The Fatty Acid Amide Hydrolase Inhibitor URB597 (Cyclohexylcarbamic Acid 3’-Carbamoylbiphenyl-3-yl Ester) Reduces Neuropathic Pain after Oral Administration in Mice

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ABSTRACT

Fatty acid amide hydrolase (FAAH) is an intracellular serine hydrolase that catalyzes the cleavage of bioactive fatty acid ethanolamides, such as the endogenous cannabinoid agonist anandamide. