Dicinnamoylquinides in roasted coffee inhibit the human adenosine transporter

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Abstract

Preliminary screening of a minor, non-xanthine constituent of roasted coffee, 3,4-diferuloyl-1,5-quinolactone (DIFEQ), showed inhibition of the adenosine transporter at low micromolar concentration. DIFEQ is a neutral derivative of the chlorogenic acids, i.e. isomeric mono- and di-substituted coumaroyl-, caffeoyl-, and feruloyl-esters of quinic acid, formed in the roasting process of coffee. Displacement of the adenosine transporter antagonist [^{3}H](S)-(nitrobenzyl)-6-thioinosine binding by DIFEQ in cultured U-937 cell preparations, expressing the human adenosine transporter protein (hENT1), showed a K_i of 0.96 ± 0.13 μM. Extracts of regular and decaffeinated coffee showed binding activities equivalent to 30–40 mg DIFEQ per three cups of coffee. Acute administration of a high dose of DIFEQ (100 mg/kg i.p.) reduced open field locomotion in mice for 20 min in correlation with brain levels of DIFEQ. Both 3,4-dicaffeoyl-1,5-quinide and 3,4-dicoumaroyl-1,5-quinide, two close structural analogs of DIFEQ also present in roasted coffee, showed similar affinities for the adenosine transporter, while the corresponding 3- and 4-mono caffeoyl- and feruloyl-quinides were one to two orders of magnitudes less active. This suggests that 3,4-dicinnamoyl-1,5-quinides in coffee could have the potential to raise extra-cellular adenosine levels, thereby counteracting the stimulant effect of caffeine. © 2002 Published by Elsevier Science B.V.

Keywords: Coffee; Quinide; Adenosine transporter; Locomotion; (Mouse)

1. Introduction

The main constituents of coffee have been known for almost half a century (Clifford, 1975; Herrmann, 1989), and particularly caffeine has been the subject of extensive studies. The stimulant effect of coffee is attributed to the pharmacological activity of caffeine, acting as an antagonist at adenosine receptors in brain (Fredholm et al., 1999). Although a central stimulant drug, caffeine is not generally considered to have abuse potential (Daly and Fredholm, 1998), because pure caffeine elicits a dose-dependent, subjective feeling of anxiety, even at low doses (Kaplan et al., 1997). Beverages made from roasted coffee, on the other hand, are able to elicit a feeling of well being that seems to increase with the strength of the brew, but not with its caffeine content (Quinlan et al., 2000). We and others have postulated that brewed and instant coffees contain pharmacologically active compounds, other than caffeine, in concentrations sufficient to cause significant effects with normal consumption of coffee. Kalsner (1977) reported that decaffeinated coffee contains a vasoconstrictive substance, later proposed but never identified as a muscarinic acetylcholine receptor agonist (Tse, 1991, 1992). Voto and Schaal (1984) reported that regular and decaffeinated coffees produce bradycardia in human subjects, an effect that was not due to caffeine.

We suggest that pharmacologically active compounds may be found among derivatives of the chlorogenic acids. The chlorogenic acids, named after their green color reaction with ferrous chloride and exposure to air, are common to most plants (Clifford, 1975). Due to their relatively high abundance in coffee, these agents must be seriously considered when elucidating potential pharmacological effects of coffee intake. Green coffee beans can contain as much as 10% of dry weight of chlorogenic acids (Herrmann, 1989),

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i.e. five to eight times the average concentration of caffeine (Fredholm et al., 1999). Chlorogenic acids are mixtures of mono- and di-esters of various 4-hydroxycinnamic acids with the aliphatic alcohols of (−)-quinic acid, i.e. (R,R,S,R)-1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid, a sugar-like molecule (Clifford, 1975). Because both roasting and brewing of coffee cause extensive isomerisation of the quinic acids (Trugo and Macrae, 1984), no single such compound dominates in coffee, except for the most abundant 5-caffeoylquinic acid (formerly called 3-caffeoylquinic acid or chlorogenic acid), which constitutes 4–5% of green coffee beans (Herrmann, 1989).

The pharmacological effects of chlorogenic acids are mostly unknown. Recently, 5-caffeoylquinic acid was found to be a potent antioxidant agent in human erythrocytes (Lekse et al., 2001). Antioxidants in coffee protect against nitroso-induced genotoxicity in the mouse bone marrow model. In fact, the anti-genotoxic effect of 5-caffeoylquinic acid was synergistically enhanced when co-administered with brewed coffee (Abraham, 1996). 3,5-Dicaffeoylquinic acid is a potent inhibitor of human immunodeficiency virus (HIV-1) inte-grase, an enzyme required for infection with AIDS (Robinson et al., 1996). Brewed and instant coffee is unique in that the high temperature of the roasting process causes some chloro-genic acids in green coffee beans to lose a molecule of water to form an internal ester bond, thereby transforming up to half of the total amount into a mixture of quinolactones (quinides) that lack the carboxylic acid moiety (Hucke and Maier, 1985). Since the quinides are neutral compounds, they would be taken up in blood and brain even more readily than the original quinic acids (Olthof et al., 2001).

