Ketoconazole Inhibits the Cellular Uptake of Anandamide via Inhibition of FAAH at Pharmacologically Relevant Concentrations

Emmelie Björklund, Therése N. L. Larsson, Stig O. P Jacobsson, Christopher J. Fowler*

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

Abstract

**Background:** The antifungal compound ketoconazole has, in addition to its ability to interfere with fungal ergosterol synthesis, effects upon other enzymes including human CYP3A4, CYP17, lipoxygenase and thromboxane synthetase. In the present study, we have investigated whether ketoconazole affects the cellular uptake and hydrolysis of the endogenous cannabinoid receptor ligand anandamide (AEA).

**Methodology/Principal Findings:** The effects of ketoconazole upon endocannabinoid uptake were investigated using HepG2, CaCo2, PC-3 and C6 cell lines. Fatty acid amide hydrolase (FAAH) activity was measured in HepG2 cell lysates and in intact C6 cells. Ketoconazole inhibited the uptake of AEA by HepG2 cells and CaCo2 cells with IC50 values of 17 and 18 μM, respectively. In contrast, it had modest effects upon AEA uptake in PC-3 cells, which have a low expression of FAAH. In cell-free HepG2 lysates, ketoconazole inhibited FAAH activity with an IC50 value (for the inhibitable component) of 34 μM.

**Conclusions/Significance:** The present study indicates that ketoconazole can inhibit the cellular uptake of AEA at pharmacologically relevant concentrations, primarily due to its effects upon FAAH. Ketoconazole may be useful as a template for the design of dual-action FAAH/CYP17 inhibitors as a novel strategy for the treatment of prostate cancer.

Introduction

The endocannabinoid system, comprising the cannabinoid CB receptors, their endogenous ligands arachidonoyl ethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), and their synthetic and metabolic enzymes, are involved in a variety of regulatory pathways including the control of pain, appetite, reproduction, bone turnover and control of cancer [1–5]. AEA is removed from the extracellular space by a process of cellular uptake followed by enzymatic metabolism, primarily to arachidonic acid via the hydrolytic enzyme fatty acid amide hydrolase. The mechanism(s) whereby AEA crosses the plasma membrane are a matter of controversy [6], but once within the cell, a variety of carrier proteins (fatty acid binding proteins, heat shock protein 70 and albumin) transport this lipid either to sequestration sites, to intracellularly located binding sites on receptors, and/or to the catabolic enzymes [7,8]. A fatty acid amide hydrolase [FAAH]-like transporter protein has also been suggested to act as an AEA transporter protein [9] but this has been disputed recently in this journal [10].

The main catabolic enzyme for AEA is the enzyme FAAH, which hydrolyses the endocannabinoid to give arachidonic acid and ethanolamine [11]. In addition to FAAH, AEA can act as a substrate for other enzymes, including cyclooxygenase-2 and lipoxygenases [12], and evidence is accruing to suggest that such pathways may have important pathophysiological relevance [13,14]. AEA is also metabolised by several members of the CYP enzyme family including CYP3A4 and CYP4F2. CYP3A4 in human liver microsomes, for example, is responsible for the production of a family of epoxyeicosatrienoic acid ethanolamides, and the 5,6- derivative is a potent agonist at cannabinoid-2 receptors [15,16].

Ketoconazole is a member of theazole family of antifungal reagents which exert their therapeutic effects by blocking fungal ergosterol synthesis via inhibition of sterol 14α-demethylase (CYP51) [17]. However, ketoconazole has additional effects upon other enzymes, including several human CYP isoforms (primarily CYP3A4, but also CYP17 involved in steroid biosynthesis [18,19]) and upon the activity of 5-lipoxygenase [20]. Given that AEA interacts with CYP3A4 and lipoxygenases, there is an overlap between the targets for this endocannabinoid and for ketoconazole, raising the possibility that ketoconazole may interact directly with the endocannabinoid system. In the present study, we demonstrate that ketoconazole can affect AEA uptake and hydrolysis at pharmacologically relevant concentrations.
**Methods**

