British Journal of Pharmacology (2008) 155, 244–252
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0007–1188/08 $32.00
www.brjpharmacol.org

RESEARCH PAPER

Inhibition of fatty acid amide hydrolase by kaempferol and related naturally occurring flavonoids

L Thors, M Belghiti and CJ Fowler

Department of Pharmacology and Clinical Neuroscience, Umeå University, Umeå, Sweden

Background and purpose: Recent studies have demonstrated that the naturally occurring isoflavone compounds genistein and daidzein inhibit the hydrolysis of anandamide by fatty acid amide hydrolase (FAAH) in the low micromolar concentration range. The purpose of the present study was to determine whether this property is shared by flavonoids.
Experimental approach: The hydrolysis of anandamide in homogenates and intact cells was measured using the substrate labelled in the ethanolamine part of the molecule.
Key results: Twenty compounds were tested. Among the commonly occurring flavonoids, kaempferol was the most potent, inhibiting FAAH in a competitive manner with a K_i value of 5 μM. Among flavonoids with a more restricted distribution in nature, the two most active toward FAAH were 7-hydroxyflavone (IC_{50} value of 0.5–1 μM depending on the solvent used) and 3,7-dihydroxyflavone (IC_{50} value 2.2 μM). All three compounds reduced the FAAH-dependent uptake of anandamide and its metabolism by intact RBL2H3 basophilic leukaemia cells.
Conclusions and implications: Inhibition of FAAH is an additional *in vitro* biochemical property of flavonoids. Kaempferol, 7-hydroxyflavone and 3,7-dihydroxyflavone may be useful as templates for the synthesis of novel compounds, which target several systems that are involved in the control of inflammation and cancer.
British Journal of Pharmacology (2008) **155**, 244–252; doi:10.1038/bjp.2008.237; published online 16 June 2008

Keywords: anandamide; cannabinoid; fatty acid amide hydrolase; flavonoids; kaempferol; apigenin

Abbreviations: AEA, anandamide (arachidonoylethanolamide); DMSO, dimethylsulphoxide; EtOH, ethanol; FAAH, fatty acid amide hydrolase; OMDM-1, (9*Z*)-*N*-(1-((*S*)-4-hydroxybenzyl)-2-hydroxyethyl)-9-octadecenamide; PPARγ, peroxisome proliferator activated receptor γ; URB597, 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate

Introduction

Flavonoids are a family of naturally occurring compounds that are currently of considerable interest in view of their possible preventative effects in diseases such as cancer and stroke (Keli *et al*., 1996; Havsteen, 2002; Gates *et al*., 2007). In addition to the well-known effects of the flavonoids, such as their antioxidant (in micromolar concentrations) and phytoestrogen actions (in nanomolar concentrations) (Kuiper *et al*., 1998; Furusawa *et al*., 2005), the compounds affect a variety of cellular processes (see Havsteen, 2002). Thus, for example, the flavone apigenin (structure shown in Figure 1a), which is present in many food sources, such as parsley, celery and teas, and which has been shown to act as a chemopreventative agent both *in vitro* and *in vivo* in experimental models (Patel *et al*., 2007; Shukla *et al*., 2007), also inhibits, when used in micromolar concentrations, a variety of enzymes, including fatty acid synthase (Brusselmans *et al*., 2005), PDE 1–3 (Ko *et al*., 2004), PI3-kinaseα (Agullo *et al*., 1997) and COX-2, the latter secondary to its ability to activate peroxisome proliferator activated receptor γ (PPARγ) (Liang *et al*., 2001). Similarly, the flavonole kaempferol (structure given in Figure 1a), which is found, for example, in broccoli and endives and whose intake may also be beneficial in certain types of cancer (Nöthlings *et al*., 2007), has a myriad of biological effects when used at micromolar concentrations, including activation of PPARγ (Liang *et al*., 2001) and inhibition of interleukin-4-induced STAT6 activation (Cortes *et al*., 2007).

The finding that both apigenin and kaempferol activate PPARγ is worthy of comment. PPARγ is a ligand-activated transcription factor that, in addition to its role in adipocyte differentiation, fatty acid and lipid metabolism, and insulin sensitivity, also has potentially important roles in inflammation and cancer (Moraes *et al*., 2006; Wang *et al*., 2006). There appears to be an overlap between this system and the endogenous cannabinoid (endocannabinoid) system (itself

Correspondence: Professor CJ Fowler, Department of Pharmacology and Clinical Neuroscience, Umeå University, Umeå SE-901 87, Sweden.
E-mail: cf@pharm.umu.se
Received 29 January 2008; revised 18 March 2008; accepted 15 May 2008; published online 16 June 2008

