Effects of URB597, an inhibitor of fatty acid amide hydrolase (FAAH), on analgesic activity of paracetamol

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Abstract

OBJECTIVES: Paracetamol is converted to an active metabolite AM404 via fatty acid amide hydrolase (FAAH). The aim of the present study was to ascertain whether a FAAH inhibitor URB597 antagonizes paracetamol analgesic activity (and to asses by this way the role of FAAH in analgesic activity of paracetamol).
METHODS: The interaction between a FAAH inhibitor URB597 and paracetamol was investigated in the writhing test in mice using an isobolographic analysis.
RESULTS: URB597 or paracetamol alone and in combinations produced dose-dependent antinociceptive effects. ED50 values were estimated for the individual drugs and an isobologram was constructed. The observed ED50 value for the URB57-paracetamol combination was 0.097 (0.062–0.247) mg/kg. This value did not differ significantly from the theoretical additive ED50 value for the URB597-paracetamol combination which was 0.108 (0.059–0.198) mg/kg. Thus, inhibition of FAAH by URB597 was not followed by the lack of analgesic activity in paracetamol.
CONCLUSION: The present results suggest that the analgesic activity of paracetamol is not dependent solely on FAAH metabolic conversion to AM404 and that paracetamol exerts analgesic activity also by additional mechanisms.

Abbreviations:

AM404	- N-(4-hydroxyphenyl)arachidonylamide
CL	- confidence limit
ED_{50}	- the fifty percent effective dose
FAAH	- fatty acid amido hydrolase
i.p.	- intraperitoneally
MPE	- maximum possible effect
PPQ	- phenyl-p-quinone
SEM	- standard error of the mean
TRPV1	- transient receptor potential vanilloid 1
URB597	- 3-carbamoyl biphenyl-3-yl ester of cyclohexyl carbamic acid

ORIGINAL ARTICLE

INTRODUCTION

Paracetamol is a wide spread analgesic used for a long time. However, its mechanism of action is still not well understood. Recent findings have showed that paracetamol is converted in an active metabolite N-(4-hydroxyphenyl)arachidonylamide AM404 via fatty acid amide hydrolase (FAAH) in the nervous system (Bertolini *et al.* 2006; Hogestatt *et al.* 2005; Zygmunt *et al.* 2000). AM404 inhibits cellular anandamide uptake (Beltramo *et al.* 1997; Fegley *et al.* 2004), activates TRPV1 receptors (Zygmunt *et al.* 2000) and exerts antinociceptive properties (Borsani *et al.* 2007; Hasanein 2009; Mitchell *et al.* 2007). AM404 thus might represent an important molecule in the mechanism of analgesic action of paracetamol.

The enzyme FAAH is responsible for the degradation of endogenous cannabinoids, in particular anandamide (Cravatt *et al.* 1996), which have antinociceptive activity. Inhibitors of FAAH have been shown to be analgesic in animal models of acute and chronic pain (Lichtman *et al.* 2004; Maione *et al.* 2006; 2007; Jayamanne *et al.* 2006). Among them, a relatively selective inhibitor of FAAH, 3-carbamoyl biphenyl-3-yl ester of cyclohexyl carbamic acid (URB597), has been shown to cause analgesia in visceral pain model (the writhing test) in mice.

The aim of the present study was to ascertain whether a FAAH inhibitor URB597 antagonizes paracetamol analgesic activity. If this would be so, then an active metabolite of paracetamol (AM404) formed by FAAH might be responsible for all of paracetamol analgesic action. We investigated an interaction between a FAAH inhibitor URB597 and paracetamol in the writhing test in mice using an isobolographic analysis. If analgesic action of paracetamol would depend only on its active metabolite AM404 formed by FAAH, then analgesic activity of the combination of paracetamol with a FAAH inhibitor URB597 should not exceed that of URB597 alone. This would manifest as a sub-additivity in the isobolographic analysis.

MATERIALS AND METHODS

Animals

Male CD1 mice (Harlan Laboratories, Italy) weighing 20–25 g were used. Animals were housed on a 12 h light-dark cycle at 22 ± 2 °C with access to food and water *ad libitum*. Animals were (i) acclimatized to the laboratory for at least 1 h before testing, (ii) were used only once during the protocol, and (iii) were euthanized, by an anesthetic overdose, immediately after algesiometric testing. The duration of the experiments was as short as possible and the number of animals was the minimum number compatible with consistent effects of drug treatments (6 or 7 mice per experimental group). Testing of control animals was interspersed concurrently with testing of drug-treated animals.

All procedures involving animals strictly adhered to the guidelines proposed by the Committee on Research and Ethical Issues of IASP for investigations in experimental pain in animals (Zimmermann, 1983).

