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preliminary communication

Flavonoids as Inhibitors of Human Butyrylcholinesterase Variants

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Summary

The inhibition of butyrylcholinesterase (BChE, EC 3.1.1.8) appears to be of interest in treating diseases with symptoms of reduced neurotransmitter levels, such as Alzheimer's disease. However, *BCHE* gene polymorphism should not be neglected in research since it could have an effect on the expected outcome. Several well-known cholinergic drugs (*e.g.* galantamine, huperzine and rivastigmine) originating from plants, or synthesised as derivatives of plant compounds, have shown that herbs could serve as a source of novel target-directed compounds. We focused our research on flavonoids, biologically active polyphenolic compounds found in many plants and plant-derived products, as BChE inhibitors. All of the tested flavonoids: galangin, quercetin, fisetin and luteolin reversibly inhibited usual, atypical, and fluoride-resistant variants of human BChE. The inhibition potency increased in the following order, identically for all three BChE variants: luteolin<fisetin<quercetin<galangin. The determined enzyme-inhibitor dissociation constants (K_i) ranged from 10 to 170 μmol/L. We showed that no significant change in the inhibition potency of selected flavonoids exists in view of BChE polymorphism. Our results suggested that flavonoids could assist the further development of new BChE-targeted drugs for treating symptoms of neurodegenerative diseases and dementia.

Key words: cholinesterase, reversible inhibition, galangin, quercetin, fisetin, luteolin

Introduction

Flavonoids, which belong to a large family of biologically active polyphenolic compounds, are found in many plants and plant-derived products that are components of the everyday human diet (fruits, vegetables, chocolates, herbs, red wine, tea, beer, *etc.*). A large number of effects have been attributed to flavonoids, but the most common activity observed for almost all of these substances is potent antioxidant and free-radical scavenging (*1*,*2*). Also, flavonoids are capable of modulating gene expression (*3*) and the activity of many enzymes including tyrosine- and cyclin-dependent kinases (CDKs) (*4*,*5*). Furthermore, many enzymes possess a high affinity towards binding flavonoids (*6*), while the subgroup of flavonoids known as phytoestrogens, which shares a similar chemical structure to estrogens, is reported to improve cognitive function in Alzheimer's disease patients (*7*). Indeed, today's neurodegenerative disease therapy is based on acetylcholinesterase (AChE) inhibitors (*e.g.* galantamine, huperzine and rivastigmine) which originate from plants, or are synthesised as derivatives of plant compounds, suggesting that herbs could potentially serve as sources for novel therapeutics (*8*,*9*).

Recently, we have evaluated flavonoid interactions with butyrylcholinesterase (BChE; EC 3.1.1.8) focusing on the structural aspects of BChE inhibition (*10*). Although the physiological function of BChE remains unclear, BChE serves as a co-regulator of cholinergic neurotransmission because it can efficiently hydrolyse the neurotransmitter acetylcholine (*11*,*12*), which is primarily the role of AChE. Therefore, the inhibition of BChE may have a consider-

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able part in treating symptoms of neurodegenerative diseases and dementia (*13*) characterised by reduced acetylcholine concentration. However, the efficacy of any drug that targets BChE activity can be affected by human *BCHE* gene polymorphism. Namely, 66 mutations of the *BCHE* gene have been identified to date (*14*), and several BChE variants possess different catalytic properties or are expressed at lower levels than usual BChE (*15,16*). For example, people with atypical BChE are unable to hydrolyse the muscle relaxant succinylcholine and can experience prolonged apnoea if this relaxant is administered (*17*). The most frequent atypical and fluoride-resistant human BChE variants can be distinguished from the usual BChE by the lower degree of inhibition by dibucaine, sodium fluoride and dimethylcarbamate Ro 02--0683 (*18,19*). The atypical variant carries *BCHE* mutation D70G, while the fluoride-resistant variant carries mutations T243M or G390V. The positions affected by these mutations are far from the active site and are not directly involved in the catalysis. However, they could affect the inhibition selectivity of some ligands (*20,21*) or the cholinesterase stereoselectivity in interaction with chiral compounds (*22*).

In this study, we have evaluated the inhibition of human BChE variants, usual, atypical and fluoride-resistant, by four flavonoids: galangin, quercetin, fisetin (flavonols), and luteolin (flavon). These flavonoids were chosen based on our recent study on human BChE and AChE inhibition (*8*). The aim of this study is to establish if BChE polymorphism affects inhibition potency of flavonoids.

