

Inhibition of cAMP-Phosphodiesterase by Biflavones of *Ginkgo biloba* in Rat Adipose Tissue

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This work compares the inhibition of cAMP-phosphodiesterase in rat adipose tissue by a mixture of *Ginkgo biloba* biflavones with the effect of individual dimeric flavonoids. The degree of enzyme inhibition by *G. biloba* biflavones was amentoflavone > bilobetin > sequoiaflavone > ginkgetin = isoginkgetin. Sciadopitysin was almost inactive.

Regressive abiotrophic pannicular disease or liposclerosis (so-called cellulitis) has a complex pathology which is promoted by venous stasis and/or chronic venous insufficiency.¹ A reduction of the sclerotic phenomenon can be accomplished by local application of compounds which can affect either skin microcirculation and lipolysis in adipose tissue such as agents with antiinflammatory, antiaedematous and lipolytic effects. Lipolysis is regulated by cyclic AMP levels which are modulated either by activation of adenylate cyclase or inhibition of phosphodiesterase (PDE). Thus one possibility of increasing the lipolysis in fat cells is to develop compounds capable of stimulating the synthesis of cAMP or compounds that reduce cAMP destruction by inhibiting PDE. Within the past decade it has become clear that there is a large family of PDE isoenzymes which are tissue specific and PDE-3B isoenzyme is localized in adipose tissue.² These multiple forms of PDE are differently regulated in relation to their function(s) in the body,² thus responses to a particular effector can differ from one organ to other.

Dimeric flavonoids³ from *Ginkgo biloba* L. (Ginkgoaceae) were shown to inhibit beef heart PDE⁴ and histamine release from mast cells.⁵ A fraction from *G. biloba* enriched in dimeric flavonoids has been shown to have antiinflammatory and vasokinetic properties.^{6,7} Therefore, *G. biloba* biflavones could be a good candidate for cosmetic formulations against cellulitis, if they increase lipolysis by inhibition of cAMP-PDE in adipose tissue. *G. biloba* biflavones are a mixture of amentoflavone and other biflavonoids, such as bilobetin, sequoiaflavone, ginkgetin, isoginkgetin and sciadopitysin, which differ from each other in the position and degree of methylation of the hydroxyl groups.³ This study was undertaken to check whether *G. biloba* biflavones were able to inhibit cAMP-PDE in rat adipose tissue and to compare the inhibitory effect of *G. biloba* biflavones as a mixture of biflavones to that of its individual components.

The basal level of PDE activity in adipocyte homogenate was 10.9 ± 0.59 pmol/min/mg of protein (mean \pm SE, $n = 7$). Maximal PDE inhibition (>90%) was obtained with 100 μ M papaverine, 200 μ M kaempferol, and 1 mM aminophylline. Dose-inhibition curves of *G. biloba* biflavones and individual components are reported in Figure 1. The complete enzyme inhibition ($92 \pm 3\%$) was obtained with a concentration of *G. biloba* biflavones of 22.7 μ M. Among biflavones only sequoiaflavone (3.6 μ M) elicited complete inhibition ($94 \pm 1\%$) of the enzyme, while ginkgetin and

isoginkgetin only caused 50% inhibition, even at a concentration which was four times higher than that of sequoiaflavone. Maximal inhibition of amentoflavone and bilobetin was 85 ± 2 and $84 \pm 3\%$, respectively. Inhibition by sciadopitysin was low and not concentration dependent: 10% inhibition for the range of concentrations tested (0.05–3 μ g/mL). The IC₅₀ values calculated from the curves are as follows: *G. biloba* biflavone mixture, 1.9 ± 0.38 μ g/mL (1.23 μ M); amentoflavone, 0.16 ± 0.02 μ g/mL (0.27 μ M); bilobetin, 0.23 ± 0.02 μ g/mL (0.36 μ M); sequoiaflavone, 0.56 ± 0.04 μ g/mL (1.01 μ M). The compounds isoginkgetin, ginkgetin and sciadopitysin contribute very little to the inhibitory effect of *G. biloba* biflavones which showed an IC₅₀ higher as compared to that of the single components: amentoflavone, bilobetin and sequoiaflavone. Alternatively, *G. biloba* biflavones could contain compounds which hinder the effect of biflavones. According to our results the inhibitory activity of dimeric flavonoids is directly related to the number of the free hydroxyl groups. These results are in agreement with those reported by Beretz et al.⁴ who found that higher substituted biflavones were less active than amentoflavone in the inhibition of beef heart PDE. On the other hand, it appears that platelet PDE was better inhibited by amentoflavone hexaacetate than amentoflavone.⁸ Adipocyte PDE has a different sensitivity to inhibitors, compared to PDE extracted from other tissues. Amentoflavone was more potent on adipocyte-derived PDE than on human platelet PDE.⁸ *G. biloba* biflavones exhibited complete PDE inhibition at concentrations much lower than that of the well-known inhibitors papaverine, aminophylline, and the flavonoid kaempferol.

