength). Calibration was performed by adding methylumbelliferone (0.5 or 1 nmol/ml) to each sample, at the end of the reaction. When required, microsomal membranes were permeabilized with the pore-forming antibiotic, alamethicin (0.1 mg/mg microsomal protein) [13].

For NPGase assay, the microsomes (0.5 mg protein/ml) were incubated in MOPS–KCl buffer at 37 °C for 10 min in the presence of NPG. Alamethicin (0.1 mg/mg microsomal protein) was used for permeabilizing the microsomal membranes. The reaction was terminated by adding three volumes of ice-cold methanol, and the samples were immediately frozen until further analysis. Samples were centrifuged (10 min, 4 °C, at 20,000 × *g*) and the protein-free supernatants were analyzed by HPLC.

All the studied inhibitors were added to the microsomal suspension 1–2 min prior to the substrate (MUG or NPG).

2.4. *High performance liquid chromatography (HPLC)*

Protein-free samples were analyzed by HPLC (Waters Alliance 2690) using a Nucleosil 100 C18 column (5 μm 25 × 0.46) (Teknokroma). 4-Nitrophenol was detected at 316 nm wavelength (Waters Dual λ Absorbance Detector 2487). The flow rate was 0.9 ml/min, the eluent consisted of Solvent A: 0.1% TFA and Solvent B: 0.1% TFA in methanol. Half a minute equilibration at 100% Solvent A was followed by an 8 min gradient from 0 to 60% Solvent B, 2 min at 40% Solvent A–60% Solvent B, then re-equilibration into 100% Solvent A. The elution time (14 min) and response factor (1000 area/pmol) of 4-nitrophenol were determined by injecting standards.

2.5. *Calculations*

MUGase and NPGase activities were expressed as nmol product (methylumbelliferone or 4-nitrophenol, respectively) produced in 1 min by 1 mg microsomal protein in intact or permeabilized microsomes. The rate of methylumbelliferone production was calculated from the slope of the fluorescence trace, i.e. the change in the intensity of fluorescence in 1 min, using the calibration described above. The amount of 4-nitrophenol produced during the incubation period was calculated as the difference between the final and initial (less than 5% of the final) 4-nitrophenol content of the samples. Difference between the enzyme activities measured in intact and permeabilized microsomal vesicles, expressed as the percentage of the latter, is usually referred to as the latency of the enzyme [14]. It was calculated by the formula shown in Fig. 3.

The diagrams were made and the derived parameters (K_m, V_{max}, IC_{50} and K_i) were calculated from the primary data by GraphPad Prism 4.01 software. The kinetic parameters (K_m and V_{max}) were calculated by non-linear curve fitting in the activity versus substrate concentration diagram, using the Michaelis–Menten equation. The results were checked also by line fitting in the Lineweaver–Burk (double reciprocal) plots and significant difference was not found. The IC_{50} and K_i values were calculated by fitting sigmoidal dose-response curves in the semi-logarithmic plot (enzyme activity versus logarithm of inhibitor concentration) and by finding log EC_{50} according to [15].

3. Results

3.1. *Glucosidase II activity in intact and permeabilized rat liver microsomal vesicles*

The hydrolytic activity of glucosidase II enzyme was measured at different concentrations (from 5 to 300 μM) of the artificial substrate MUG both in intact and alamethicin-permeabilized microsomes (Fig. 2). In permeabilized microsomes, MUGase activity followed Michaelis–Menten kinetics, with a K_m of 55.2 ± 3.1 μM and a V_{max} of 39.0 ± 0.7 nmol/min/mg protein. Lower activities were measured in intact microsomes than in permeabilized ones at any substrate concentration investigated (Fig. 2). The latency of MUGase activity was the most remarkable at lower substrate concentrations (e.g. 60% at 5 μM MUG), and inversely correlated to substrate concentration (Fig. 3).

Glucosidase II enzyme was also studied using another specific substrate, NPG, at concentrations ranging from 50 μM to 10 mM. In permeabilized microsomes, the substrate-concentration-dependence of the activity (Fig. 4) was consistent with Michaelis–Menten kinetics (K_m = 2.2 ± 0.1 mM; V_{max} = 15.3 ± 0.2 nmol/min/mg protein) with a much lower affinity to the substrate NPG, than in the case of MUG (see above). NPGase activity was virtually the same in intact and permeabilized microsomal vesicles at any substrate concentration investigated, i.e. the NPGase enzyme activity showed no latency.