**Compounds**

Ketoconazole, sulpaphenazole, quinidine and nefazodone were obtained from Sigma-Aldrich Inc, St Louis, MO, U.S.A. Non-radioactive AEA, URB597 (cyclohexylocarbamic acid 3′-carboxamoylphényl-3-yl ester) and [JZL184 (4-nitrophenyl-1-(4-dibenzoyl[d][1,3]dioxol-5-yl)(hydroxy)methyl)piperdine-1-carboxylate] were obtained from Cayman Chemical Co., Ann Arbor, MI, U.S.A. AM404 (X-(4-hydroxyphenyl)arachidonamide) and OMDM-2 ([9Z]-X-[1-((R)-4-Hydroxybenzyl)-2-hydroxyethyl]-9-octadecenamide) were obtained from Tocris Bioscience, Ellisville, MO, U.S.A. [Arachidonyl 5,6,8,9,11,12,14,15-3H]AEA (for FAAH experiments, specific activity 1.48 TBq mmol\(^{-1}\)), [palmitoyl 9,10-3H] palmitoylethanolamide ([3H]PEA, specific activity 2.2 TBq mmol\(^{-1}\)) and [glycerol-2,3,2,4-3H]2-AG (for hydrolysis experiments, specific activity 1.48 TBq mmol\(^{-1}\)) were obtained from American Radiolabeled Chemicals Inc., St Louis, MO, U.S.A.

**Cell cultures**

All cells were grown in 75 cm\(^2\) flasks at 37°C with 5% CO\(_2\) in humidified atmospheric pressure. Cells were split into new flasks once or twice per week. All culture media were supplemented with 1 IU mL\(^{-1}\) penicillin, 1 µg mL\(^{-1}\) streptomycin and 10% foetal bovine serum. CaCo-2 human epithelial colorectal adenocarcinoma cells (passage 31–97), originating from the American Type Culture Collection (Porton Down, UK) and cultured in William's E Medium Glutamax. CaCo-2 human epithelial colorectal adenocarcinoma cells (passage 104–124) were obtained from Health Protection Agency Culture Collections (Porton Down, UK) and grown in William's E Medium Glutamax. C6 glioma cells (passage range 18–22) obtained from ECACC Collection of Cell Cultures (ECACC; Porton Down, UK) were cultured in Ham's F-10 medium, 2% non-essential amino acids (NEAA), and 2 mM L-glutamine. SH-SY5Y human neuroblastoma cells (passage 28) obtained from European Collection of Cell Cultures (ECACC; Porton Down, UK) were cultured in Ham's F-10 medium, 2 mM L-glutamine, 10% foetal bovine serum and penicillin + streptomycin. Media and supplements were obtained from Invitrogen Life technologies (Stockholm, Sweden).

**Uptake assays**

The procedure was initially developed by Rakhshan et al. [21] and modified by Sandberg and Fowler [22]. On the day before the experiments, cells were plated in transparent 24-well plates at a density of 2×10\(^5\) cell per well in 400 µL of medium. After incubation over night in an incubator at 37°C and 5% CO\(_2\), cells were washed twice with 400 µL warm KRH-buffer (Krebs-Ringer HEPES buffer pH 7.4 in MiliQ-water; 120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl\(_2\), 10 mM HEPES, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\)) with 1% BSA first, then in the same manner without BSA. KRH-buffer (340 µL) with 0.1% fatty acid free BSA was added together with 10 µL of test compounds (ketonozazole, sulpaphenazole, quinidine, nefazodone or URB597 (cyclohexylcarbamic acid 3′-carboxamoylphényl-3-yl ester)) or vehicle. The concentration of solvent (DMSO or EtOH) was kept constant at ≤1% in all assays, also in the blanks. After 10 minutes of preincubation at 37°C, 50 µL of the tritiated substrate ([3H]PEA, labelled in the arachidonate part of the molecule, [3H]2-AG at a final concentration of 100 nM in KRH-buffer was added and the cells were incubated for 4 minutes at 37°C, unless otherwise shown. To stop the reaction, plates were placed on ice and washed three times with 500 µL KRH-buffer containing 1% BSA. Finally 500 µL 0.2 M NaOH was added and plates were incubated at 75°C for 15 minutes to solubilise cells. Following cooling to room temperature, 300 µL from each well was transferred to a scintillation vial and 4 ml of scintillation liquid was added. The tritium content was then measured by scintillation spectroscopy with quench correction. In a typical experiment where ~30000 d.p.m. of ligand is added to the wells, the total recovery of tritium after an incubation time of 5 min is roughly 1000–2000 d.p.m. for experiments with cells (depending of course upon the cell type used), whilst the recovery is in the region of 100 d.p.m. is found for wells alone.