Boublik et al. (1983) reported that approximately one-fifth of the concentration contained in a cup of coffee displaced 50% of the binding of the opiate antagonist [^{3}H]naloxone. The molecular entity that caused this activity has yet to be determined, but mass spectrometry on an active HPLC fraction suggested an isomer of feruloylqui-nide (Wynne et al., 1987). No other pharmacological data on the quinides are available, except that these compounds being α-hydroxy acid derivatives were found to have anti-urease activity useful for treating dermatological conditions (Huynh-Ba et al., 1994; Yu and Van Scott, 1996). Because of stereochemical requirements, only chlorogenic acids that lack a cinnamoyl group in the 5-position can form γ-quinides, i.e. internal 1,5-lactones (Hanson, 1965). This excludes the most abundant 5-caffeoylquinic acid. 1-Cinna-moylquinic acids have never been found in coffee, leaving γ-quinides with cinnamoyl esters in the 3- or 4-positions as the only candidates. Di-cinnamoyl esters would be more likely than mono esters to have favorable properties for crossing the blood–brain barrier. For this reason, and for synthetic expediency, 3,4-diferuloyl-1,5-quinide (DIFEQ) was chosen as a suitable model compound to evaluate the potential psychopharmacological activities of such constit-uents of roasted coffee. We have synthesized DIFEQ from commercially available starting materials. Preliminary

screening (NovaScreen) revealed that DIFEQ exhibits low micromolar affinity for the human equilibrium-sensitive (es) adenosine transporter. We have therefore investigated the binding characteristics of DIFEQ, its mono- and di-caffeoyl and di-coumaroyl analogs, and coffee extracts at the human adenosine transporter protein, and the acute behavioral effects of DIFEQ in mice. A preliminary report of this study was presented at the 19th International Conference on Coffee Science, Trieste, Italy (Martin et al., in press).

2. Materials and methods

2.1. Drugs

A synthetic sample of DIFEQ was prepared in five steps from quinic acid and ferulic acid according to the methods of Wynne et al. (1986) as described by Huynh-Ba (1995). By using similar methods, 3-caffeoyl-, 3,4-dicaffeoyl- and 3,4-dicoumaroyl-1,5-quinides were also prepared (Huynh-Ba, 1995). 4-Caffeoyl-1,5-quinide was a gift from Dr. Huynh-Ba, Nestle. (S)-(4-Nitrobenzyl)-6-thioinosine (NBTI), 8-cyclopentyl-1,3-dipropylxanthine (CPX) and 2-[4-(2-car-boxyethyl)phenyl]ethylamino-5′-N-ethylcarboxamidoade-nosine (CGS-21680) were obtained from RBI, Natick, MA. The corresponding tritiated ligands were obtained from NEN, Boston, MA. Polyoxyethylene-sorbitan mono-oleate (Tween 80) and other solvents and reagents were obtained from Sigma-Aldrich, Milwaukee, WI.

2.2. Radioligand binding

Initial binding assays with DIFEQ to determine its affin-ities for adenosine receptors and transporter sites in brain were performed by NovaScreen (Hanover, MD). DIFEQ was dissolved in dimethylsulfoxide (DMSO) (10 mM) and diluted in appropriate buffer to 100 and 10 μM final con-centrations. Inhibition of [^{3}H]adenosine transport in homo-genates of human myeloid leukemia (U-937) cell culture, expressing the human (es) transporter, was performed according to Gu et al. (1993). Rate of transport was deter-mined in HEPES buffer at 37 °C for 30 min (with choline replacing sodium). Nonspecific transport was defined with 1 μM NBTI. Full displacement curves for the inhibition of the adenosine transporter were obtained by using eight concen-trations of each quinide, increasing by a factor of 2 and ranging from 0.25–32 μM. Nonspecific binding was defined with 10 μM NBTI. Affinity of the quinides for adenosine A1 receptors was determined by displacement of [^{3}H]CPX bind-ing according to Bruns et al. (1987) in Madin–Darby canine kidney (MDCK) cell culture, expressing the human adeno-sine A$_1$ receptor. Affinity of the quinides for adenosine A$_{2A}$ receptors was determined by displacement of [^{3}H]CGS-21680 binding according to Jarvis et al. (1989) in human embryonic HEK 293 cell culture, expressing the human adenosine A$_{2A}$ receptor.