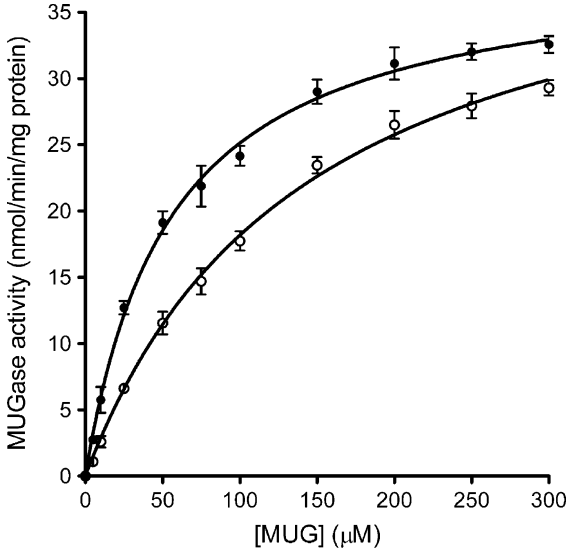

Fig. 2 – Activity of glucosidase II in rat liver microsomes measured with MUG as substrate. MUG was added at different concentrations to intact (○) or alamethicin-permeabilized (●) microsomes (0.1 mg protein/ml), and the initial rate of methylumbelliferone production was detected fluorimetrically as described in Section 2. Data are mean ± S.E.M. from three experiments.

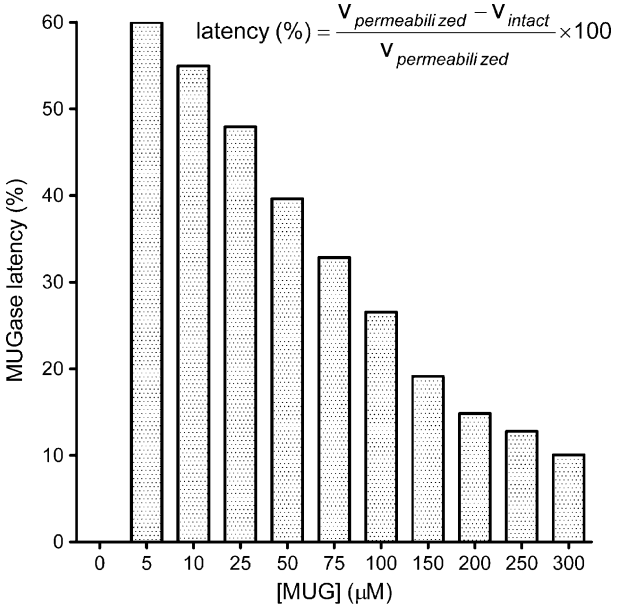

Fig. 3 – Latency of glucosidase II at different concentrations of the substrate MUG. Latency was calculated using the formula shown in the figure, from the enzyme activities measured in intact and permeabilized liver microsomal vesicles (data shown in Fig. 2).

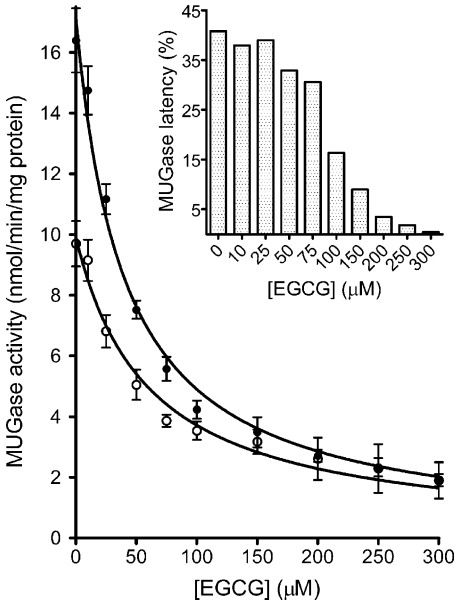

Fig. 5 – Inhibition of glucosidase II by EGCG. MUGase activity of intact (○) or alamethicin-permeabilized (●) rat liver microsomes (0.1 mg protein/ml) was measured at 40 μM substrate concentration. EGCG was administered 1–2 min prior to the substrate. The latency of MUGase activity as a function of EGCG concentration is shown in the inset. Data are mean ± S.E.M. from three experiments.

