INFLUENCE OF FLAVONOIDS ON LCK AND FYN TYROSINE KINASES USING ELISA METHOD WITH COMPARISON OF DIFFERENT SUBSTRATES:

Poly Glu:Tyr (4:1) AND M3-01 PEPTIDE

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Abstract

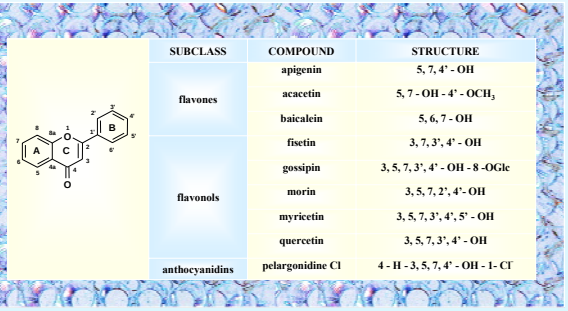
Tyrosine phosphorylation represents unique signaling process in the cell as an answer to the different extracellular signals. The enzymes that carry out this modification are protein tyrosine kinases (PTKs) which catalyze transfer of γ-phosphate of ATP on phenole –OH group of tyrosine on protein substrates. The largest subfamily of nonreceptor PTKs is the Src family. Humane Fyn and Lck tyrosine kinases are two of the nonreceptor kinases involved in T-cell signaling transport. Flavonoids are biologically active polyphenolic compounds naturally occurring in many plants. This group of compounds has been recognized as inhibitor of Fyn and Lck protein kinases. In conducted experiments, myricetin showed the highest inhibitory effect, with ATP non-competitive mechanism of inhibition. In contrast, competition with ATP has been proven in the example of staurosporine. Inhibitory activity of flavonoids on Fyn and Lck kinases was measured *in vitro* by ELISA (*Enzyme-Linked Immunosorbent Assay*) method. Affinity of these enzymes on two different substrates, polypeptide polymer Poly Glu:Tyr (4:1) and peptide M3-01 was tested. Development of efficient protein kinase inhibitors is important not just for the treatment of diseases, but also as a tool for investigation of the physiological roles of protein kinases.

Key words: tyrosine kinases, Lck, Fyn, flavonoids, ELISA.

Introduction

Many flavonoids are known for their anti-inflammatory, antiallergic, antitumoral, cardioprotective and immunomodulatory activities [5]. Some of these biological activities are assumed to be connected with antioxidant properties of these compounds, which are displayed by limiting the production of reactive oxygen species and/or scavenging them [5]. Flavonoids have also been shown to inhibit several enzymes including hipoxygenases and protein kinases [5]. Enzymatic activity of Lck and Fyn in presence of selected flavonoid compounds was measured by highly sensitive and precise ELISA method. Procedure for this method was optimized in PLIVA - RESEARCH INSTITUTE Ltd.

Table 1. Tested flavonoid compounds.



SUBCLASS	COMPOUND	STRUCTURE
flavones	apigenin	5, 7, 4' - OH
	acacetin	5, 7 - OH - 4' - OCH_3
	baicalein	5, 6, 7 - OH
flavonols	fisetin	3, 7, 3', 4' - OH
	gossipin	3, 5, 7, 3', 4' - OH - 8 -OGlc
	morin	3, 5, 7, 2', 4'- OH
	myricetin	3, 5, 7, 3', 4', 5' - OH
	quercetin	3, 5, 7, 3', 4' - OH
anthocyanidins	pelargonidine Cl	4 - H - 3, 5, 7, 4' - OH - 1- Cl

Materials and Method

Experiments were conducted in 96-well Dynex Immulon 2 hb microtitar plates (flat bottom, transparent). As a tyrosine kinase substrate, two peptides were used: polypeptide polymer Poly Glu:Tyr (4:1) (Sigma, P-0275) and M3-01 peptide (Eötvos Lorand Institute, PEP M3-01). Tested flavonoids are listed in Table 1. Compounds were selected from PLIVA's Compound Library. All of selected flavonoid compounds, as well as staurosporine (Sigma, S-4400), are literary known and commercially available (Sigma, InterBioScreen, Alexis etc.). Lck and Fyn kinases were expressed in Sf9 baculovirus, isolated and purified in PLIVA – RESEARCH INSTITUTE Ltd. BSA was obtained from Sigma (A-2153) and peroxidase-labeled anti-phospho-tyrosine antibody from Calbiochem (525320). Developed coloration was measured spectrophotometric, absorbantion at 490 nm was read.