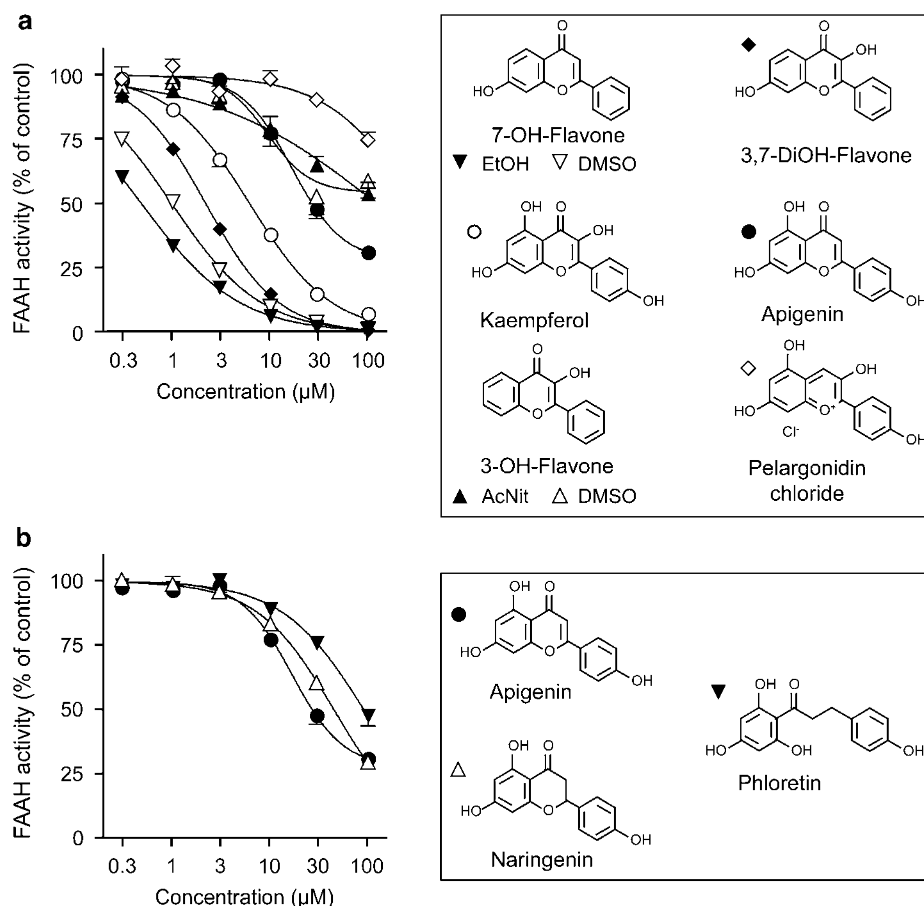

Figure 1 Inhibition by flavonoids and related compounds of the hydrolysis of 0.5 µM [^{3}H]-AEA by rat brain homogenates. In panel a, example compounds related to kaempferol are shown. In panel b, saturated and ring-opened analogues of apigenin are shown. Shown are means ± s.e.mean (when not enclosed by the symbols), $n = 3$, except for the dimethylsulphoxide samples for 7-hydroxyflavone ($n = 6$). The dose–response curve for apigenin is the same in both panels. AcNit, acetonitrile; AEA, anandamide; DiOH, dihydroxy; FAAH, fatty acid amide hydrolase; OH, hydroxy.

important in inflammation and cancer) (Klein, 2005; Bifulco *et al*., 2006), as the endocannabinoid ligands anandamide (arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol interact with PPARγ (O'Sullivan, 2007). The reverse is also true, as several compounds that activate PPARγ are also capable of inhibiting fatty acid amide hydrolase (FAAH) (Lenman and Fowler, 2007), the enzyme primarily responsible for the hydrolysis of AEA (Deutsch and Chin, 1993) and a current target for drug development for disorders such as inflammation, inflammatory pain and possibly cancer chemotherapy (Bifulco *et al*., 2004; Holt *et al*., 2005; D'Argenio *et al*., 2006; Jayamanne *et al*., 2006; Izzo *et al*., 2008). This overlap also includes the phytoestrogen genistein, the isoflavone analogue of apigenin, which activates PPARγ (at micromolar concentrations; Dang *et al*., 2003) and is a potent competitive inhibitor of FAAH (K_i value 2.8 µM) (Thors *et al*., 2007a). The related isoflavone compound daidzein (the isoflavone analogue of 4′,7′-dihydroxyflavone, a flavonoid found in alfalfa roots; Maxwell *et al*., 1989) also inhibits rat brain FAAH in a competitive manner, with a K_i value of 1.7 µM (Thors *et al*., 2007b) and activates PPARγ (Chacko *et al*., 2007). Taken together, these findings raise the possibility that flavonoids, such as apigenin and kaempferol, inhibit FAAH. This possibility has been investigated in the present study. In particular, two questions have been asked. Firstly, do the commonly occurring flavonoids inhibit FAAH at concentrations that can be attained after dietary ingestion? Secondly, what are the structural requirements for inhibition of FAAH by flavonoids?

Methods

Assay of FAAH activity in rat brain homogenates and RBL2H3 cells

Frozen brains (without cerebella) from adult Wistar or Sprague–Dawley rats were thawed and homogenized in 20 mM HEPES, 1 mM $MgCl_2$ (pH 7.0), and thereafter centrifuged at ~35 000 *g* for 20 min (4 °C). After resuspension in buffer, recentrifugation and a second resuspension in buffer, the pellets were incubated at 37 °C for 15 min in order to hydrolyse all endogenous FAAH substrates. Following recentrifugation, the pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 3 mM $MgCl_2$ and frozen at −80 °C in aliquots until used for assay. FAAH was assayed by the method described by Boldrup *et al*. (2004)

using 0.5 μM (unless otherwise stated) [^{3}H]-AEA, labelled in the ethanolamine part of the molecule. Blank values were obtained by the use of buffer rather than homogenate.

For experiments using intact RBL2H3 cells, the cells were cultured in minimum essential medium with Earl's salts, 15% foetal bovine serum and 100 U mL^{-1} penicillin + 100 μg mL^{-1} streptomycin (termed 'medium' below), and aliquots (containing 2×10^5 cells) were added to 24-well plates and allowed to affix to the wells overnight. [^{3}H]-AEA hydrolysis by the cells was measured as described previously (Paylor *et al*., 2006), using a preincubation time of 10 min, an incubation time of 20 min and an assay substrate concentration of 100 nM. Blank values were obtained from wells on the same culture plates that had not been seeded with cells. The final concentration of solvent in these experiments did not exceed 0.5% (vol/vol).

Assay of AEA uptake by adherent RBL2H3 and PC3 cells

The assays for RBL2H3 cells (cultured as described above) and for PC3 prostate cancer cells (cultured in Hams F-10, 2 mM L-glutamine, 10% foetal bovine serum and 100 U mL^{-1} penicillin + 100 μg mL^{-1} streptomycin) were carried out as described by Thors *et al*. (2007b), using a preincubation time of 10 min and an incubation time of 5 min with 100 nM [^{3}H]-AEA, labelled in the arachidonoyl part of the molecule. The final concentration of solvent in these experiments did not exceed 0.5% (vol/vol).

Assay of URB597-sensitive accumulation of tritium in RBL2H3 cell membranes following incubation of cells in suspension with [^{3}H]-AEA

RBL2H3 cells were pelleted by centrifugation and resuspended in medium in Eppendorf tubes to a concentration of 2×10^5 cells per tube (5×10^5 cells mL^{-1}). The cells were then preincubated with either the test flavonoids or URB597 for 10 min at 37 °C before the addition of [^{3}H]-AEA (assay concentration 100 nM, substrate labelled in the arachidonoyl part of the molecule) in medium and incubation for a further 10 min (final assay volume: 500 μL). After incubation, the cells were sedimented using a microcentrifuge (1 min, 1000*g*) and washed twice with ice-cold medium. Aliquots (200 μL) of the suspensions were placed in 96-well plates and the radioactivity retained by the cells was separated from that in the medium by filtration through polyethylenimine-coated FilterMAT filters (Skatron Instruments Inc., Sterling, VA, USA) using a Micro cell harvester (Skatron Instruments Inc.) and a 30 s period of washing with deionized water (setting 4), which ruptures the cells. The URB597-sensitive accumulation of tritium was defined as the radioactivity recovered on the filter papers minus the corresponding radioactivity for the cells treated with 100 nM URB597.

Statistical analyses

The values of pI_{50}, IC_{50}, Hill slope (n_H, given here as the positive rather than the negative values), K_m and V_{max} (nonlinear regression analyses), linear regressions and confidence limits were determined using the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA, USA). The pI_{50}, n_H and IC_{50} values were calculated using the built-in programme 'sigmoidal dose–response (variable slope)' from the data expressed as percentage of control, using top (that is, uninhibited) values of 100% and bottom (residual activity) values that were either set to zero or allowed to float. The two curves were compared using Akaike's Informative Criteria. When this statistical analysis suggested that the simple 0% residual inhibition model fitted the curve more appropriately, this alone was presented in the results. In the other three cases, both models are presented. When K_m and V_{max} values were calculated by the other analyses (direct linear plot, Hanes–Woolf, Lineweaver–Burk, Eadie–Hofstee and Johansen–Lumry plots), the Enzyme Kinetics v1.4 computer programme for Macintosh (Trinity Software, Campton, NH, USA) was used.

Compounds

Anandamide (ethanolamine-1-^{3}H; specific activity: 2.22 TBq mmol^{-1} for the FAAH experiments) and anandamide (arachidonoyl 5,6,8,9,11,12,14,15-^{3}H; specific activity: 7.4 TBq mmol^{-1} for the uptake and membrane tritium accumulation experiments) were purchased from American Radiolabeled Chemicals Inc. (St Louis, MO, USA). All of the flavonoid compounds used in the present study were obtained from Sigma-Aldrich (St Louis, MO, USA), with the exception of 7,4′-dihydroxyflavone and 5-deoxykaempferol, which were obtained from Extrasynthese (Genay, France). Non-radioactive AEA and URB597 (3′-carbamoyl-biphenyl-3-yl-cyclohexyl-carbamate) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). OMDM-1 ((9Z)-*N*-(1-((*S*)-4-hydroxybenzyl)-2-hydroxyethyl)-9-octadecenamide) was obtained from Tocris Bioscience (Ellisville, MO, USA). RBL2H3 cells (passage range 23–36) were obtained from the American Type Culture Collection (Manassas, VA, USA). PC3 cells (passage range 27–34) were obtained from Professor Anders Bergh (Department of Medical Biosciences, Umeå University, Umeå, Sweden).

Results

Structure–activity relationships for the inhibition of FAAH by flavonoids

A total of 20 flavonoids and related compounds were tested for their ability to inhibit 0.5 μM [^{3}H]-AEA hydrolysis by rat brain homogenates. Unless otherwise stated, the compounds were initially dissolved in dimethylsulphoxide (DMSO). The data for flavonoids, with the exception of 3-hydroxyflavone, are shown in Table 1, and selected examples are given in Figure 1a. In most cases, data were best fitted by a curve with no residual activity, that is, the maximum attainable inhibition was 100%, and the pI_{50}, and hence IC_{50}, values can be considered to be robust. For apigenin and galangin, there was an apparent residual activity of ~25%, which may reflect a solubility issue at the highest concentration tested (100 μM) (see Figure 1 for apigenin). Certainly, the Hill slopes were nearer to unity when the data were calculated assuming no residual inhibition (Table 1). 3-Hydroxyflavone (shown in