**FAAH activity measurements**

For the cell-free assays, cells were washed twice with 10 ml of cold PBS (phosphate-buffered saline), followed by the addition of 5 ml PBS and the flasks were placed on ice. The cells were detached by using a rubber policeman and collected to a 15 ml tube, and thereafter 5 ml PBS was used to rinse the flask. The cells were centrifuged for 5 min at 200 g at 4°C, and the pellet was resuspended in 1 ml 10 mM Tris-HCl buffer, pH 9. Aliquots were stored at −80°C until assayed for FAAH activity. On the day of experiment, the aliquots were thawed and the protein content was measured with bovine serum albumin used as standard [23]. The FAAH activity assay was performed essentially as described by Boldrup et al. [24]. Briefly, 175 µL of the protein solution containing 2.5 µg protein in 10 mM Tris-HCl buffer, pH 9, was added to each test tube. [3H]AEA (substrate labelled in the ethanolamine part of the molecule, 25 µL, final concentration 0.5 µM, in a buffer containing 1% w v\(^{-1}\) fatty acid free BSA and 10 mM Tris-HCl buffer pH 9) was added and incubated for 10 min in 37°C. Reactions were stopped by adding 80 µl of charcoal in 320 µl of 0.5 M HCl followed by vortex mixing, and the test tubes were placed on ice for 5 minutes, and then adjusted to room temperature for 30 min before centrifugation for 10 min. Aliquots (200 µl) of the supernatant were transferred into scintillation vials and 4 ml of scintillation liquid were added. The tritium content was then measured by scintillation spectroscopy with quench correction. Blank values were obtained using buffer in place of cell lysate.

For the FAAH assays in intact C6 glioma cells, the initial part of the assay was essentially the same as for the uptake assays, albeit with [3H]AEA labelled in the ethanolamine rather than the arachidonate part of the molecule. Following incubation for 10 min at 37°C, reactions were stopped by addition of 120 µl of charcoal in 480 µl of 0.5 M HCl and aliquots (600 µl) transferred to test tubes. The samples were then centrifuged and aliquots (200 µl) of the supernatant were collected and analysed as described above. Blank values were obtained in the absence of cells. The same assay was used for 2-AG hydrolysis, using [3H]2-AG labelled in the glycerol rather than the arachidonate part of the molecule.

**Statistical analyses**

One-way ANOVAs, pI\(_{50}\) and IC\(_{50}\) values were determined using the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA, USA). The pI\(_{50}\) and thereby IC\(_{50}\) values, were calculated using the built-in equation \(\log (\text{inhibitor})\) vs. response – variable slope (four parameters) from the data expressed as % of vehicle controls using top (i.e. uninhibited)
values of 100% and bottom (residual activity) values that were either set to zero or allowed to float. In cases where the residual activity was >0, the two models were compared using Akaike’s Informative Criteria, to determine which model was the most likely.