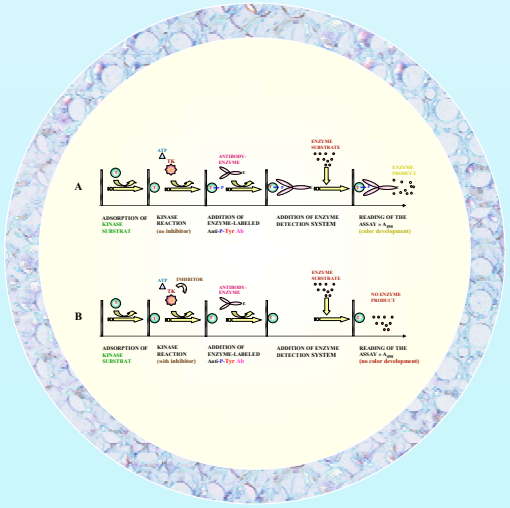


Fig. 1. ELISA method.

A) Method is based on tyrosine phosphorylation, on selected peptide substrate, by tyrosine kinase which is beeing tested. Phosphorylated tyrosine is than marked with specific, enzyme-labeled anti-phosphotyrosine antibody. Further on, enzyme detection system is added and catalytic activity starts. As a result of enzyme catalytic activity color development occurs. Intensity of developed coloration is measured spectrophotometric, absorbantion at 490 nm is read. Intensity of developed coloration is proportional to the amount of phosphorylated tyrosine.

B) Inhibitory activity of tested compounds is detected by absence of color development in the last step of the assay, in other words, absence of tyrosine phosphorylation by kinases, absence of enzyme-labeled anti-phospho-tyrosine antibody binding, and in the end, absence of enzyme catalytic activity.

a)

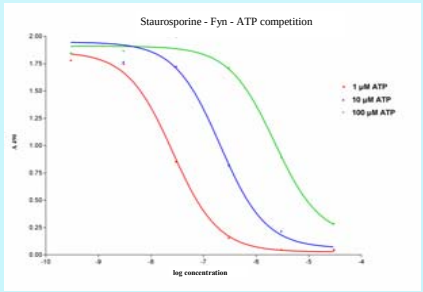
b)

Table 2. Inhibitory activity of flavonoid compounds on Fyn and Lck kinases; substrates: Poly Glu:Tyr (4:1), M3-01 peptide.
EC_{50} (compound concentration for 50 % inhibition of kinase) was defined as a final result. E_{50} was determined using graph: f [log (conc. of tested compound)] = A490. EC_{50} represents the point of inflection on the curve. Test was also conducted with staurosporine, known kinase inhibitor in micromolar concentration, as a type of control.

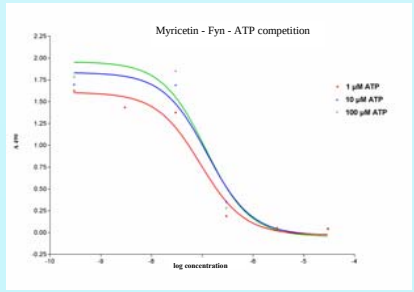
Compound	EC_{50} / Fyn / µmol/L		EC_{50} / Lck / µmol/L	
	Poly Glu:Tyr (4:1)	pep M3-01	Poly Glu:Tyr (4:1)	pep M3-01
Staurosporine	0.014	0.015	0.024	0.049
Apigenin	8.55	4.76	14.58	57.46
Gossipin	17.54	29.28	>100	>100
Morin	11.86	15.31	14.41	102.8
Myricetin	1.05	0.79	1.2	3.45
Quercetin	1.87	1.79	8.3	3.33
Acacetin	20.3	5.78	>100	>100
Baicalein	14.71	13.54	>100	61.96
Fissetin	29.82	8.63	>100	>100
PelargonidineCl	28.55	111	>100	>100

Table 3. Inhibitory activity of myricetin and staurosporine on Fyn kinase in presence of different concentration of ATP; substrates: Poly Glu:Tyr (4:1).
Possible ATP-competitive mechanism of inhibition of flavonoids on Fyn kinase was tested with only one selected, the most active flavonoid compound, myricetin, in presence of different concentrations of ATP (1 µmol/L, 10 µmol/L, 100 µmol/L final). The ATP binding site is proven to be most tractable target for kinase inhibitor drug development [4]. Staurosporine, known ATP-competitive kinase inhibitor, was also used here as a type of control.