Table 1 Inhibition of rat brain AEA hydrolysis by flavonoids

	Hydroxy substituent (●) at position										
	3	*5*	*6*	*7*	*2′*	*3′*	*4′*	*5′*	*Hill slope (−ve value)*	*pI$_{50}$*	*IC$_{50}$ (μM)*
Flavone									D 43 ± 2% inhibition at 100 μM		
									E 45 ± 2% inhibition at 100 μM		
5-OH-flavone		●							D 14 ± 2% inhibition at 100 μM		
									E 27 ± 3% inhibition at 100 μM		
7-OH-flavone				●					D 0.96 ± 0.03	6.01 ± 0.01	0.99
									E 0.87 ± 0.03	6.32 ± 0.01	0.48
3,7-DiOH-flavone	●			●					1.18 ± 0.03	5.66 ± 0.01	2.2
7,4′-DiOH-flavone				●			●		D 1.12 ± 0.10	5.08 ± 0.04	8.3
									E 0.90 ± 0.09	5.25 ± 0.05	5.6
Chrysin		●		●					9 ± 2% inhibition at 100 μM		
Galangin	●	●		●					a. 1.23 ± 0.16	4.50 ± 0.05	31
									b. 2.17 ± 0.39	4.73 ± 0.05	19
5-Deoxykaempferol	●			●			●		1.07 ± 0.05	5.19 ± 0.02	6.4
Baicalein		●	●	●					44 ± 8% inhibition at 100 μM		
Apigenin		●		●			●		a. 0.98 ± 0.09	4.45 ± 0.04	35
									b. 1.67 ± 0.23	4.78 ± 0.05	17
Kaempferol	●	●		●			●		1.05 ± 0.05	5.21 ± 0.02	6.2
Fisetin	●			●		●	●		1.51 ± 0.19	5.06 ± 0.04	8.7
Luteolin		●		●		●	●		1.22 ± 0.22	4.07 ± 0.06	86
Morin	●	●		●	●		●		0.95 ± 0.09	4.04 ± 0.04	92
Quercetin	●	●		●		●	●		1.32 ± 0.12	4.46 ± 0.03	35
Myricetin	●	●		●		●	●	●	28 ± 3% inhibition at 100 μM		

Abbreviations: AEA, anandamide (arachidonoylethanolamide); D, DMSO; E, ethanol.
All values were calculated from 3–6 experiments using at least five concentrations of test compound. For the two compounds marked a and b, the curve fitting indicated that a model with a residual activity (b: 28 ± 4% for apigenin, 24 ± 5% for galangin) fitted the data better than a model with no residual activity (a). For all the other compounds, the model 'a' fitted the data better, and so only this is given. When the derived IC$_{50}$ value was higher than the highest concentration tested (100 μM), the inhibition at this concentration is given.

Figure 1a) was an extreme example of the difficulties associated with analysing compounds with residual activities. When the compound was dissolved in DMSO, the results were better fitted with a curve with a residual activity of 46 ± 5% ($-n_H$ value 1.98 ± 0.98, pI$_{50}$ value 5.03 ± 0.11, IC$_{50}$ value 0.94 μM) than with a curve assuming 100% inhibition (pI$_{50}$ value <4). However, when the compound was dissolved in acetonitrile, the curve of best fit was that for 100 % inhibition (pI$_{50}$ value <4). The raw data for the two solvents are not that dissimilar (see Figure 1a), illustrating the need to be cautious in interpreting curve-fitting data. In consequence, we have not assigned an IC$_{50}$ value for 3-hydroxyflavone.

At the extremes, flavone, with no hydroxy groups, and myricetein, with six hydroxy substituents, did not inhibit FAAH to any great extent (Table 1). A single hydroxy substituent at position 5 did not produce an active compound, whereas 7-hydroxyflavone, with an IC$_{50}$ value of 0.99 μM (in DMSO) and 0.48 μM (in ethanol (EtOH)) was the most active toward FAAH among all the compounds in the series. Addition of a second hydroxy group to this compound either reduced (3,7-dihydroxyflavone, IC$_{50}$ value 2.2 μM; 7,4′-dihydroxyflavone, IC$_{50}$ value 5.6–8.3 μM) or obliterated (5,7-dihydroxyflavone (chrysin)) the activity. For the trihydroxy compounds, 5-deoxykaempferol (3,7,4′-trihydroxyflavone) was the most active toward FAAH (IC$_{50}$ value 6.4 μM), followed by the 5,7,4′- (apigenin) and 3,5,7- (galangin) derivatives (IC$_{50}$ values ~30 μM), whereas the 5,6,7-derivative (baicalein) was a poor inhibitor of FAAH. Additional hydroxy substituents did not improve the ability of the compounds to inhibit FAAH; for the three compounds with four hydroxy substituents, kaempferol (IC$_{50}$ value 6.2 μM) had the same potency as 5-deoxykaempferol, fisetin (3,7,3′4′-derivative, IC$_{50}$ value 8.7 μM) was equipotent with the 7,4′- and 3,7,4′- (5-deoxykaempferol) derivatives, whereas luteolin (IC$_{50}$ value 86 μM) was a poor inhibitor. Replacement of the benzopyran-4-one ring of kaempferol with a flavylium chloride structure (pelargonidin chloride) led to a loss of activity (Figure 1a). Morin and quercetin, with five hydroxy substituents, were weak FAAH inhibitors, with IC$_{50}$ values of 92 and 35 μM, respectively (Table 1).

Flavone compounds are often strongly coloured (flavonoids are found in petal pigments; Havsteen, 2002), a case in point being 7-hydroxyflavone in DMSO. However, addition of 1, 10 or 100 μM of 7-hydroxyflavone (in either DMSO or EtOH) to homogenates first after the incubation with [^{3}H]-AEA and placing of the samples on ice had no effect on the observed rate of hydrolysis (data not shown), indicating that the colouration did not affect the assay results.

The effects of ring saturation and ring opening of apigenin were investigated. Naringenin, the flavanone analogue of apigenin, was roughly equipotent, with a pI$_{50}$ value of 4.34 ± 0.02 (IC$_{50}$ value 45 μM, $-n_H$ value 1.10 ± 0.05)

(Figure 1b). Ring opening of apigenin to give phloretin resulted in a reduction of activity toward FAAH (pI_{50}, 4.04 ± 0.04; IC_{50}, 92 μM; $-n_H$, 1.03 ± 0.10) (Figure 1b).