Cell cultures (American Type Culture Collection, Manassas, VA) were grown in high glucose Dulbecco's modified Eagle's medium supplemented with NaHCO$_3$ (40 mM), 10% fetal bovine serum (Gibco), 400 mg/l geneticin, and 0.1 mM hypoxanthine. The cells were cultured at 37 °C under a CO$_2$/air (5:95), v/v in tissue culture flasks (75 cm^2, Falcon). When reaching confluence (70–80%), cells were trypsinized and harvested by centrifugation. Harvested cells were re-suspended in 50 mM Tris buffer at 4 °C (0.1 mg/ml), the cell membranes were disrupted by Polytron homogenization (12,500 rpm for 25 s), and the suspension was centrifuged at 30,000 $\times$ g for 60 min. The supernatant was discarded and the remaining pellet was re-suspended in 50 mM Tris buffer to 0.2–0.5 mg protein/ml and frozen at -80 °C until used. Protein content was measured by the Lowry method using bovine serum albumin as the standard. Inhibition of [^{3}H]NBTI binding in homogenates of U-937 cell culture, expressing the human *es* transporter was performed according to Marangos et al. (1982). The cell membranes in final concentration of 0.03 mg protein/ml were incubated with [^{3}H]NBTI at 2–4 nM concentration in 50 mM Tris buffer (pH 7.5) at 4 °C for 1.5 h in a total volume of 1.0 ml. Non-specific binding was defined with 10 μM NBTI. Each determination was carried out in triplicate. Bound and free [^{3}H]ligand were separated by vacuum filtration through fiberglass filters (Schleicher & Schuell, Keene, NH), pre-soaked with 0.3% polyethylenimine for 10 min, using a Brandel M-24R cell harvester. The filters were washed three times for 10 s with ice-cold 50 mM Tris buffer and placed in 10 ml scintillation fluid (Cytoscint, ICN). Beta spectrometry was performed using a Beckman L5801 instrument at 47% counting efficiency. IC$_{50}$ values and Hill slopes (n_H) were calculated by linear regression analysis of log–logit transformation of competition binding data. K_i values of the competing ligand were calculated from IC$_{50}$ values using the Cheng–Prusoff equation, $K_i = \mathrm{IC}_{50}/(1 + L/K_d)$, where L is the concentration of the [^{3}H]ligand and K_d is the equilibrium dissociation constant obtained from Scatchard analysis. Data are expressed as mean $\pm$ standard error of the mean (S.E.M.).

2.3. Preparation of coffee extract

Twenty-five grams of roasted Columbian coffee (*Coffea arabica*) was finely ground and suspended with stirring in 200 ml hot water at 75 °C. After 5 min, the pulp was removed by filtration. Ten grams of instant coffee (Taster's Choice) was dissolved in the same amount of hot water. The coffee solutions were chilled to room temperature with crushed ice (50 g), acidified with 12 N hydrochloric acid (1 ml) to pH 5, and extracted with ethyl acetate (2 $\times$ 200 ml). Foaming was suppressed by addition of ethanol (10 ml). The organic layers were combined and washed with water (2 $\times$ 100 ml), dried with anhydrous sodium sulfate (50 g), and the solvent was removed by evaporation to give an oily residue containing extractable quinides and caffeine (if present).

2.4. Pharmacokinetic studies

Measurements of DIFEQ in blood plasma and brain homogenate of mice were performed by high performance liquid chromatography (HPLC), using a modification of the method for caffeine (Biaggioni et al., 1988). A whole mouse brain was homogenized in 15 ml 0.1 M hydrochloric acid (Dounce, 20 strokes, tight pestel), 15 ml ethyl acetate was added and mixed by another 20 strokes, then centrifuged at 10,000 $\times$ g for 10 min at 4 °C (Sorvall Model RC-5B). The supernatant organic layer was separated and the solvent evaporated by a stream of air. A disposable 1 ml C18 cartridge (Alltech 204900) was pre-conditioned by flushing with 1 ml of methanol followed by 1 ml water. The residue was dissolved in 1.2 ml 50% dimethylsulfoxide, diluted with 4.8 ml water, and the mixture was added to the cartridge through a 0.45 μm syringe filter (Millex 32). The cartridge was washed with 2 $\times$ 1 ml of water. Elution with 0.2 ml 100% methanol and evaporation of the solvent by a stream of air (5 min) gave a semi-dry sample that was reconstituted with 0.1 ml HPLC buffer. Separation on a C18 reverse phase column (Alltech Hypersil BDS 3 μm) in 26% ethanol in 100 mM NaH$_2$PO$_4$ at pH 4.4 showed a retention time of 8.5 min at a flow rate of 1.5 ml/min. The UV peak was recorded at 310 nm wavelength on a Dynamax UV-1 instrument (Rainin). The corresponding di-desmethoxy analog, 3,4-dicoumaroyl-1,5-quinide (prepared from coumaric acid as described for DIFEQ), retention time 11.8 min, was used as internal standard to estimate recovery of DIFEQ. Ten microliter of a 0.1 mM solution of 3,4-dicoumaroyl-1,5-quinide (1 nmol) was added to each dissected brain before homogenization.

2.5. Behavioral studies

Twenty-four 8-month-old C57BL6/J mice (12 male and 12 female) were housed in groups of three to five animals per cage, separated by gender. The animals had free access to food and water and were on a 12 h light/dark cycle, with light on at 6 am. Each animal was used only once. The European Community guidelines for the use of animals were adhered to, and the protocol was approved by the Vanderbilt Animal Care Committee. Locomotor activity was measured using four identical open field activity monitors (Med Associates, Georgia, VT), measuring 27 $\times$ 27 cm and equipped with three vectors of infrared photo beam emitters and detectors spaced 1.5 cm apart. Horizontal locomotor activity was detected by two arrays of infrared beams positioned 1.8 cm above the floor of the open field arena. An additional vector was attached 5.7 cm above the floor of the arena to detect vertical activity (rearing). Activity was measured in four animals simultaneously and was expressed as the total distance traveled in cm or the total number of vertical rearings for each 5 min block of the 90 min session. All behavioral sessions were counter balanced with respect to dose, gender, and apparatus, and conducted during the