Materials and Methods

Chemicals

The studied flavonoids (Fig. 1) (Sigma-Aldrich Co., St. Louis, MO, USA) were a gift from Dr. Dubravko Jelić (Fidelta Co., Zagreb, Croatia) and Prof. Gordana Rusak (Faculty of Science, Univesity of Zagreb, Zagreb, Croatia). Propionylthiocholine iodide (PTCh) and 5,5´-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma--Aldrich Co. Stock solutions (10–100 mM) were prepared in DMSO (galangin), while quercetin, fisetin and luteolin were disolved in 96 % ethanol. PTCh stock solution (10 mM) was prepared in water, while DTNB was prepared in 0.1 M sodium phosphate buffer (pH=7.4). The solutions of all compounds were prepared immediately prior to use.

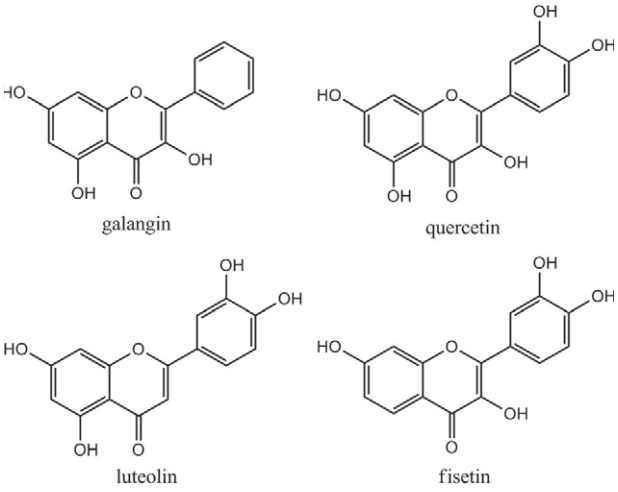

Fig. 1. Chemical structure of the tested flavonoids

Enzymes

The source of BChE was the native human serum. Specimens of the fluoride-resistant BChE variant were provided by Dr. Robert T. Evans (Cholinesterase Investigation Unit at St. James's University Hospital, Leeds, UK) and specimens of the atypical BChE variant by Dr. Oksana Lockridge (University of Nebraska Medical Center, Eppley Institute, Omaha, NE, USA). The determination of BChE variants was carried out earlier by measuring the percentage of inhibition by dibucaine, sodium fluoride and Ro 02-0683 (*18,19*). Final serum dilution was 100--fold.

Enzyme activity measurements

The enzyme activity was measured with PTCh as a substrate in 0.1 M sodium phosphate buffer, pH=7.4, using the modified Ellman spectrophotometric method (*23,24*) with DTNB as the thiol reagent (final concentration of 0.30 mM). Measurements were carried out at 25 °C and 412 nm using the Cary 300 spectrophotometer with a temperature controller (Varian Inc., Mulgrave, Victoria, Australia).

Enzyme inhibition

The reversible inhibition of BChE by flavonoids was measured by determining the decrease of enzyme activity towards PTCh (0.05–1.0 mM) in the presence of flavonoids. The reaction mixture contained BChE suspended in the phosphate buffer, DTNB, flavonoid and PTCh. To avoid its instability in the buffer, the flavonoid was added to the mixture immediately prior to starting the enzyme reaction with the substrate PTCh. At least three different concentrations of flavonoids (Table 1) for each substrate concentration were used in at least two experiments. All of the possible side-reactions of the enzyme with DMSO and ethanol, or PTCh with DMSO, ethanol and flavonoids were negligible.

The dissociation constants of the enzyme-flavonoid complex (K_i) describing the reversible inhibition by flavonoids were determined as described previously (*8,20*). The determination of kinetic constants was carried out using the GraphPadPrism program (GraphPad Software, Inc., La Jolla, CA, USA).

Results and Discussion

All four selected flavonoids (galangin, quercetin, fisetin, and luteolin; Fig. 1) reversibly inhibited the tested BChE variants. Experimentally determined flavonoid-enzyme dissociation inhibition constants (K_i) ranged from 5 to 200 μmol/L (Table 1). The inhibition potency increased in the following order for all of the BChE variants: luteolin<fisetin<quercetin<galangin.