Compound	1 µmol/L ATP EC_{50} / Fyn / µmol/L	10 µmol/L ATP EC_{50} / Fyn / µmol/L	100 µmol/L ATP EC_{50} / Fyn / µmol/L
staurosporine	0.026	0.206	2.2
myricetin	0.091	0.126	0.118



Graph 1. Inhibitory activity of staurosporine on Fyn kinase in presence of different concentration of ATP; substrate: Poly Glu:Tyr (4:1).



Graph 2. Inhibitory activity of myricetin on Fyn kinase in presence of different concentration of ATP; substrate: Poly Glu:Tyr (4:1).

c)

d)

e)

f)

Table 4. Flavonoids in herbs [2, 5]. Healing effects of many herbs, which are used in traditional and modern medicine, are also connected to the flavonoid presence [2, 5]. Examples of flavonoids in herbs are listed in the table below.

HERB	ACTIVE FLAVONOID COMPONENT	USAGE
calendula flowers [a] *(Calendula officinalis)*	isoquercitrin, rutin, narcissin	sun burns gum inflammation wound healing immunomodulatory properties
ginkgo leaf extract [b] *(Ginkgo biloba)*	flavonol glycosides	Alzheimer disease headache (effects blood circulation in brain)
chamomile flowers [c] *(Matricaria chamomilla)*	apigenin and luteolin glycosides	gastrointestinal complaints skin and mucosa irritation gum inflammation insomnia
licorice root [d] *(Glycyrrhiza glabra)*	*glycyrrhizin*- glycosides , flavanones, chalcones, isoflavones	dyspeptic problems

Table 5. Flavonoids in food [1]. Numerous of flavonoid compounds are components of everyday human diet (fruits, vegetables, chocolates, herbs, red wine, tea, beer) [1, 5]. It has been reported that the intake of flavonoids from diet is about 20 mg to 1 g every day [6]. Food combined with mentioned desirable biological properties, so called functional-food, have stimulated even greater interest for flavonoid compounds. Examples of flavonoids in food are listed in the table below.

FOOD	COMPOUND	SUBCLASS	AMOUNT PER 100 g OF FOOD
cherries (sweet, raw)	pelargonidin	anthocyanidin	0.8 mg
chocolate (dark)	catechin epicatechin	flavan-3-ols	12 mg 41.2 mg
tea leaves (black, dry)	catechin epicatechin	flavan-3-ols	157 mg 293.3 mg
wine (red)	malvidin catechin	anthocyanidin flavan-3-ol	4.2 mg 8.9 mg
grapefruit (raw)	naringenin	flavanone	78.1 mg
celery (raw)	apigenin quercetin	flavone flavonol	6.1 mg 3.5 mg
cranberry (raw) [e]	quercetin myricetin	flavonol flavonol	14 mg 4.3 mg
garlic (raw) [f]	quercetin	flavonol	22.6 mg
orange (raw)	hesperetin	flavanone	39 mg
orange juice (raw)	hesperetin	flavanone	13.9 mg
kale (raw)	kaempferol	flavonol	14.6 mg

Conclusions

- **Flavonoid compounds have shown inhibitory activity towards Fyn and Lck tyrosine kinases. It has been detected that Fyn kinase is more sensitive to flavonoid inhibition than Lck. However, both kinases have been inhibited by myricetin at lower concentrations than quercetin (0.79 µmol/L – 3.45 µmol/L versus 1.79 µmol/L – 8.35 µmol/L).**
- **Significant difference in kinase affinity for tyrosine phosphorylation between two structurally diverse substrates, Poly Glu:Tyr and M3-01 peptide (N-KVEKIGEGTYGVVYK-OH), has not been observed. Therefore, even using different substrates by this method, obtained values for the inhibition of Fyn and Lck are accurate and reliable.**
- **ATP-competitive mechanism of inhibition of myricetin on Fyn tyrosine kinase has not been observed. This indicates that presumably myricetin inhibits Fyn by binding on a different site of the protein, distinctive from the ATP-binding site. Since all tested flavonoid compounds show structural similarity, we can conclude that the mechanism of inhibition is probably the same for all analogues.**
- **ATP-competitive mechanism of inhibition has been obtained in the case of staurosporine – standard inhibitor of all known protein kinases.**

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