Results

Effect of ketoconazole upon the uptake of [3H]AEA by HepG2, CaCo2 and PC-3 cells

The ability of ketoconazole to affect the uptake of [3H]AEA by HepG2 and CaCo2-2 cells is shown in Fig. 1A and B. The compound concentration-dependently inhibited uptake by both cells with pIC50 values (with IC50 values in parentheses) of 4.77±0.09 (17 μM) and 4.73±0.08 (18 μM) for HepG2 and Caco2 cells, respectively. Ketoconazole was also found to inhibit the uptake of the related non-cannabinoid lipid [3H]PEA and of [3H]2-AG by HepG2 cells, with pIC50 values of 5.45±0.16 (IC50 value 3.5 μM) and 4.64±0.06 (IC50 value 23 μM), respectively (Fig. 2A and B). AEA binds avidly to wells, although the level of binding is very low relative to the levels of cellular uptake under the assay conditions used here. In the present study, for example, the tritium recovered after incubation of AEA with the CaCo2 cells for 4 min was ~12-fold higher than for the wells alone. However, the binding of AEA to the wells has been found to be inhibited by uptake inhibitors such as AM404 [25] and OMDM-2 [26] at concentrations similar to those needed to block cellular uptake [27,28]. The same was found to be true for ketoconazole, which inhibited the binding of AEA to wells with a pIC50 value of 4.09±0.19 (IC50 value 82 μM) (Fig. 1). The compound also inhibited the binding of [3H]PEA and [3H]2-AG to the wells over the same concentration range (Fig. 2).

The interaction of AEA with the culture wells and its inhibition by ketoconazole is a potential confounding factor in the interpretation of the data. In the uptake experiments, subtraction of well data from the cell data is not appropriate - the cells cover the wells and thus reduce the available well surface area for binding, see [29]. As pointed out above, the binding to wells in absolute terms is low compared with the cellular uptake under the conditions used here. Nonetheless, the ability of the compounds to reduce the retention of ligand by the wells is an issue because it indicates that the compound can affect non-specific associations of the endocannabinoids, and may do the same in cells. One way of circumventing this issue is to investigate the time-dependency of the uptake. The rapid and reversible non-specific association of AEA with cell plasma membranes and with wells will not change over time, since there is little depletion of the ligand in the medium. In contrast, the accumulation of label over time will reflect the true cellular uptake. We have previously shown in P19 mouse embryonic carcinoma cells that there is a linear relationship between the amount of [3H]AEA retained by the cells and the incubation time, measured between 45 s and 15 min. The slope of the lines reflect the rate of cellular uptake, and this was found to be saturable with respect to the AEA concentration (Km value 1 μM) [22]. A similar result was found for ND7/23 mouse neuroblastoma x rat dorsal root ganglion neuron hybrid cells [30]. In contrast, the extrapolated initial association of AEA with the cells at t=0 showed no saturability [22,30]. Thus, by following the accumulation of [3H]AEA over time and measuring the slope, effects of a compound upon the cellular uptake of AEA can be separated from its non-specific effects upon the association of the endocannabinoid with the available surface, be it cells or wells. Using this approach, we found that ketoconazole significantly reduced the rate of cellular accumulation of [3H]AEA by the HepG2 cells, as did the FAAH inhibitor URB597 [31] and OMDM-2 (Fig. 3A,B). For the ten experiments where slopes were determined (the six shown in Fig. 3, and four undertaken for ketoconazole and URB597, but not OMDM-2, using time points of 1, 4, 7 and 10 min), the slopes seen with ketoconazole, OMDM-2 and URB597 were 37% (29–46%), 68% (42–95%) and 36% (27–46%) (means and 95% confidence interval) of the corresponding vehicle controls, respectively. Under these conditions, the rate of increase of retention of [3H]AEA by the wells alone over time was extremely low. These data indicate that ketoconazole can produce both a non-specific effect upon the retention of [3H]AEA but also an inhibition of the cellular uptake of the ligand.

The above conclusion was reinforced by experiments in C6 rat glioma cells, where the effects of ketoconazole, and for comparative purposes the prototypical endocannabinoid reuptake inhibitor AM404 upon the uptake of AEA were compared with a functional measure of intracellular accumulation, namely the production of [3H]ethanolamine from the intracellurally FAAH-catalysed hydrolysis of [3H]AEA labelled in this part of the molecule. C6 glioma cells have a high expression of FAAH and are thus a useful system to study the hydrolysis of internalised AEA. Both compounds inhibited the production of [3H]ethanolamine over a broadly similar concentration range as required for inhibition of the uptake of [3H]AEA [labelled in the arachidonoyl part of the molecule] (Fig. 4A and B). The pIC50 values (with IC50 values in parentheses) for inhibition of uptake and hydrolysis, respectively, were: ketoconazole, 4.69±0.04 (20 μM) and 4.53±0.07 (29 μM; based on an inhibitable fraction of 54±7%); AM404, 4.93±0.09 (12 μM) and 5.35±0.07 (4.5 μM). In contrast, ketoconazole did not affect the hydrolysis of 2-AG over the concentration range tested (Fig. 4C).