last 6 h of the light portion of the light–dark cycle. Mice were transported to the behavioral testing room at least 30 min before testing. Because of poor solubility of DIFEQ in water (<0.1 mg/ml), DIFEQ was dissolved in 50% Tween-80 in distilled water, diluted with water to 10% Tween-80, and sterilized using a 0.45-μm pore syringe filter (Millipore). Both DIFEQ and Tween-80 solutions were stored in the dark at 4 °C until time of use. After a single intraperitoneal injection of either drug (25, 50, 100, or 300 mg/kg) or vehicle in a total volume of 10 ml/kg, the animals were placed in their home cage for 10 min, then placed in the center of the open field arena, where activity was continuously monitored for 90 min. Scores for locomotor activity and rearing were analyzed using repeated measures analysis of variance (RMANOVA). Follow-up analyses were conducted using Bonferroni–Dunn statistics and two-group RMANOVA.

3. Results

3.1. Synthesis

Synthesis of 10 g DIFEQ in 31% overall yield was accomplished from 15 g quinic acid and 25 g ferulic acid. Since the protected quinide was esterified in both the available 3- and 4-positions, the condensation reaction did not require the low temperature conditions of Huynh-Ba (1995), but was performed at ambient temperature. Melting point of DIFEQ was 132–134 °C after crystallization from a mixture of ethyl acetate and diisopropyl ether (1:2). The ^{1}H and ^{13}C NMR spectra of DIFEQ in DMSO-d_6 were consistent with the structure of DIFEQ (Fig. 1). HPLC showed a single peak at 8.5 min with 98% chemical purity. Minor impurities ($<1\%$) were identified as the corresponding 3- and 4-monoferuloyl analogs. Synthesis of 2 g 3,4-dicaffeoyl-1,5-quinide in 24% overall yield was accomplished from 3 g quinic acid and 8 g caffeic acid as reported (Huynh-Ba, 1995). HPLC showed a major peak at 3.5 min with 86% chemical purity. Synthesis of 1 g 3,4-dicoumaroyl-1,5-quinide in 42% overall yield was accomplished from 1 g quinic acid and 2 g coumaric acid as reported (Huynh-Ba, 1995). HPLC showed a single peak at 12.3 min with 98% chemical purity. Gram quantities of 3-caffeoyl-, 1-
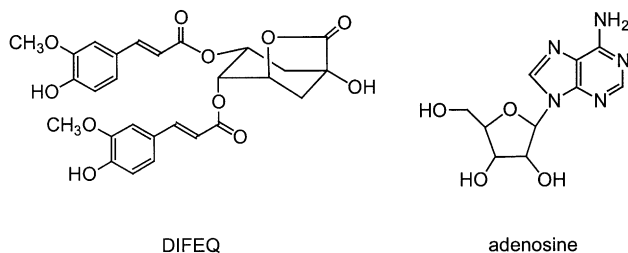
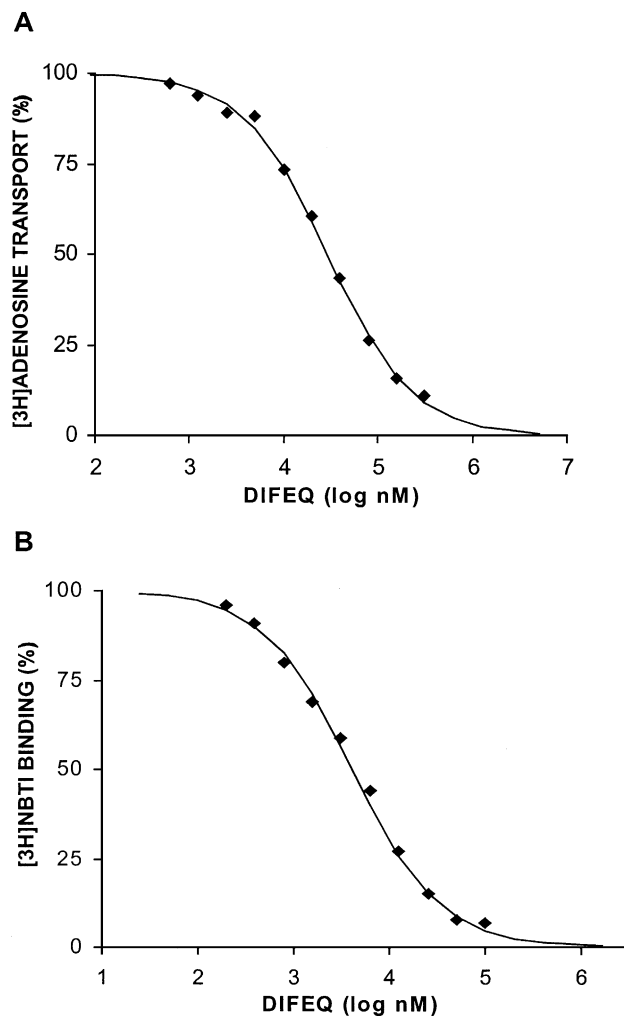Fig. 1. Structures of (1R,3R,4S,5R)-3,4-diferuloyl-1,5-quinide (DIFEQ) and adenosine.