The writhing test

The writhing test was selected as a model of acute visceral pain, because it is easily reproducible, widely accepted, and a well established model of visceral pain used in laboratories. The writhing test was carried out as previously described (Jain *et al.* 2002). Briefly, mice were intraperitoneally (i.p.) injected with 10 ml/kg of a 0.7% acetic acid solution, 60 min after the i.p. administration of the drug or drug combination to be tested. The 60 min interval between the administration of acetic acid and drugs was established during preliminary experiments as the optimal interval for achieving the maximal effect of URB597.

Groups of mice (6–7) were injected with the acetic acid solution and then placed in a Plexiglas cage (20 × 30 × 20 cm) for observation. A writhe was defined as a wave of contraction of abdominal muscles followed by dorsal flexion and extension of the hind limbs. The number of writhes in a 20 min period was counted, starting immediately after administration of the acetic acid. Antinociception was expressed as percent inhibition in the number of writhes observed in saline treated control animals during the 20 min period. Up to three animals were studied simultaneously by one observer.

Study design of analgesic activity measurement

Sixty minutes before the beginning of the writhing test, animals were intraperitoneally treated with: (i) the vehicle (5% dimethylsulfoxide in saline, 0.9% NaCl), (ii) increasing doses of URB597 (0.1, 0.3, 0.5 and 1.0 mg/kg), (iii) increasing doses of paracetamol (1, 10, 100, 300 and 1 000 mg/kg) and (iv) URB597-paracetamol combinations to asses the antinociceptive effect via isobolographic analysis. Thus treatment (iv) consisted of URB597-paracetamol combinations in decreasing doses of URB597 mixed with (+) paracetamol: 0.19 + 70.7 mg/kg, 0.095 + 35.37 mg/kg, 0.048 + 17.68 mg/kg and 0.024 + 8.83 mg/kg. All drugs were dissolved in 5% dimethylsulfoxide in saline (0.9% NaCl). Mice were randomly assigned to treatment groups. Each mouse was tested only once; however each test involved each mouse receiving two intraperitoneal injections during the experiment (the first contained an isovolumetric injection of the individual analgesic or combination and was given 60 min before the second injection, which contained the acetic acid solution needed to induce the writhing behavior).

Data analysis

Results are presented as mean ± SEM of the dose resulting in 50% of the effect (ED_{50}) values with 95% confidence intervals. At least six animals were tested at each of four doses to determine a dose-response curve

for each drug. Seven animals were tested at each of four doses to determine the dose-response curve for URB597-paracetamol combination. Antinociceptive activity (% MPE) was calculated using the following equation: % MPE = [(mean writhes in control group – mean writhes in drug(s) treated group) / mean of writhes in control group) × 100].

Dose-response curves were constructed using least-squares linear regression and ED_{50} ± standard error (SE) values were calculated according to Tallarida (Tallarida, 2000). The interaction between URB597 and paracetamol was characterized using isobolographic analysis assuming that the combinations were constituted with equally-effective doses of each drug. Thus, from the dose-response curves of each individual agent, the dose resulting in 50% of the effect (ED_{50}) could be determined. Therefore, we estimated the ED_{50} of URB597 and paracetamol. Subsequently, a dose-response curve was obtained by concurrent delivery of URB597 and paracetamol in fixed-ratios based on the ED_{50} values of each individual agent. To construct the curve, groups of animals received intraperitoneally a single dose of each one of the following drug combinations: (i) (URB597 ED_{50} + paracetamol ED_{50}); (ii) [(URB597 ED_{50} + paracetamol ED_{50})/2]; (iii) [(URB597 ED_{50} + paracetamol ED_{50})/4]; or (iv) [(URB597 ED_{50} + paracetamol ED_{50})/8]. The experimental ED_{50} value for the combination was calculated from these curves.

The theoretical additive ED_{50} was estimated from the dose-response curve of each drug administered individually, which presupposes that the observed effect of the combination is the sum of the effects of each individual drug. This theoretical ED_{50} value is then compared with the experimentally derived ED_{50} value to determine if there is a statistically significant difference (Tallarida 2001; 2006; 2007). The theoretical and experimental ED_{50} values of the studied combination were also evaluated by calculating the interaction index (γ) as follows: γ = [ED_{50} of combination (experimental) / ED_{50} of combination (theoretical)]. An interaction index not significantly different from one corresponds to an additive interaction whereas values greater than or less than one imply antagonistic or synergistic interactions, respectively (Tallarida 2002).

Statistical significance between the theoretical additive ED_{50} and the experimentally derived ED_{50} value was evaluated using the Student's t-test. An experimental ED_{50} significantly lower than the theoretical additive ED_{50} was considered to indicate a synergistic interaction. Statistical significance was considered to be achieved when $p<0.05$.

Drugs

The FAAH inhibitor (cyclohexyl carbamic acid 3-carbamoyl biphenyl-3-yl ester (URB597)) was provided by Alexis Biochemicals (USA). Paracetamol was provided by Kulich a.s. (Czech Republic). Drugs were freshly dissolved in 5% dimethylsulfoxide in saline (0.9% NaCl). Drugs were prepared and given in a volume of 10 ml/kg. Control groups received an equal volume of 5% dimethylsulfoxide in saline.

RESULTS

Dose-response relationship of the antinociceptive effect of URB597 and paracetamol

Acetic acid administration produced a typical pattern of writhing behavior. Dose-response curves obtained from the writhing test with URB597 and paracetamol are depicted in Figure 1. The ED_{50} value and 95% confidence limit (CL) for URB597 (i.p.) was 0.20 (0.05–0.35) mg/kg. The ED_{50} value and 95% CL for paracetamol (i.p.) was 89.07 (38.87–148.65) mg/kg.

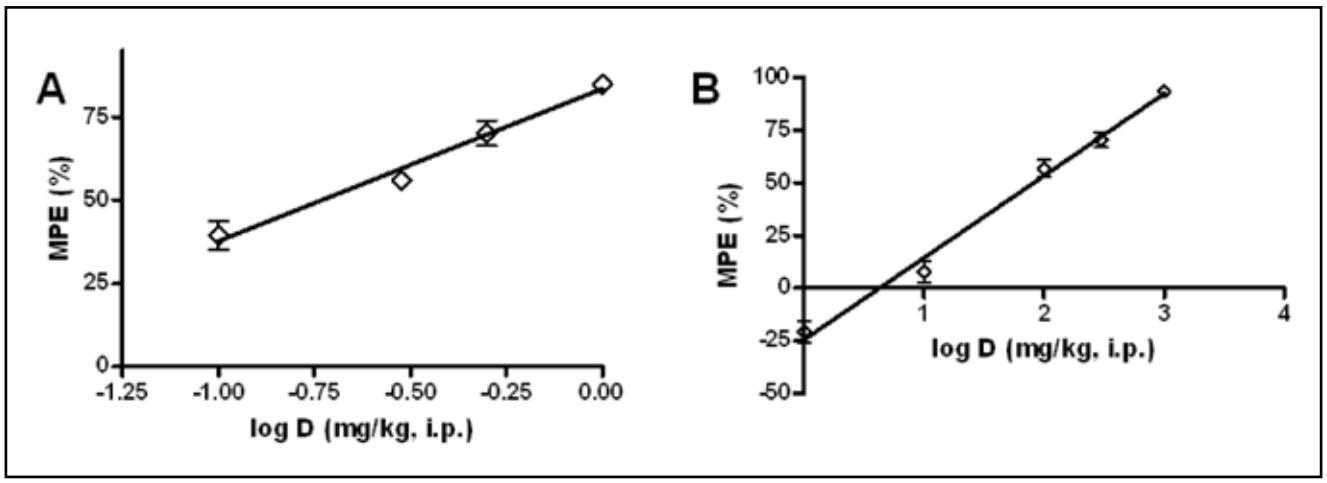

Fig. 1. Dose-response curves for the antinociceptive activity after i.p. administration of URB597 (**A**) and paracetamol (**B**) in mice. Antinociceptive activity (reduction in writhes) was expressed as percent of the maximum possible effect (% MPE) that was calculated by using the following equation: % MPE = [100 × (mean writhes in control group – mean writhes in drug treated group)]/ mean of writhes in control group. Each point represents data for six animals per group ± SEM.

Interaction between URB597 and paracetamol

The antinociceptive activity of i.p. co-administration of fixed ratio combinations of ED_{50} fractions of URB597 with paracetamol was assessed by calculating the ED_{50} value of the mixture from the corresponding dose-response curves. Fixed-dose ratio combinations were prepared, as described in the methods section. The experimental ED_{50} and 95% CL values were calculated as 0.097 (0.062–0.247) mg/kg. This value was not significantly lower than the theoretical ED_{50}/CL values expected for an additive interaction, which were 0.108 (0.059–0.198) mg/kg. As can be seen in Figure 2, the experimental ED_{50} value is located below but in the vicinity of the additive dose line.

The interaction index (γ) for the URB597-paracetamol combination was 0.90 ± 0.13, which is not statistically different from one. These data indicate that the interaction between the antinociceptive actions of URB597 and paracetamol are additive.

DISCUSSION

The present isobolographic analysis indicates that a FAAH antagonist URB597 interacts in an additive manner with paracetamol in the visceral pain model (the writhing test) in mice. These results may have some implications for research of mode of action of paracetamol.