Since PDE inhibition in cell free systems does not imply a corresponding stimulation of lipolysis, the next target will be assaying a *G. biloba* biflavone mixture and single dimeric flavonoids on lipolysis rate in intact fat cells.

Experimental Section

Materials. [8-¹⁴C]Adenosine (51.0 mCi/mmol) and [2,8-³H]-adenosine 3',5'-cyclic phosphate (37.4 Ci/mmol) were purchased from Amersham International plc (Little Chalfont, UK) and Dupont NEN (Dupont Italiana, Cologno Monzese, Italy), respectively. cAMP, collagenase type II, papaverine chloride, and aminophylline were from Sigma Chemical Co. (St. Louis, MO). *G. biloba* biflavones⁹ (35.7% purity), bilobetin (87.8% purity), sequoiaflavone (7-*O*-methylamentoflavone, 98.9% purity), ginkgetin (94.5% purity), isoginkgetin (91.3% purity), and sciadopitysin (98.0% purity) were a kind gift of Indena SpA (Milan, Italy). The purity of biflavones was determined by HPLC.¹⁰ According to Bombardelli et al.,¹⁰ the proportion of the various biflavonoids in the *G. biloba* mixture was as

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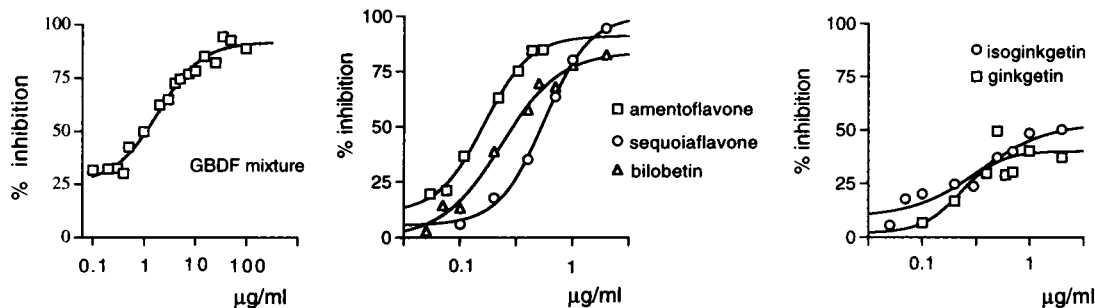


Figure 1. Dose–inhibition curves of *G. biloba* biflavones as a mixture and individual components. The compounds were added immediately before the starting of the incubation of the enzyme. Each point is the mean of 6 replications.

follows: 34% ginkgetin, 35% isoginkgetin, 17% bilobetin, 8% sciadopitysin, 2% amentoflavone, and 4% sequoiaflavone. Amentoflavone (99% purity) was purchased from Extrasynthèse (Lyon, France).

Bioassay. Phosphodiesterase preparations were obtained under the following conditions. Isolated fat cells were prepared by the collagenase method¹¹ from epididymal adipose tissue of Sprague–Dawley rats (Charles River, Calco, Italy) weighing 150–175 g. The freshly isolated cells were washed by centrifugation with 0.25 M sucrose/10 mM Tris-HCl buffer, pH 7.4, homogenized, and fractionated.¹² The homogenate was centrifuged at 20000g for two min at 4 °C. The fat cake was removed, the pellet was resuspended with the supernatant and the suspension was used for the enzyme assay. The phosphodiesterase activity was tested as described by Kono et al.¹² Compounds to be assayed were added in DMSO (less than 1% solvent/sample), just prior the starting of the incubation. *G. biloba* biflavones were tested at concentration ranging from 0.1 to 100 µg/mL, single biflavones in the range of 0.05–8 µg/mL. The molar concentration was calculated taking into account the product purity and the molecular weight. For *G. biloba* biflavones an average molecular weight of 550 was calculated. Papaverine chloride (2–500 µM), kaempferol (40–400 µM), and aminophylline (0.1–1 mM) were used as reference compounds. Adenosine formed during the incubation was separated from the substrate by column chromatography with AG 1-X2 (200–400 mesh, from Bio-Rad). The recovery of adenosine was evaluated by addition of [¹⁴C]adenosine (2000 cpm) before the column chromatography. The radioactivity of the eluate containing adenosine was determined by liquid

scintillation counting. Dose–inhibition curves were analyzed as reported elsewhere, and each point represents the mean of 6 replications.¹³

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