Fig. 2. (A) Inhibition of 10 μM [^{3}H]adenosine transport and (B) inhibition of 0.6 nM [^{3}H]NBTI binding by DIFEQ in U937 cell preparations, expressing the human adenosine es transporter. Nonspecific transport and binding were defined by 1 and 10 μM NBTI, respectively (the former performed by NovaScreen).

feruloyl-, 3-feruloyl-, and 4-feruloyl-1,5-quinides were prepared by the appropriate modifications of the methods above (Huynh-Ba, 1995).

3.2. Radioligand binding

Preliminary screening using two concentrations of DIFEQ indicated that DIFEQ has low micromolar affinity for the adenosine A_1 receptor and for the rat adenosine transporter (data not shown). Displacement of [^{3}H]CPX binding by DIFEQ and 3,4-dicoumaroyl-1,5-quinide in membranes from MDCK cells expressing the human adenosine A1 receptor showed $K_i = 33 \pm 5$ μM ($n=4$) and 28 ± 1 μM ($n=2$), respectively. Extrapolation of the percent inhibition by DIFEQ of [^{3}H]CGS-21680 binding in HEK-293 cell homogenate indicated $K_i > 800$ μM for the adenosine A_{2A} receptor. Binding affinity of DIFEQ for the human adenosine transporter and inhibition of adenosine transport by
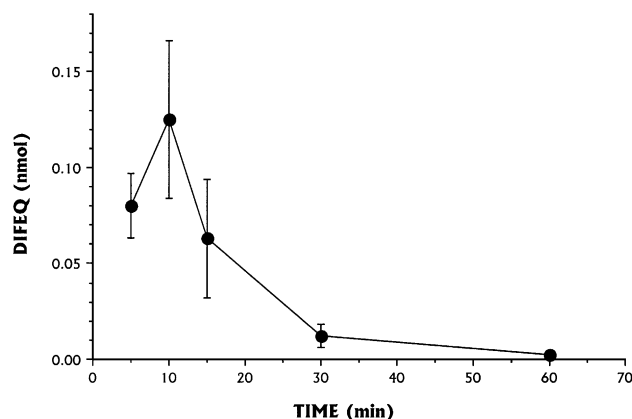
Fig. 3. Uptake and elimination of DIFEQ (100 mg/kg i.p.) in the mouse brain. The data are expressed as total DIFEQ (nmol) mean ± S.E.M. per brain from two to three animals at each time point.

DIFEQ are shown in Table 1 (also see Fig. 2). The adenosine transport assay showed $K_i = 1.3 \pm 0.4$ μM ($n = 2$). Displacement of [³H]NBTI binding at the human adenosine transporter protein by DIFEQ showed $K_i = 0.96 \pm 0.13$ μM and n_H of 0.94 ± 0.02. The affinities of the other mono- and dicinnamoylquinides for the adenosine transporter are shown in Table 2. Both 3,4-dicaffeoyl- and 3,4-dicoumaroyl-1,5-quinides are equally potent as DIFEQ. Mono substituted 3- or 4-cinnamoyl-1,5-quinides showed weak or no affinity. The lack of affinity of caffeine for the adenosine transporter ($K_i > 120$ μM, data not shown) was consistent with reported values (Marangos et al., 1982). DIFEQ did not show appreciable affinity for dopamine, serotonin, norepinephrine, choline, gamma-aminobutyric acid, or glutamate transporters ($K_i > 60$ μM).

3.3. Coffee extracts

Regular and decaffeinated coffee from 25 g whole beans gave 0.82 and 0.76 g extracts, respectively. Each extract was dissolved in methanol (100 mg/ml) and serial diluted with buffer and subjected to the binding assay for the adenosine transporter. Due to the dark color of stock solutions, scintillation at the higher concentrations was partly quenched, resulting in incomplete displacement curves. Inhibition of [³H]NBTI binding based on the linear range of the log–logit curves showed activities equivalent to $K_i = 12.6$, 12.4, and 15.6 mg/l for coffee extracts from regular whole beans and from instant regular and decaffeinated coffee, respectively.

Table 1
Inhibition of adenosine transport and displacement of [³H]NBTI binding by DIFEQ at the human adenosine transporter

Receptor	Tissue	Radioligand	Blocker	K_i (μM)	Slope (n_H)
Transport[a]	U-937 cell	[³H]adenosine	NBTI	1.28 ± 0.37	1.13 ± 0.05
Binding[b]	U-937 cell	[³H]NBTI	NBTI	0.96 ± 0.13	0.94 ± 0.02

[a] Average result of two experiments in duplicates (performed by NovaScreen).
[b] Average result of six experiments in triplicates.

Table 2
Affinities of mono- and di-cinnamoylquinides in roasted coffee for the human adenosine transporter

Quinide	Affinity (K_i μM)[a]	Hill slope (n_H)	n[b]
5-Caffeoylquinic acid[c]	>100		1
4-Caffeoyl-1,5-quinide	53 ± 15	0.98 ± 0.03	4
3-Caffeoyl-1,5-quinide	>100		1
3,4-Dicaffeoyl-1,5-quinide	2.4 ± 0.7	0.99 ± 0.03	2
3,4-Dicoumaroyl-1,5-quinide	0.96 ± 0.07	1.22 ± 0.07	3

[a] Displacement of [³H]NBTI binding in U-937 cell preparations, expressing the human adenosine transporter.
[b] Number of separate experiments.
[c] Unable to form 1,5-quinide.

The corresponding value for DIFEQ was 0.50 mg/l (1 μM). Thus, the 800 mg extracts (corresponding to three 200 ml cups of coffee) contain 1/25 of DIFEQ equivalents, or approximately 32 mg.

3.4. Pharmacokinetic studies

Analysis of 0.5 ml mouse plasma spiked with 1 nmol of DIFEQ showed a robust signal peaking at 8.5 min retention time. The estimated signal-to-noise ratio was 10,000 giving a detection limit of 0.3 pmol DIFEQ. Uptake and elimination of DIFEQ in the brain of pigmented mice sacrificed 10, 15, 30, and 60 min after administration of DIFEQ (100 mg/kg i.p.) is shown in Fig. 3. This showed that 100 mg/kg i.p. resulted in 66 ± 22 ng DIFEQ (0.13 nmol) after 10 min in a 0.5 g mouse brain (0.003%) with an elimination half-life of 14 min. Recoveries of DIFEQ and 3,4-dicoumaroyl-1,5-quinide in brain tissue were 25% and 11%, respectively. Plasma levels of DIFEQ were similar to those in brain (data not shown). Calculation of the lipophilicity of DIFEQ by SciLogP v1.5 software (Scivision, Burlington, MA) resulted in a log P value of 3.61. This means that DIFEQ is 4000 times more soluble in 1-octanol than in water, predicting a high ability of DIFEQ to cross the blood–brain barrier.
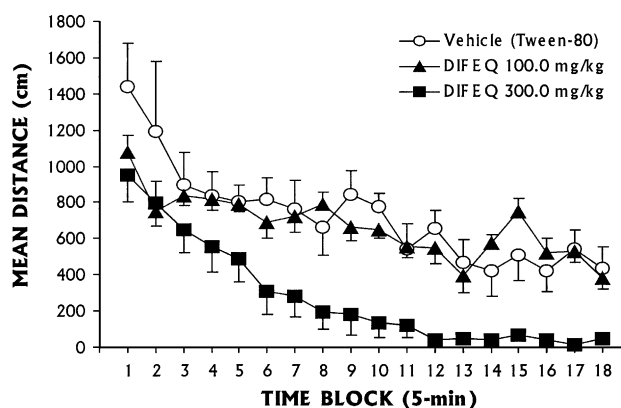

Fig. 4. Effect of DIFEQ (100 and 300 mg/kg i.p.) and vehicle (10% Tween 80) on horizontal locomotor activity in C57BL6/J mice. Total distance traveled in cm per animal for each 5 min interval is expressed as mean ± S.E.M. ($n = 8$ animals per treatment groups).

3.5. Behavioral studies

Injection of 10% Tween 80 vehicle (10 ml/kg) had no behavioral effects, as expected (Castro et al., 1995). As illustrated in Fig. 4, administration of DIFEQ significantly affected open field activity in mice ($F(2,21) = 10.1$; $P = 0.0008$). The highest dose of DIFEQ (300 mg/kg) resulted in a profound suppression of locomotor activity that lasted the entire duration of the 90-min testing session. When the animals did move, no signs of altered locomotor behavior were observed. Follow-up statistical analyses showed that mice under DIFEQ 300 mg/kg had significantly reduced locomotor activity compared to both Tween-80 (critical difference = 282.8; mean difference = 448.3; $P = 0.0005$) and DIFEQ 100 mg/kg (critical difference = 282.8; mean difference = 393.5; $P = 0.0016$). At 100 mg/kg there appeared to be a slight suppression of locomotor activity early in the session. During the first 10 min after being placed in the open field arenas, mice given 100 mg/kg DIFEQ displayed locomotor activity levels similar to those seen in mice given 300 mg/kg. However, by 15 min into the session the activity of mice given 100 mg/kg was more similar to that observed in vehicle-injected mice. The overall difference in activity level between mice under DIFEQ 100 mg/kg and vehicle was not statistically significant (critical difference = 282.8; mean difference = 54.8; $P = 0.612$). The repeated-measures interaction effect between these two doses was also not statistically significant ($F(17,238) = 1.5$; $P = 0.105$). In pilot data (not shown) obtained with doses of DIFEQ lower than 100 mg/kg, there were no differences in activity levels between drug-injected and vehicle-injected mice.

DIFEQ affected rearing behavior in a manner similar to that observed with distance traveled ($F(2,21) = 10.8$; $P = 0.0006$). At the 100 mg/kg dose, DIFEQ induced a small reduction in rearing that lasted much of the testing session; however, it did not reach significance overall (critical difference = 27.6; mean difference = 9.1; $P = 0.401$). There was a substantial reduction in rearing behavior in mice given the 300 mg/kg dose of DIFEQ (data not shown). However, when comparing the difference in locomotor behavior between DIFEQ and control treatment, only the 100 mg/kg dose showed a linear correlation with the brain levels of DIFEQ.

4. Discussion

The present study shows that DIFEQ not only binds to the human equilibrative sensitive (*es*) adenosine transporter (hENT1), but inhibits the re-uptake of adenosine with a potency that is approximately three times higher than the antagonist binding affinity of caffeine at the human adenosine A_{2A} receptor (Fredholm et al., 1999). In brain (Jennings et al., 2001) and periphery the adenosine transporter salvages extra-cellular adenosine for use in the biosynthesis of purine derivatives inside the cell (Thorn and Jarvis, 1996; Baldwin

et al., 1999). This increased extra-cellular level of adenosine causes stimulation of adenosine receptor subtypes, similar to the effects seen with adenosine receptor agonists (Dunwiddie and Masino, 2001). Adenosine uptake inhibitors, such as dipyridamole, mioflazine, and draflazine (Hammond, 2000), have been used as cardioprotective agents in heart surgery and after heart attacks (Rongen et al., 1995; Jacobson et al., 2000).

The behavioral studies in mice show a lasting locomotor suppressant effect of DIFEQ but only at a relatively high dose. In spite of the moderate to high lipophilicity of DIFEQ ($\log P = 3.61$) which should ensure its ready uptake, levels of DIFEQ in plasma (and brain) in the mouse was very low, i.e. 0.003% of injected dose after 10 min. In a series of radio-ligands for the dopamine D2 receptor, optimal uptake in rat brain was found with compounds having an apparent lipophilicity of $\log P = 1.8–2.4$ (Kessler et al., 1991). The adenosine transport inhibitor, KF21652 (3-[1-(6,7-dimethoxyquinazolin-4-yl)piperidin-4-yl]-1,6-dimethyl-2,4(1*H*,3*H*)-quinazolinedione), with similar lipophilicity as DIFEQ ($\log P = 3.14$) and also lacking aliphatic amino groups, demonstrated 0.58% uptake in the mouse brain 15 min after intravenous administration (Ishikawa et al., 2001). While the low plasma levels can explain the low brain levels of DIFEQ in the mouse, the levels in blood were not anticipated. Acute caffeine (10 mg/kg i.p.) produces a 4 µM (2.7%) peak concentration in plasma of the mouse at 15 min with a half-life of 1.4 h (Shi et al., 1994). Caffeine has a considerable lower lipophilicity than DIFEQ, but similar to that of the major chlorogenic acid component in coffee, 5-caffeoylquinic acid ($\log P = -0.51$), of which only one third is absorbed in the human intestine and enters the blood stream (Olthof et al., 2001). One explanation for the low uptake of DIFEQ in mouse plasma and brain could be the poor solubility of DIFEQ in water (< 0.1 mg/ml). Another explanation could be that DIFEQ is more rapidly metabolized in the mouse than caffeine, presumably by ubiquitous esterases. The precise reason for the reduction in locomotion in mice by high doses of DIFEQ is unknown, but the behavior after the 100 mg/kg dose mirrors the brain levels of DIFEQ, and could be an effect of adenosine A_{2A} receptor stimulation in brain by elevated adenosine concentrations (Florio et al., 1997). This effect of DIFEQ is opposite of the molecular and behavioral actions of caffeine (El Yacoubi et al., 2000). Caffeine is an antagonist at the human adenosine A_1 and A_{2A} receptors, with K_i values of 12 and 2.4 µM, respectively, and both the stimulant and anxiogenic effects of caffeine are mediated by inhibition of adenosine A_{2A} receptors in striatum (Fredholm et al., 1999). However, in light of the short duration of DIFEQ in brain and plasma of the mouse, the profound and long lasting decrease in locomotor activity at the 300 mg/kg i.p. dose is unlikely to be centrally mediated and may, in fact, be the result of general acute toxicity of DIFEQ. Compared with the LD_{50} of caffeine in the rat, i.e. 200 mg/kg i.p. (Fredholm et al., 1999), DIFEQ does not seem particularly toxic. However, at the 300 mg/kg i.p. dose

of DIFEQ, one of eight animals did not recover from the treatment.