3.2. *Concentration-dependent inhibition of glucosidase II with EGCG*

The effect of EGCG on glucosidase II activity was studied using MUG (at 40 μM concentration) as substrate. Fig. 5 shows the concentration-dependent inhibitory effect of EGCG (from 10 to 300 μM) on MUGase activity of intact and alamethicin-permeabilized microsomes (lower and upper curves, respectively). The maximal inhibition was nearly 90% in permeabilized microsomes and the calculated IC_{50} and K_i values were 50.9 and 29.5 μM, respectively. It is remarkable that the latency of MUGase activity was inversely related to EGCG concentration; in fact, it was not evident at inhibitor concentrations higher than 150 μM (see inset, Fig. 5).

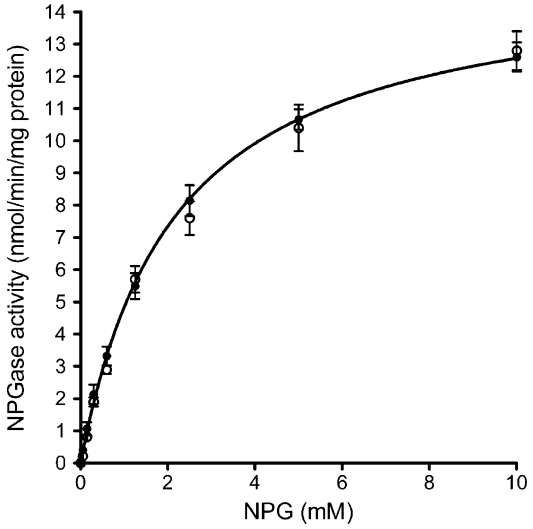

Fig. 4 – Activity glucosidase II in rat liver microsomes measured with NPG as substrate. NPG was added at different concentrations to intact (○) or alamethicin-permeabilized (●) microsomes (0.5 mg protein/ml), and the initial rate of 4-nitrophenol production was detected by HPLC as described in Section 2. Data are mean ± S.E.M. from three experiments.

3.3. *Kinetic analysis of the inhibition*

The effect of EGCG on glucosidase II was further studied by determining how the kinetics of MUGase and NPGase activities changed in the presence of the inhibitor. The alamethicin-permeabilized microsomes were treated with 100 μM EGCG, and the initial enzyme activity was measured in the presence of increasing concentrations of either MUG (from 5 to 300 μM) or NPG (from 50 μM to 10 mM). EGCG remarkably reduced both MUGase and NPGase activities at all the substrate concentrations investigated (Fig. 6A and B). The Lineweaver–Burk plots (Fig. 6C and D) showed that EGCG treatment resulted in a four-fold decrease in V_{max}, while K_m values remained unaltered (Table 1).

3.4. *Concentration-dependent effect of various tea catechins on glucosidase II*

The effect of EGCG on glucosidase II activity was compared with that of four other tea catechins (ECG, GC, EGC and GCG), of propyl-gallate (PG) and of the known glucosidase II inhibitor NBDJ. The investigated compounds were added to