Mode of inhibition of FAAH by kaempferol and 7-hydroxyflavone
The modes of inhibition of [^{3}H]-AEA hydrolysis by kaempferol and 7-hydroxyflavone are shown in Figure 2. Neither compound showed a time-dependent inhibition of AEA hydrolysis (left insets to Figures 2a and b). In the experiments with kaempferol, the data were clear-cut. In the absence of kaempferol, hydrolysis was saturable, and the K_m and B_{max} values obtained using nonlinear regression analysis of the data were 1.74 μM and 1.51 nmol mg^{-1} protein min^{-1}, respectively. Almost identical values were seen when the mean values were re-analysed using other plots (direct linear plot, Hanes–Woolf, Lineweaver–Burk, Eadie–Hofstee, Johansen–Lumry; data not shown). For 3 μM of kaempferol, the K_m and B_{max} values obtained using nonlinear regression analysis of the data were 2.78 μM and 1.53 nmol mg^{-1} protein min^{-1}, respectively, and once again very similar values were found with the other plots. These values indicate a competitive mode of interaction, from which a K_i value of 5.0 μM could be calculated. At the 6 μM and particularly 9 μM concentrations of kaempferol, the apparent K_m and V_{max} values varied more between the analysis methods, which is to be expected for a situation where a K_m value is greater than the highest assay concentration used. However, visual inspection of the Lineweaver–Burk plot (shown as the right inset of Figure 2a) confirmed the competitive nature of the interaction between kaempferol and FAAH. Indeed, when the data were assessed using a fixed B_{max} value of 1.512 nmol mg^{-1} protein min^{-1} for all concentrations of kaempferol, the theoretical curves fitted the observed data very well (main graph, Figure 2a). The K_m^{app} values using this constrained model were 1.74, 2.73, 3.82 and 4.73 μM for 0, 3, 6 and 9 μM of kaempferol, respectively, and from these values, K_i was calculated to be 5.2 μM. These K_m and K_i values would give an IC_{50} value of 6.7 μM at an assay AEA concentration of 0.5 μM according to the Cheng–Prusoff relation, which is in good agreement with the value of 6.2 μM found for kaempferol in the initial experiments (Table 1).

In the case of 7-hydroxyflavone (using EtOH as solvent), inhibition by 0.4 and 0.8 μM again appeared to be competitive in nature, whereas the situation for 1.2 μM was less clear. However, constraining the B_{max} value to that seen in the absence of 7-hydroxyflavone (1.112 nmol mg^{-1} protein min^{-1}) once again fitted the data points well at all concentrations of this compound (Figure 2b), suggesting that a competitive mode of inhibition is a reasonable interpretation of the data. The K_i value determined from the K_m^{app} values calculated from these constrained plots (1.22, 2.13, 2.53 and 3.09 μM at 0, 0.4, 0.8 and 1.2 μM of 7-hydroxyflavone, respectively) was 0.9 μM.

Effects of flavonoids on the cellular uptake and hydrolysis of AEA by adherent cells
The ability of adherent RBL2H3 cells to hydrolyse AEA was measured by quantification of the water-soluble products following incubation with 100 nM [^{3}H]-AEA, labelled in the ethanolamine part of the molecule (Figure 3). Kaempferol, 3,7-dihydroxyflavone and 7-hydroxyflavone at concentrations $\geqslant$3 μM significantly inhibited the hydrolysis of AEA by the RBL2H3 cells, whereas apigenin had only minor effects (Figure 3).

The reduction of the rate of hydrolysis by the flavones can, in theory, be due to the inhibition of the cellular

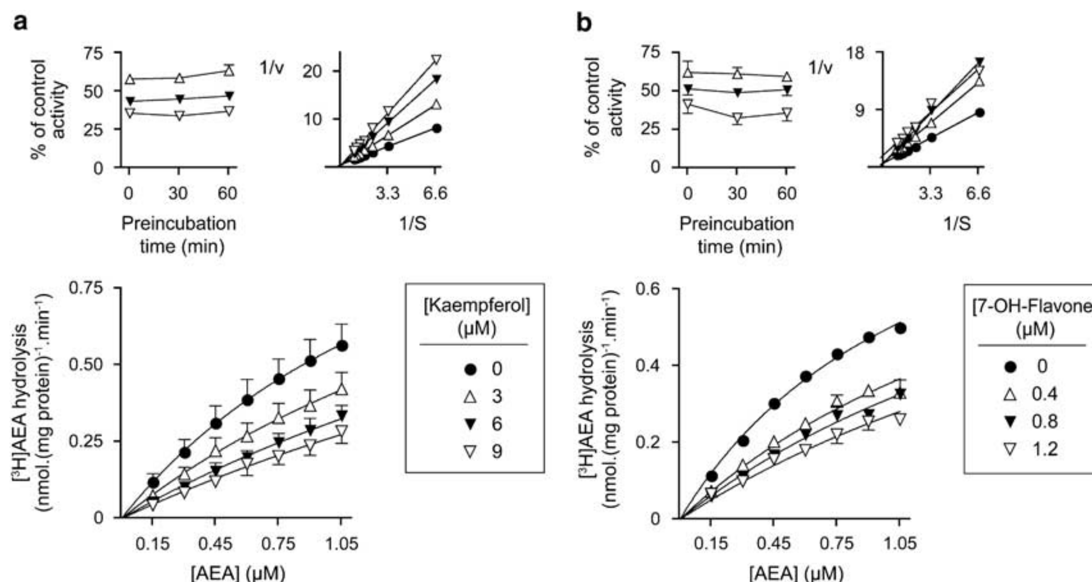

Figure 2 Inhibition of AEA hydrolysis by (**a**) kaempferol and (**b**) 7-hydroxyflavone. In the main graphs, the curves are those of best fit from analyses where V_{max} values were fixed at 1.512 and 1.112 nmol mg^{-1} protein min^{-1} for **a** and **b**, respectively. In the insets to the left of each panel, the values are percentage control for 0.5 μM AEA hydrolysis, following preincubation periods for the times shown. In the insets on the right, the mean data from the main graphs are replotted as double-reciprocal plots to show the competitive nature of the inhibition. Results are means (± s.e.mean, where appropriate and when not enclosed by the symbols), n = 3. AEA, anandamide.