In order to determine whether other compounds able to interact with CYP enzymes also affect [3H]AEA uptake, the CYP3A inhibitor nefazodone, the CYP2D6 inhibitor quinidine and the CYP2C9 inhibitor sulfaphenazole were investigated. Quinidine and sulfaphenazole had at best minor effects upon the cellular accumulation of AEA into HepG2 and CaCo2 cells. However, robust inhibition of the uptake of AEA into both HepG2 and Caco2 cells and of the association of AEA by the wells was seen with nefazodone (Fig. 1). The pIC50 values (with IC50 values in parentheses) for nefazodone were: HepG2 cells, 5.09±0.09 (9.7 μM); CaCo2 cells, 4.40±0.10 (40 μM); wells, 4.28±0.11 (52 μM). As with ketoconazole, nefazodone inhibited the production of ethanolamine following intracellular hydrolysis of AEA in C6 cells over a similar concentration range as required for inhibition of AEA uptake (Fig. 4). Nefazodone also inhibited the uptake of [3H]PEA by HepG2 cells, with a pIC50 value of 5.03±0.17 (IC50 value 9.3 μM), but, as with ketoconazole, inhibited the association of the ligand to wells over the same concentration range (data not shown).

Ketoconazole and nefazodone inhibit FAAH activity

In many cell types using the experimental protocol used here, the uptake of AEA is driven by the activity of FAAH, since this removes the intracellularly accumulated AEA and hence preserves its gradient across the cell plasma membrane [32,33]. This appears to be the case for the HepG2 and CaCo-2 cells, since URB597 produced a large reduction in the observed rate of AEA uptake in both cells (Fig. 1A,B, Fig. 3C). In the HepG2 cells, after a preincubation phase of 10 min with the test compounds and an incubation of 4 min with AEA, the inhibition produced by ketoconazole was not additive to that produced by URB597 (Fig. 5), suggesting that both compounds are acting along the same pathway. URB597 also reduced the uptake of PEA into the
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Figure 1. Inhibition of [3H]AEA uptake by ketoconazole, nefazodone, quinidine and sulphenazole. The panels show the data for A, HepG2 cells; B, CaCo2 cells; C, PC-3 cells and D, adsorption to wells alone. The cells (or wells) were preincubated with the compounds for 10 min prior to addition of [3H]AEA (assay concentration 100 nM) and incubation for a further 4 min. For comparative purposes, the effect of the selective FAAH inhibitor URB597 (''U'', 1 μM) is indicated. Shown are means ± s.e.m. (when not enclosed by the symbols), n = 3–9 except for 100 μM nefazodone in the HepG2 cells, where n = 2.

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Figure 2. Inhibition of A. [3H]PEA and B. [3H]2-AG uptake by ketoconazole. The panels show the data for the uptake into HepG2 cells (filled symbols) or adsorption by wells (open symbols). The cells (or wells) were preincubated with ketoconazole for 10 min prior to addition of [3H]PEA or [3H]2-AG as appropriate (assay concentration 100 nM) and incubation for a further 4 min. For comparative purposes, the effects of the selective FAAH inhibitor URB597 (''U'', 1 μM) and (for 2-AG) the selective MGL inhibitor JZL184 (''J'', 100 μM) are indicated. Shown are means ± s.e.m. (when not enclosed by the symbols), n = 3.

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HepG2 cells, whereas the uptake of 2-AG was not affected by either this compound or by the MGL-selective inhibitor JZL184 [34] (Fig. 2B).