The additivity (the simple sum of the two individual effects) between the antinociceptive action of FAAH inhibitor URB597 and paracetamol suggests that analgesic activity of paracetamol is not mediated only by AM404, the active metabolite of paracetamol formed by FAAH (Bertolini *et al.* 2006; Hogestatt *et al.* 2005; Sinning *et al.* 2008). If this would be so, then the analgesic activity of the combination of URB597 with paracetamol would not exceed the analgesic activity of URB597 alone.

AM404 has been reported to show antinociceptive effects and a cannabinoid activity by various not fully understood mechanisms, such as by inhibiting anandamide transport from the synaptic cleft into neurons and glia (Beltramo *et al.* 1997; Fegley *et al.* 2004), activating cannabinoid receptors (Hasanein 2009; Mitchell *et al.* 2007), inhibiting cyclooxygenases (Hogestatt *et al.* 2005). It also activates vanilloid receptors (De Petrocellis *et al.* 2000; Zygmunt *et al.* 2000). AM404 may inhibit FAAH (Glaser *et al.* 2003). URB597, which is an irreversible FAAH inhibitor (Lichtman *et al.* 2004), caused analgesia in the phenyl-p-quinone (PPQ) model of visceral pain in mice (Haller *et al.* 2006) as well as in other pain models in rodents (Hasanein *et al.* 2008; De Novellis *et al.* 2008; Mallet *et al.* 2008). The antinociceptive efficacy of URB597 was also confirmed in this study.

AM404 appears to be predominantly responsible for cannabinoid activity of paracetamol but inhibition of AM404 synthesis by URB597 was not followed by the lack of analgesic activity in paracetamol. The antinociceptive effects of paracetamol and URB597 were complementary (additive) in the present study. The effect of the URB597/paracetamol combination was the sum of the URB597 effect alone and paracetamol effect alone. This suggest that paracetamol exerts its analgesic activity also by other mechanisms, such as by inhibition of cyclooxygenases, interaction with serotonergic system (Mallet *et al.* 2008; Ruggieri *et al.* 2008), L-arginine-NO pathway (Bjorkman *et al.* 1994).

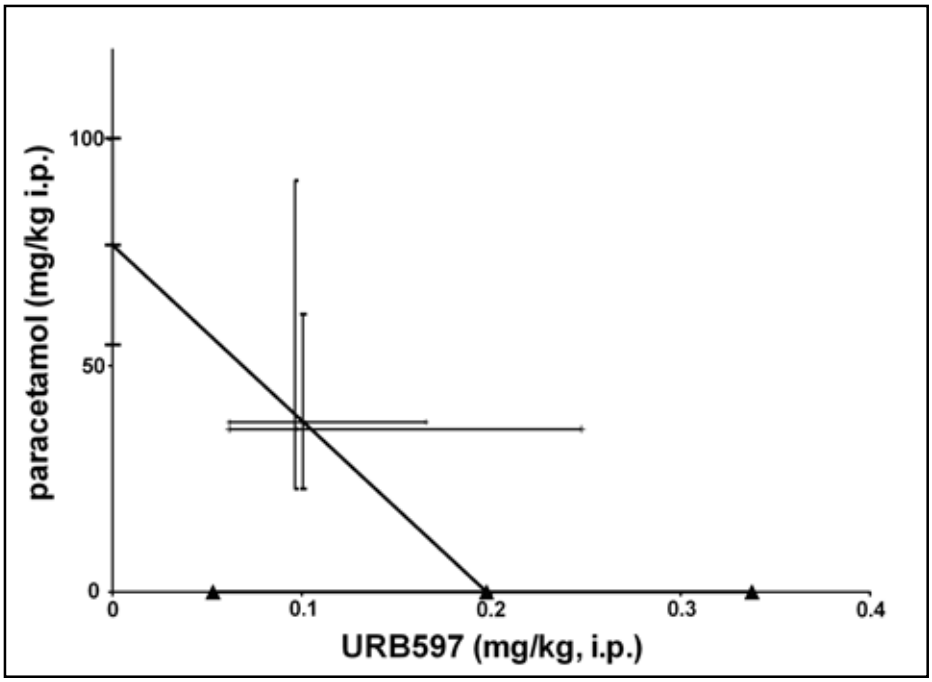

Fig. 2. Isobologram for the i.p. co-administration of URB597 and paracetamol. The point on the theoretical additive line corresponds to theoretical $ED_{50} \pm$ SEM, the point under the theoretical additive line corresponds to experimental $ED_{50} \pm$ SEM of the mixture. The experimental point was not significantly different from the calculated additive point, indicating an additive interaction ($p<0.05$; Student's t-test).

In conclusion, our results suggest that paracetamol reduces pain not only through its metabolite AM404, but exerts its analgesic activity also by other mechanisms.

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