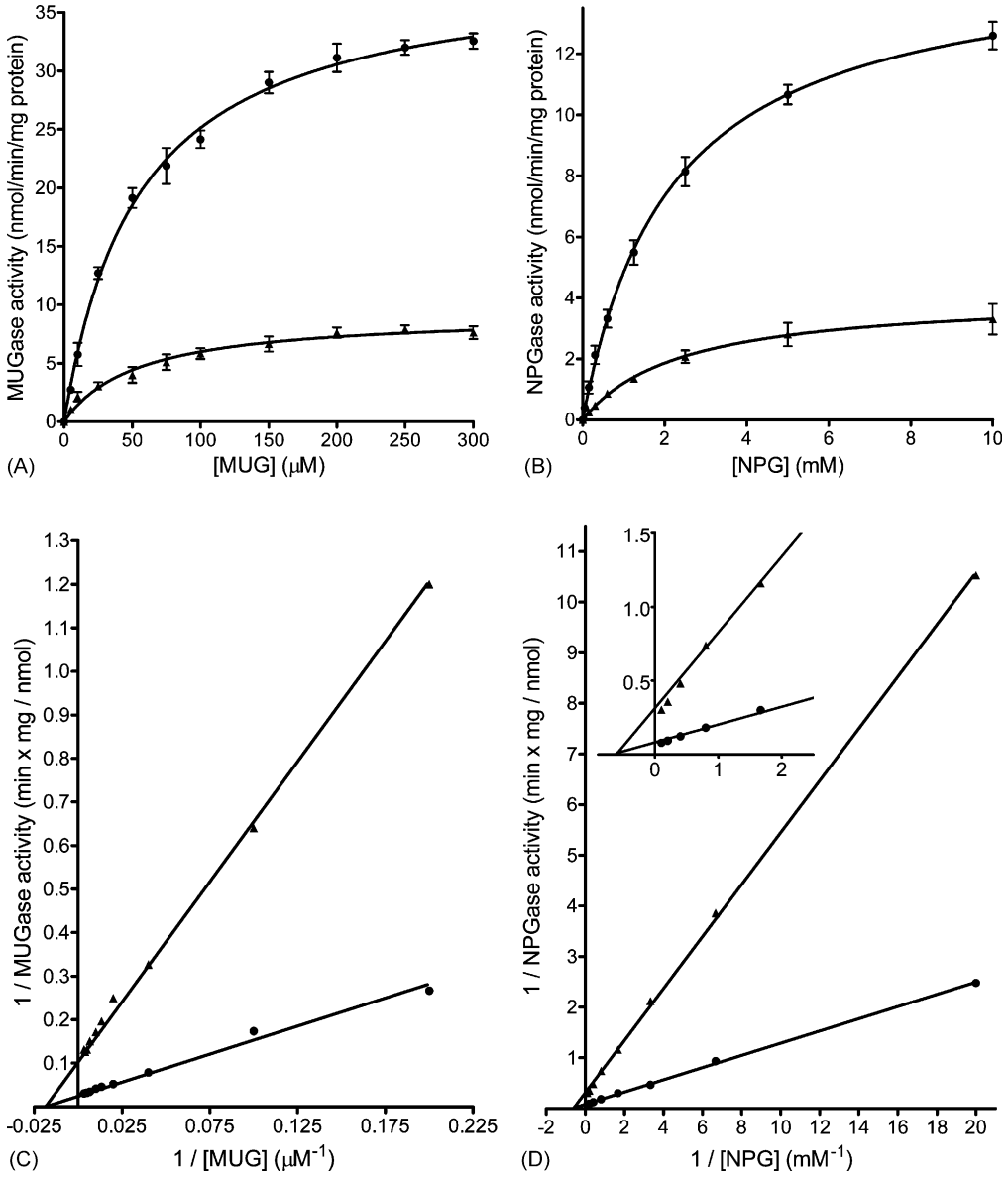

Fig. 6 – Kinetic analysis of the inhibitory effect of EGCG on glucosidase II. Activity of glucosidase II was measured in permeabilized liver microsomes at various concentrations of two different substrates: MUG (panels A and C) and NPG (panels B and D), in the absence (●) or in the presence (▲) of 100 μM EGCG. The results of three experiments were plotted as standard saturation curves (panels A and B; mean ± S.E.M.) and also in Lineweaver–Burk format (panels C and D; reciprocal of mean values).

permeabilized microsomes at various concentrations (between 10 and 300 μM), and the MUGase activity was measured in the presence 40 μM substrate. All the compounds inhibited the enzyme, although with different efficiencies (Fig. 7A). Similar results were obtained when NPGase activity was assessed at 1 mM substrate level in the presence of the same compounds (Fig. 7B). The activity was almost totally blocked at higher concentrations of GCG and ECG, similarly to the glucosidase II inhibitor NBDJ. Even the least effective tea flavonols (GC and EGC) at 200 μM concentration caused nearly 30% decrease in the activity. Compared to the other agents, the inhibitory effect of EGCG was intermediate, and it was similar to that of a non-catechin gallate, PG. The calculated IC_{50} and K_i values of each compound are reported in Table 2.