One explanation for the above findings is that ketoconazole (and nefazodone) affect AEA uptake in the cells due to a direct action upon FAAH. If this is the case, the compounds should be poor inhibitors of AEA uptake into cells lacking FAAH. Human PC-3 prostate cancer cells have a very low level of FAAH activity [35,36] and are thus useful in this respect. Both compounds, as well as URB597, produced only modest inhibition of AEA uptake into PC-3 cells (Fig. 1C).

In order to investigate the direct interaction of ketoconazole and nefazodone with FAAH, we measured FAAH activity in HepG2 and Caco2 cell lysates. Lysates from both HepG2 and Caco2 cell

Figure 3. Time course of the effects of ketoconazole and URB597 upon the accumulation of [3H]AEA by HepG2 cells. In Panels A and C, the individual values at each time point are shown (means ± s.e.m., unless enclosed by the symbols, n = 6). For both Panels, two-way ANOVA with repeated time measures for the data with the cells gave a significant (P<0.05) interaction time x treatment. Regression lines for each experiment were determined and the slopes, i.e. the rates of uptake are shown in Panels B and D as means ± s.e.m., n = 6. The vehicles used were A, ethanol; B, DMSO. *P<0.05, **P<0.01 vs. vehicle, either using Dunnett’s multiple comparisons test following significant one-way repeated ANOVA not assuming sphericity for the three conditions with cells (Panel C) or for a two-tailed paired t-test for the two conditions with cells (Panel D).

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Figure 4. Effects of ketoconazole, nefazodone, AM404 and URB597 (‘U’, 1 μM) upon A, the uptake of [3H]AEA; B, the hydrolysis of [3H]AEA and C, the hydrolysis of [3H]2-AG by C6 glioma cells. The compounds were preincubated with the cells for 10 min prior to addition of AEA or 2-AG (100 nM final concentration) and incubation for a further 10 min at 37°C. For the uptake experiments, the tritium label was on the arachidonoyl side chain, whilst for the hydrolysis experiments, the label was on the ethanolamine group (AEA) or the glycerol group (2-AG). Shown are means ± s.e.m. (when not enclosed by the symbols), n = 3, except for ketoconazole in Panel B, where n = 4.

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lines were able to hydrolyse AEA at rates not dissimilar to those seen with human SH-SY5Y neuroblastoma cells and rat C6 glioma cells (Fig. 6A). Using the HepG2 lysates, both ketoconazole and nefazodone were found to inhibit AEA hydrolysis (Fig. 6B). For nefazodone, the $pK_{50}$ value was $4.39 \pm 0.04$, corresponding to an IC$_{50}$ value of $41 \mu M$. For ketoconazole, the inhibition curve of best fit produced a maximum inhibition of $57 \pm 6\%$, with a $pK_{50}$ value for the inhibitable component of $4.47 \pm 0.09$ (IC$_{50}$ value $34 \mu M$). The lack of a complete inhibition of FAAH in the assay system used is a common phenomenon with lipophilic compounds in our hands (see e.g. [37]) and may represent a solubility issue, although it should be noted that a similar incomplete inhibition of AEA hydrolysis by intact C6 cells was found (Fig. 4B), whereas the inhibition of uptake was complete (Fig. 4A). The $pK_{50}$ values for the inhibitable component of the FAAH activity seen in the lysates are reasonably in line with the $pK_{50}$ values for the effects of the compounds upon AEA uptake in the intact cells.