Six structural isomers are possible for the stereochemical constitution of (−)-quinic acid, as well as four γ-quinides (1,5-lactones) and three δ-quinides (1,4-lactones) (Scholz-Bottcher et al., 1991). Each γ-quinide can be esterified with one, two, or three *para*-hydroxycinnamic acids. In addition, each cinnamoyl ester can carry a hydrogen atom (coumaroyl), a hydroxyl group (caffeoyl), or a methoxy group (feruloyl) in the aromatic meta-positions (Hanson, 1965; Clifford, 1975). This results in the formation of hundreds of different quinides, few of which have been individually identified and found to be present in more than 0.3% of dry weight of roasted coffee (Scholz and Maier, 1990). However, based on the amount of quinic acids found in green coffee (Trugo and Macrae, 1984; Herrmann, 1989), total quinides may represent as much as 3–5%. The actual concentration of DIFEQ in brewed coffee has yet to be determined but is probably insufficient to account for any pharmacological effects from normal coffee consumption. DIFEQ is characterized by having two identical feruloyl ester groups in the aliphatic 3- and 4-alcohol positions of the quinide moiety (Fig. 1). Other cinnamoyl esters of γ-quinides can have one hydroxy (coumaroyl) or two hydroxy substituents (caffeoyl). Permutation of these three esters in each of the 3- and 4-positions of the γ-quinide results in nine positional isomers and analogs of DIFEQ. The des-methyl precursor of DIFEQ, 3,4-dicaffeoylquinic acid, which would be transformed into the corresponding γ-quinide upon roasting, is present in 0.2–0.6% in green coffee beans (Herrmann, 1989). Table 3 shows the average percent of quinic acids found in green coffee beans from various sources and the maximal theoretical amount of their corresponding 1,5-quinides in roasted coffee. Preliminary HPLC analysis of freshly prepared coffee and extracts reveal substantial peaks with retention times similar to those of 3,4-dicaffeoyl-1,5-quinide and DIFEQ. Preliminary results from displacement of [^{3}H]NBTI binding by 3,4-dicaffeoyl- and 3,4-dicoumaroyl-1,5-quinide showed nearly identical affinities for the human adenosine transporter protein expressed in U-937 cells ($K_i = 2.4$ and 1.0 μM, respectively), as that of DIFEQ ($K_i = 0.9$ μM). This suggests that all nine structural permutations of coumaroyl, caffeoyl, and feruloyl groups in the 3- and 4-positions of the γ-quinide share this property, and that all dicinnamoylquinides together exert a cumulative effect on the adenosine transporter, potentially causing elevated adenosine levels even after a single cup of coffee. Ten grams of instant coffee containing 10% of total chlorogenic acids, of which half is made up by quinides, could contain as much as 500 mg of quinides with the capacity to enter brain. One-tenth of these (50 mg) would be dicinnamoyl-1,5-quinides with inhibitory activity on the adenosine transporter. Calculated lipophilic-ities of 3,4-dicaffeoyl- and 3,4-dicoumaroyl-1,5-quinide are $\log P = 2.57$ and $\log P = 2.85$, respectively, suggesting their ready entering into the brain. The existence of such com-pounds in coffee is supported by the binding activity of coffee extracts, which suggest the presence of 30–40 mg of DIFEQ equivalents in the average three cups of coffee, regardless of whether it is regular, instant, or decaffeinated.

It is unknown whether human metabolism of DIFEQ-like compounds is similar to that of DIFEQ in the mouse, but anti-caffeine effects with coffee have actually been observed in humans. Since adenosine mediates the somnogenic effects of prior wakefulness (Porkka-Heiskanen et al., 1997) and adenosine transport inhibitors induces sleep in the rat (O'Connor et al., 1991), the paradoxical fact that coffee intake at bedtime seems to promote sleep in some individuals (Fredholm et al., 1999) could be the result of elevated adenosine levels. On the other hand, the feeling of energetic arousal reported in healthy volunteers increased dose-dependently with increasing strength of freshly brewed coffee, but it did not correlate with the level of caffeine (Quinlan et al., 2000). In a test of psychomotor performance in 19 healthy volunteers, 100 mg caffeine given in tea or hot water, but not when given in decaffeinated coffee, raised the threshold frequency to detect a flickering light (Hindmarch et al., 1998). Thus, there seems to be a pharmacologically active agent in coffee that is not only different from caffeine but may have the opposite effect. However, in light of the

Table 3
Chlorogenic acids in green coffee (*C. arabica*), estimates of the corresponding quinides in roasted coffee, and their calculated lipophilities

Quinic acid (green beans)	Amount (%)[a]	Quinide (roasted coffee)	Amount (%)[b]	log P[c]
5-Caffeoylquinic acid	4.79	NA[d]		− 0.50
4-Caffeoylquinic acid	0.71	4-caffeoyl-1,5-quinide	0.84	0.63
3-Caffeoylquinic acid	0.51	3-caffeoyl-1,5-quinide	0.60	0.62
3,5-Dicaffeoylquinic acid	0.43	NA		
4,5-Dicaffeoylquinic acid	0.29	NA		
5-Feruloylquinic acid	0.28	NA		
3,4-Dicaffeoylquinic acid	0.20	3,4-dicaffeoyl-1,5-quinide	0.24	2.57
3,4-Diferuloylquinic acid	<0.1	3,4-diferuloyl-1,5-quinide	<0.1	3.61
3,4-Dicoumaroylquinic acid	<0.1	3,4-dicoumaroyl-1,5-quinide	<0.1	2.85

[a] Taken from Herrmann (1989).
[b] Maximum theoretical amount assuming 15% roasting loss of water.
[c] Calculated lipophilicity using SciLogP v1.5 software (Scivision).
[d] Not applicable. Only quinic acids without a 5-*O*-substituent can form a 1,5-quinide.

poor uptake of DIFEQ in the mouse and the low theoretical levels of DIFEQ-like compounds in roasted coffee (0.2%), it seems unlikely that the above effects of coffee are caused solely by these inhibitors of the adenosine transporter.

In conclusion, we report that dicinnamoylquinide derivatives of chlorogenic acid have low micromolar affinity for the human adenosine es transporter (hENT1). The potency of these compounds for inhibiting the human adenosine transporter is equal to or higher than that of caffeine for blocking adenosine receptors. The similar activities for 3,4-dicaffeoyl-1,5-quinide and 3,4-dicoumaroyl-1,5-quinide suggest that DIFEQ is a lipophilic representative of a large number of neutral, isomeric compounds formed in the roasting process of coffee. Although the combined activities of DIFEQ-like constituents of coffee may have the potential to reach pharmacologically active levels as a result of normal coffee consumption, further studies are needed to establish whether they exert a modulating effect on the stimulant effect of caffeine.

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