The atypical BChE was indistinguishable from the usual BChE after the inhibition with all four flavonoids, which is probably the consequence of the neutral charge

Table 1. Reversible inhibition of human BChE variants by flavonoids

c(flavonoid)/μM	K_i/μM		
	usual*	atypical	fluoride-resistant
galangin 10–140	6.9±2.2	13±2.5	30±8.4
quercetin 40–140	68±7.9	88±9.7	55±6.9
fistein 60–200	90±10	97±13	99±17
luteolin 40–200	166±32	152±21	117±17

The enzyme-flavonoid dissociation inhibition constant (K_i±standard error) was determined from at least three experiments
*according to Katalinić *et al.* (*10*)

of flavonoids since it is known that mutation in the atypical variant (D70G) reduces the inhibition potency of many charged ligands and BChE inhibitors (*19*,*20*,*25*,*26*). However, in the case of galangin and quercetin, the fluoride-resistant variant slightly differed from the usual BChE. Galangin was three times less potent as an inhibitor of the fluoride-resistant variant than as an inhibitor of the usual BChE, and the inhibition was non-competitive since it was characterised by a substrate concentration-independent inhibition. On the other hand, quercetin inhibition potency slightly increased compared to the usual BChE. These changes could be attributed to the fluoride--resistant variant mutations T243M or G390V which, even when far from the enzyme active centre, could cause conformational changes resulting in the change of flavonoid interaction within the BChE active site.

In a previous study, we attributed flavonoid potency for inhibiting usual BChE to the number of OH groups and their side of the phenyl ring or perhaps to the lack of a C-8 hydroxyl group (*8*), which can also be asserted for the atypical and fluoride-resistant variants. A docking study showed that flavonoids bind to the BChE active site by forming multiple residue π-π interactions and hydrogen bonds (*8*). Since all aromatic residues within the BChE active site of atypical and fluoride-resistant variants are identical, there is no change in the flavonoid interactions and the inhibition potency between the most frequent variants of BChE.

Together with previous studies, the results presented here suggest that flavonoids could assist further development of new active drugs for treating symptoms of neurodegenerative diseases and dementia (*8*,*27*). In our opinion, the most promising candidate is the flavonol galangin, a major flavonoid found in the rhizome of *Alpinia officinarum* (*27*), due to its binding selectivity, characterised by a ratio of inhibition between BChE and AChE, which was particularly noticeable in the case of usual BChE (a 12 times higher preference for binding to BChE than to AChE) (*8*). The selectivity was only slightly decreased for the fluoride-resistant BChE variant. Furthermore, apart from its antioxidant and free-radical scavenging activity, galangin is capable of modulating the hypoxia-induced factor (*28*). Nevertheless, the research in the field of flavonoids as cholinesterase inhibitors is becoming more and more interesting as new data are being published (*29*). For example, this recent study by Cho *et al.* (*29*) points out several geranylated flavonoids isolated from *Paulownia tomentosa* fruits as potent cholinesterase inhibitors (in μM range), more potent than their ungeranylated parent compounds.

However, developing flavonoids as plant-derived drugs still presents a challenge, as their bioavailability is a major concern, especially with regard to oral administration. Flavonoids are synthesised in plants mainly as glycosides and it has been established that bound sugars are of great importance for compound absorption from the gastrointestinal tract (*30*). Furthermore, flavonoid circulation in the blood is relatively short as they are quickly metabolized and secreted. On the other hand, flavonoids possess lower toxicity compared to other compounds that originate from plants (such as alkaloids) and are able to cross the blood-brain barrier. These facts make a strong case in favour of flavonoids as drugs acting on specific targets during treatment of neurodegenerative diseases (*31*,*32*).

Conclusion

Flavonoids as natural compounds present a great potential to protect against a variety of human diseases, particularly cardiovascular diseases and cancer. BChE is involved in the metabolism of various drugs, toxins, and synthetic poisons and its inhibition may have a considerable role in treating neurodegenerative diseases. However, its activity can be affected by *BCHE* gene polymorphism. We have shown that quercetin, galangin, fisetin and luteolin are equally potent reversible inhibitors of the usual, atypical and fluoride-resistant variants of BChE. The most potent inhibitor of all three variants, galangin, could be a promising lead in the search for new BChE inhibitors.

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