**Discussion**

In the present study we have demonstrated that ketoconazole inhibits the uptake of the endocannabinoid AEA into a variety of cell lines, and that this effect can be most simply explained by the ability of the compound to inhibit FAAH. At first sight, the potency of the compound as an inhibitor of AEA uptake (IC$_{50}$ value $\sim 20 \mu M$ for the FAAH-containing cells) is modest compared with the nanomolar affinity of the compound towards CYP51, CYP3A4 and CYP17 [17–19], and so might be considered a “pharmacological curiosity” without additional relevance. However, the potency of the compound towards AEA uptake is very similar to that seen for inhibition of 5-lipoxygenase, in cell-free preparations (28 $\mu M$) [20]. Thromboxane synthase is also inhibited in vitro by ketoconazole (IC$_{50}$ value $40 \mu M$ [20,38]). In intact rat peritoneal polymorphonuclear leukocytes, leukotriene B4 and 5-hydroxyeicosatetraenoic acid production from arachidonic acid was inhibited with IC$_{50}$ values of 30 and 26 $\mu M$, respectively [20]. These authors demonstrated further that oral pretreatment with ketoconazole (10–40 mg/kg) inhibited in a dose-dependent manner ovalbumin-induced bronchoconstriction in sensitised guinea pigs, suggesting that leukotriene synthesis could be inhibited in vivo by the compound [20]. After oral administration of 400 mg of ketoconazole to volunteers, plasma levels of thromboxane B2 were unchanged with respect to placebo treatment. However, following ischaemia to the arm induced by a 10 min cuff, the increase in thromboxane B2 levels found after placebo was inhibited in the ketoconazole group [39]. In Sweden, ketoconazole is available as a shampoo (20 mg/ml) and until recently as tablets (200 mg; the dose could be doubled if deemed necessary), and in HIV-healthy volunteers, a C$_{max}$ value of 5.3 $\mu g/ml$ was found following 6 days of treatment with the 200 mg dose [40]. This corresponds to a plasma concentration of about 10 $\mu M$. Of course, this number does not take into account the considerable plasma protein binding of ketoconazole, but the uptake and FAAH experiments are also undertaken in the presence of serum albumin and so are comparable. Taken together, these data are consistent with the suggestion that ketoconazole affects AEA reuptake in pharmacologically relevant concentrations.

**Figure 5. The effect of combinations of ketoconazole and URB597 upon the uptake of [3H]AEA into HepG2 cells.** The cells were preincubated with the compounds for 10 min prior to addition of [3H]AEA (assay concentration 100 nM) and incubation for a further 4 min. Shown are means ± s.e.m., n = 3.

**Figure 6. The effects of ketoconazole and nefazodone upon FAAH activity.** Panel A. Hydrolysis of [3H]AEA by lysates of HepG2 and CaCo2 cells. Shown are means ± s.e.m., n = 3. Concurrent data for human SH-SY5Y neuroblastoma and rat C6 glioma cells are shown for comparison. Panel B. Inhibition of the hydrolysis of [3H]AEA in lysates of HepG2 cells by ketoconazole and nefazodone. Shown are means ± s.e.m. (unless enclosed by the symbols), n = 3.
Ketoconazole can also affect PEA and 2-AG uptake at similar concentrations to those affecting AEA uptake. Whilst the effects upon PEA uptake are to be expected for a compound with FAAH-inhibitory properties [41] (see Fig. 2A), the effects upon 2-AG are more unexpected, since blockade of 2-AG hydrolysis does not reduce the intracellular accumulation of this endocannabinoid [42–44], a result also found here. However, AEA can inhibit 2-AG uptake [42,44] suggesting communalities in their transport mechanisms, and so it is possible that an action of ketoconazole upon, for example, a fatty acid binding protein [7] may be involved in its effects upon 2-AG uptake, assuming, of course, that the effects are not simply non-specific, such as are seen in our experiments with the wells alone. An interaction with fatty acid binding proteins should be considered, given that 100 μM ketoconazole can compete with the arachidonoyl derivative 20:4-acycloethanolamine for binding to a 47 kDa protein in cell membranes from U937 leukemia monocytoid lymphoma cells [45]. PC-3 cells express FABP5 (E-FABP) and, to a lesser extent, FABP6 (IL-FABP), but binding to a 47 kDa protein in cell membranes from U937 ketoconazole can compete with the arachidonoyl derivative 20:4-acycloethanolamine for binding to a 47 kDa protein in cell membranes from U937 leukemia monocytoid lymphoma cells [45]. Binding of arachidonic acid to fatty acid binding proteins should be considered, given that 100 μM ketoconazole can compete with the arachidonoyl derivative 20:4-acycloethanolamine for binding to a 47 kDa protein in cell membranes from U937 leukemia monocytoid lymphoma cells [45].

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