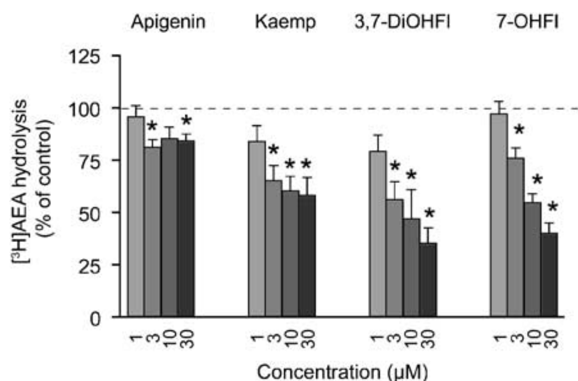

Figure 3 Inhibition by apigenin, kaempferol (kaemp), 3,7-dihydroxyflavone (3,7-DiOHFl) and 7-hydroxyflavone (7-OHFl) of the hydrolysis of 100 nM AEA by adherent RBL2H3 cells. The compounds were dissolved in dimethylsulphoxide with the exception of 7-hydroxyflavone (ethanol). Shown are means and s.e.mean, $n = 4$–5. *Indicates values where both lower and higher confidence limits were <100% (in the other cases, the values straddled 100%). AEA, anandamide.

accumulation of the compounds, a direct action on FAAH following uptake, or an effect on both these components. In order to investigate this further, the effects of 7-hydroxyflavone, 3,7-dihydroxyflavone and kaempferol on the uptake of 100 nM [^{3}H]-AEA (labelled in the arachidonate part of the molecule) were assessed in adherent RBL2H3 basophilic leukaemia cells (where the uptake is 'driven' to a large extent by FAAH) and PC3 prostate carcinoma cells (where FAAH does not contribute to the uptake) (Day *et al.*, 2001; Ruiz-Llorente *et al.*, 2004; Kaczocha *et al.*, 2006; Thors *et al.*, 2007a, b). For comparative purposes, OMDM-1, which inhibits the cellular accumulation of AEA (Ortar *et al.*, 2003), was also tested. In the RBL2H3 cells, all four compounds reduced the uptake (Figure 4). The reduction in uptake was in no case greater than that seen with the FAAH inhibitor URB597 (0.1 μM), and the combination of OMDM-1 and URB597 did not produce a greater inhibition than seen with either compound *per se*. Given that different authors find different relative potencies of uptake inhibitors relative to their direct effects on FAAH (see Ortar *et al.*, 2003; Fowler *et al.*, 2004; Hillard *et al.*, 2007), the precise point(s) of inhibition of AEA uptake produced by OMDM-1 in the RBL2H3 cells cannot be pinpointed, other than being along the uptake–hydrolysis pathway. In the PC3 cells, on the other hand, none of the compounds produced a significant reduction in AEA uptake, although a very small decrease was seen with the combination of URB597 and OMDM-1 (Figure 4). OMDM-1 also produced a small, but significant, reduction in the retention of AEA by wells alone, consistent with previous data (Fowler *et al.*, 2004). The simplest explanation for these data is that the effects of the flavonoids on the accumulation of AEA are a result of their effects on FAAH rather than on the uptake process itself.

Effects of flavonoids on the processing of AEA metabolites

McFarland *et al.* (2004) reported that in RBL2H3 cells incubated with AEA, the products of FAAH catalysis (arachidonic acid and ethanolamine) were enriched in the

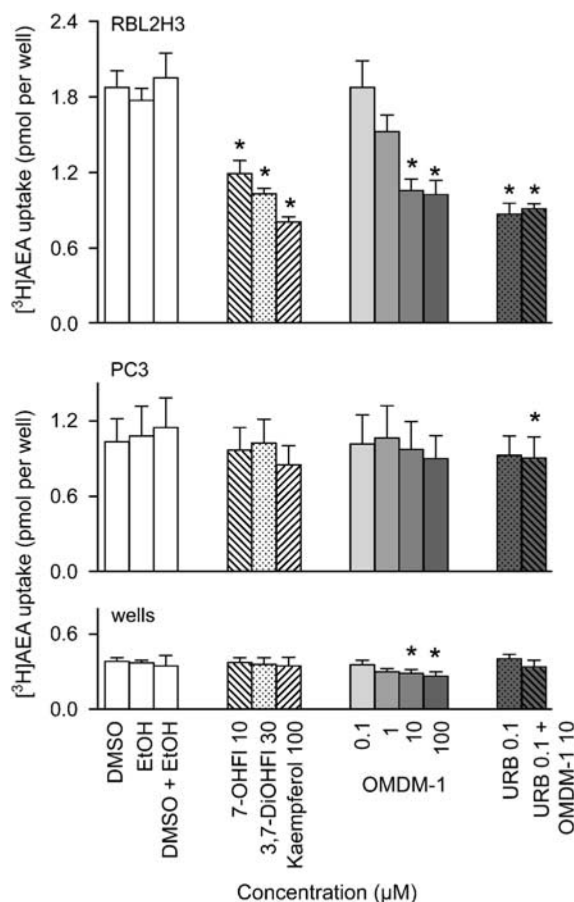

Figure 4 Uptake of 100 nM [^{3}H]-AEA by RBL2H3 cells, PC3 cells and retention by wells alone. Test compounds (in dimethylsulphoxide except for OMDM-1, which was dissolved in ethanol before dilution with assay buffer) were preincubated with the cells (or wells) for 10 min at 37 °C before the addition of AEA and incubation for a further 5 min. Shown are means ± s.e.mean, $n = 4$. *$P < 0.05$ vs the corresponding vehicle treatment, two-tailed paired *t*-test (flavonoids, URB597) or Dunnett's multiple comparison test following significant one-way ANOVA for repeated measures (OMDM-1). AEA, anandamide.