4. Discussion

The known beneficial health properties of the green (unfermented) tea are attributed to its flavonol content. These polyphenolic catechins are excellent antioxidants and have been shown to reduce proliferation, angiogenesis and induce apoptosis. These effects have been extensively investigated and molecular mechanisms have already been proposed. The most frequently studied tea flavonol, EGCG, interferes with the

Table 1 – Effect of EGCG on the kinetic parameters of glucosidase II

	MUGase	NPGase
V_{max} (nmol/min/mg protein)		
Control	39.0 ± 0.7	15.3 ± 0.2
100 μM EGCG	9.2 ± 0.4	4.1 ± 0.1
K_m (μM)		
Control	55.2 ± 3.1	2.2 ± 0.1
100 μM EGCG	54.4 ± 8.0	2.4 ± 0.1

Activity of glucosidase II was measured in permeabilized liver microsomes at various concentrations of two different substrates: MUG and NPG, in the absence (control) or in the presence of 100 μM EGCG. The kinetic parameters were calculated from the results shown in Fig. 6.

Table 2 – IC_{50} and K_i values for various inhibitors of glucosidase II

Inhibitor	MUGase		NPGase	
	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
GCG	3.502	2.027	3.702	2.545
ECG	15.14	8.763	19.06	13.10
EGCG	50.92	29.48	47.72	32.81
PG	62.67	36.28	62.73	43.12
EGC	117.7	68.15	110.5	75.99
GC	136.3	78.93	102.7	70.62
NBDJ	8.965	5.190	3.268	2.247

Each compound was added at different concentrations to permeabilized liver microsomes at 37 °C. The rate of MUG hydrolysis was measured at 0.1 mg/ml protein concentration and 40 μM substrate level for 2 min. The NPGase activity was determined at 0.5 mg/ml protein concentration and 1 mM substrate level in 25 min incubations. The IC_{50} and K_i values were calculated by GraphPad Prism 4.01 software from the results of three parallel measurements.

receptor tyrosine kinase signaling pathways, mostly by inhibiting the tyrosine phosphorylation or by suppressing the expression of the receptor proteins [1]. EGCG has also been shown to induce pro-apoptotic protein Bax and some cyclin kinase inhibitors, to downregulate anti-apoptotic protein Bcl2, cyclin D1 and cdk2 and activate several caspases [16].

Endoplasmic reticulum dysfunction can also be the origin of antiproliferative and proapoptotic effects. The folding and post-translational modification of newly synthesized lysosomal, secretory or plasma membrane proteins take place in the lumen. Glycosylation, one of the major post-translational modifications, plays a role also in the protein quality control. Inhibited glycosylation, therefore, may lead to lowered mitotic stimulation due to the reduced production of functional membrane proteins (e.g. growth factor receptors), as well as endoplasmic reticulum stress and apoptosis due to the accumulation of immature polypeptides. Accordingly, NBDJ, a potent inhibitor of glucosidase I and II, has been reported to reduce proliferation and induce apoptosis. The inhibitors of glycoprotein processing in the endoplasmic reticulum are regarded as potential anticancer agents [4,7].

In this study, the effect of some tea flavonols on glucosidase II was investigated in rat liver microsomes using two artificial glucosidase-II-specific substrates, MUG and NPG. The enzyme activity measured at different substrate concentrations in permeabilized microsomes – to allow free access of substrates and inhibitors to the luminal enzyme – showed Michaelis–Menten kinetics with both substrates. The calculated K_m values indicate that the enzyme has greater affinity to MUG than to NPG. The rate of NPG hydrolysis was the same in