lipid raft regions of the cell membrane. By measuring the accumulation of tritium label in RBL2H3 membrane fractions after incubation of intact cells in suspension with 100 nM [^{3}H]-AEA (labelled in the arachidonate part of the molecule), a simple indirect measure of FAAH activity in the cells can be obtained. Consistent with this contention, the accumulation of tritium by the RBL2H3 cells was time-dependent and was greatly inhibited by URB597 (Figure 5a), but was not significantly affected by the CB_1 and CB_2 receptor inverse agonists AM251 (1 μM) and AM630 (1 μM), respectively (data not shown). PC3 cells, with low expression of FAAH, showed no time-dependent accumulation, and URB597 was without effect (Figure 5a). Thus, URB597-sensitive tritium accumulation by the membranes is a measure of the FAAH-dependent processing of AEA by the cells. In Figure 5b, the effects of apigenin, kaempferol, 3,7-dihydroxyflavone and 7-hydroxyflavone on URB597-sensitive accumulation of tritium in RBL2H3 cell membranes are shown following incubation of the intact cells with 100 nM [^{3}H]-AEA. All four compounds inhibited URB597-

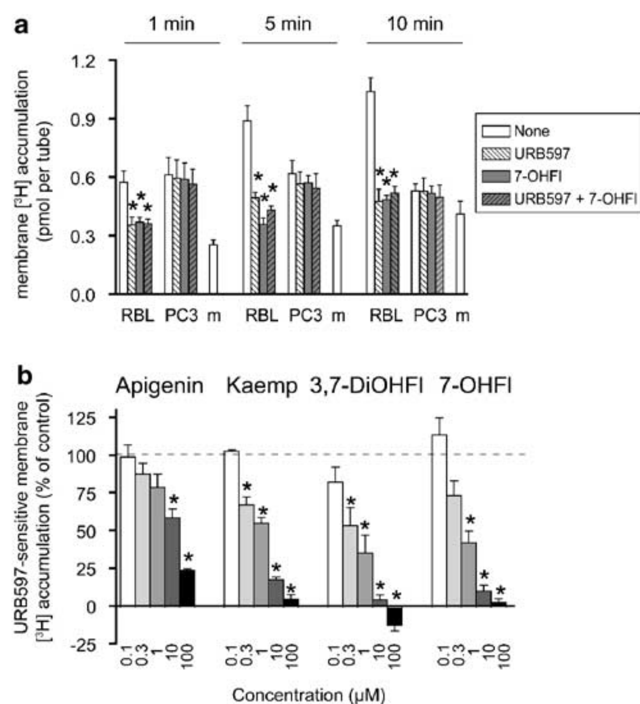

Figure 5 Accumulation of tritium label in RBL2H3 ('RBL') and PC3 cell membranes following incubation of 100 nM [^{3}H]-AEA with intact cells in suspension. (**a**) Effects of URB597 (1 μM), 7-hydroxyflavone (7-OHFl, 30 μM) and the combination of the two compounds on membrane tritium labelling. The cells were preincubated with the compounds or vehicle, as appropriate, for 10 min at 37 °C before the addition of AEA and incubated for the times shown; 'm' indicates data for assay medium alone in the absence of cells. Shown are means ± s.e.mean, $n=4$. *$P<0.05$ vs the corresponding vehicle treatment, Tukey's multiple comparisons test following significant one-way ANOVA for repeated measures performed for the incubation time point and cell line shown. In no case were the 7-hydroxyflavone, URB597 and the 7-hydroxyflavone + URB597 values significantly different from each other. (**b**) Inhibition by apigenin, kaempferol (kaemp), 3,7-dihydroxyflavone (3,7-DiOHFl) and 7-OHFl of the URB597 (100 nM)-sensitive labelling of RBL2H3 cell membranes. The compounds were dissolved in dimethylsulphoxide, with the exception of 7-hydroxyflavone (ethanol), before dilution with assay buffer. Shown are means and s.e.mean, $n=4$–5. *Indicates values where both lower and higher confidence limits were <100% (in the other cases, the values straddled 100%).

sensitive tritium accumulation. The IC_{50} values calculated from the data shown in Figure 5b were 15, 1.3, 0.8 and 0.4 μM for apigenin, kaempferol, 3,7-dihydroxyflavone and 7-hydroxyflavone, respectively. The effects of 7-hydroxyflavone and URB597 on the total accumulation of tritium were not additive (Figure 5a). It can be noted that the rank order of potency of the compounds was the same as for the cell-free homogenates. Thus, it can be concluded that kaempferol, 3,7-dihydroxyflavone and 7-hydroxyflavone inhibit FAAH in both intact cells and cell-free preparations at low micromolar concentrations, whereas apigenin is less active in this regard.

Discussion

The present study was instigated by the finding that the isoflavones genistein and daidzein were quite potent competitive inhibitors of FAAH. The finding that this property is shared by several flavonoids adds to the multitude of biological effects that these natural products possess (Havsteen, 2002). The concentrations required for inhibition of FAAH are generally in the micromolar range, which is, by any standard, modest, particularly when compared either with the most potent FAAH inhibitors available, or with the potencies of the flavones toward oestrogen receptors (Kuiper *et al*., 1998). However, the potencies are well in line with those required for other *in vitro* flavonoid biological actions. Thus, the concentrations of kaempferol required to inhibit AEA hydrolysis in both homogenates and intact cells are similar to those required for antioxidant effects (Furusawa *et al*., 2005), inhibition of EGF-receptor intrinsic tyrosine kinase and PKC (Agullo *et al*., 1997), inhibition of 20α-hydroxysteroid dehydrogenase (Brožic *et al*., 2006), inhibition of interleukin-4-induced STAT6 activation (Cortes *et al*., 2007) and activation of COX-2 (Liang *et al*., 2001). Thus, FAAH inhibition should be included among the *in vitro* properties of the flavonoids.

For the 20 compounds tested, there appear to be some structural requirements for FAAH inhibition, of which a hydroxy substituent at the position 7 of the benzopyran-4-one ring is probably the most important. In terms of drug design based on such compounds, the 7-hydroxy- and 3,7-dihydroxyflavones may be useful starting points for synthesis of compounds that are more potent FAAH inhibitors, while retaining other potentially useful biological properties, such as activation of PPARγ (Liang *et al*., 2001). Such compounds could, in theory, be useful for the treatment of inflammatory disorders and cancers, given that both components have potential in this regard (Bifulco *et al*., 2004; Holt *et al*., 2005; D'Argenio *et al*., 2006; Jayamanne *et al*., 2006; Moraes *et al*., 2006; Wang *et al*., 2006; Izzo *et al*., 2008).

With respect to the effects of the flavonoids on AEA uptake, metabolism and processing of metabolites, two observations are worth commenting on. Firstly, with respect to the effects on FAAH, higher concentrations are required for the adherent cells than for the cell-free homogenates. This pattern has been seen with other FAAH inhibitors (Bisogno *et al*., 1998) and presumably indicates that the cell membrane is a permeability barrier to the compounds. However, the compounds were rather efficaceous in preventing the FAAH-dependent accumulation of tritium in the membranes following incubation of RBL2H3 cells in suspension with AEA. The simplest explanation for this finding, given that the rank order of potencies were the same as for the cell-free homogenates, is that the cells in suspension, although viable (as checked using trypan blue), are more permeable to the flavonoids than when they are adherent.

Although this investigation has primarily been concerned with the structural requirements for the inhibition of FAAH by flavonoids, the nature of the compounds begs the question as to whether dietary flavonoid intake is sufficient to inhibit FAAH *in vivo*. At the outset, it should be pointed out that extrapolation of *in vitro* data, such as reported here, to the situation in man is difficult, to put it mildly, but 'ballpark' estimates can be considered. In plants, flavones are often, but not exclusively, present as glycosides, but

aglycones are produced after ingestion. The two most potent (with respect to FAAH inhibition) compounds were 7-hydroxyflavone and 3,7-dihydroxyflavone, but these compounds, although naturally occurring (in *Dracaena cochinchinensis*, *Clerodendron phlomoidis* and *Platymiscium praecox* Mart., found in China, India and Brazil, respectively; Braga De Oliveira *et al*., 1972; Roy and Pandey, 1994; Tu *et al*., 2003) cannot be described as commonly occurring compounds. The mean dietary intake of kaempferol by adults is $\sim 5\,\mathrm{mg\,day^{-1}}$, whereas that of quercetin is $\sim 16\,\mathrm{mg\,day^{-1}}$; intakes of apigenin, myricetin, fisetin and luteolin are much lower, although there is naturally a large inter-individual variation (Arai *et al*., 2000; Sampson *et al*., 2002; Johannot and Somerset, 2006). Following intake of a bowl of endive soup, containing 8.65 mg of kaempferol equivalent, a mean peak plasma kaempferol concentration of $\sim 0.1\,\mu\mathrm{M}$ was found for eight healthy subjects (DuPont *et al*., 2004). In another study, the plasma concentrations of kaempferol and quercetin following ingestion of concentrated black tea (providing 27 and 49 mg of these flavonoids, respectively) were found to be 15 and $29\,\mu\mathrm{g\,L^{-1}}$, corresponding to ~ 0.05 and $\sim 0.1\,\mu\mathrm{M}$, respectively (de Vries *et al*., 1998). These values for kaempferol are considerably lower than the concentrations needed for inhibition of FAAH activity in either cell-free homogenates or intact cells, and suggests that inhibition of FAAH following ingestion of dietary flavonoids is unlikely. Inhibition of FAAH may, however, occur following localized exposure to flavones. Tobacco leaves contain kaempferol glycoside (Pang *et al*., 2007), raising the possibility that a local inhibition of FAAH in the lungs occurs after cigarette consumption. Given that AEA, which is often produced at high levels following cellular damage (Kondo *et al*., 1998; Hansen *et al*., 2001), induces cough (Jia *et al*., 2002), a high local concentration of kaempferol would hardly be beneficial to smokers.

A separate question is whether inhibition of FAAH contributes to the pharmacological actions of flavonoids in experimental animals. As an example, kaempferol and quercetin (administered orally at a dose of $50\,\mathrm{mg\,kg^{-1}}$ as glycosides) produce antinociceptive effects in a model of visceral pain (acetic acid writhing) and anti-inflammatory effects in a carrageenan model (Toker *et al*., 2004). Such effects are also seen with URB597 (Holt *et al*., 2005; Naidu and Lichtman, 2007), so it is at least theoretically possible that inhibition of peripheral FAAH can contribute to such actions. However, the fact that kaempferol and quercetin glycosides were equally effective in the models at the dose given (Toker *et al*., 2004) would argue against a contribution mediated by FAAH. A definitive answer requires the measurement of local AEA levels in the tissues.

In conclusion, the present study has demonstrated that flavonoids inhibit FAAH at the concentrations used (with the notable exception of oestrogen receptors) to demonstrate other biological effects *in vitro*, with kaempferol being the most potent of the common naturally occurring compounds. Although it is unlikely that dietary ingestion of flavonoids will result in FAAH inhibition, the compounds may be useful templates for synthetic strategies for the discovery of novel anti-inflammatory and chemotherapeutic agents.

Acknowledgements

We thank Britt Jacobsson and Ingrid Persson for their expert technical assistance in the FAAH screening and adherent RBL2H3 AEA hydrolysis assays, respectively. The study was supported by grants from the Swedish Research Council (Grant no. 12158, medicine) and the Research Funds of the Medical Faculty (Umeå University). MB is a recipient of a grant from the EU Leonardo da Vinci programme.

Conflict of interest

The authors state no conflict of interest.

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