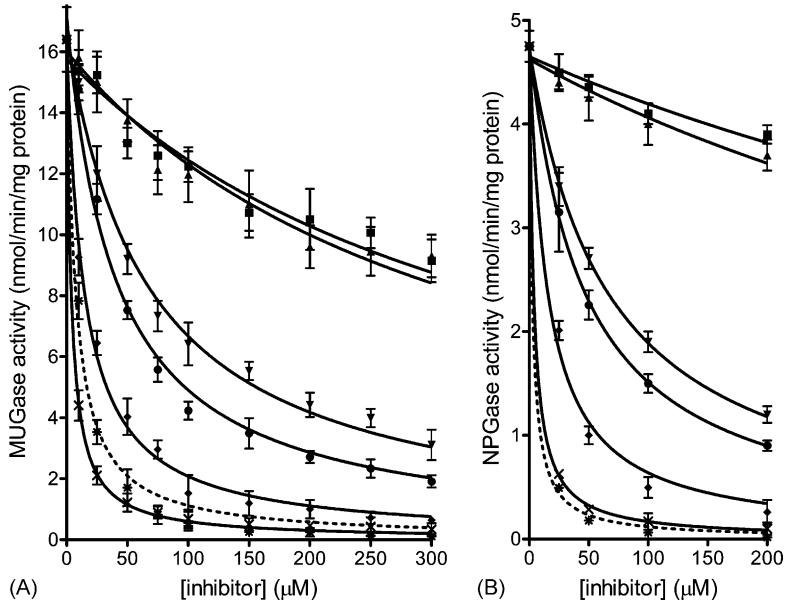

Fig. 7 – Effect of various tea flavonols and propyl gallate on glucosidase II. MUGase activity (panel A: at 40 μM MUG and 0.1 mg protein/ml) and NPGase activity (panel B: at 1 mM NPG and 0.5 mg protein/ml) were measured in alamethicin-permeabilized liver microsomes. The indicated compounds ((■) EGC; (▲) GC; (▼) PG; (●) EGCG; (◆) ECG; (×) GCG) were administered 1–2 min prior to the substrate. The effect of NBDJ (asterisk and dashed line) is shown for comparison. Data are mean ± S.E.M. from three experiments.

intact and permeabilized vesicles, while the MUGase activity was higher in permeabilized microsomes. This phenomenon (referred to as latency) has been often observed in case of luminal enzymes [14]. It indicates that the transport of MUG across the membrane (i.e. its access to the active center) limits the rate of MUG hydrolysis. As shown in Fig. 3, the latency of MUG hydrolysis was inversely correlated to the substrate concentration. Apparently, when the enzyme became saturated, the transport caught up with its activity. It can be concluded that MUG transport has lower affinity and higher capacity than the enzyme. The affinity of glucosidase II to NPG was remarkably lower, thus the transport could much easier keep pace with the enzyme, which can explain the lack of latency.

EGCG inhibited the enzyme in a concentration-dependent manner, with non-competitive kinetics. The effect was observed both in intact and permeabilized microsomes. However, higher concentrations of EGCG practically abolished the latency of MUGase activity (see inset, Fig. 5). In other words, MUG transport was no longer rate-limiting when the enzyme activity was strongly inhibited. These results suggest that EGCG exerts a pure inhibition on glucosidase II enzyme leaving MUG transport apparently unaffected in native microsomes.

The comparison of the concentration-dependent effect of different tea catechins and propyl gallate revealed that the presence of the gallate group in the compound is necessary for an efficient inhibition of glucosidase II. EGC and GC, which lack the gallate group, were much less effective than the other investigated compounds. It is remarkable that the configuration of the gallo moiety also largely influenced the inhibitory effect: the IC_{50} and K_i values of GCG were comparable with those of NBDJ, a widely used glucosidase inhibitor, while EGCG was less efficient.

The EGCG concentrations required for an 80–90% inhibition were above 100 μM. The reduction of glucosidase II activity at this extent, therefore, can only be achieved by pharmacological doses of the compound. However, it should be noted that other tea flavonols were much more effective, for example, GCG caused a nearly 80% inhibition at 10 μM level. Consistently, tea flavonols are usually applied at 10–100 μM concentrations in cellular models [1–3], and specific effects on cell signaling and apoptotic control have been observed under these circumstances. Although the maximum EGCG concentration achieved by regular green tea consumption in human plasma was about 4 μM [17], it can be supposed that the flavonols might reach an efficient concentration in the cells by accumulation, and hence result in a significant decrease in glucosidase II activity. The connection between such a reduction of glucosidase II activity and the antiproliferative, antiangiogenic and apoptotic effects requires further investigations. In this respect, the alterations in the glycoprotein processing and quality control, as well as the potential generation of endoplasmic reticulum stress – and consequent apoptotic stimuli – should also be taken into account.

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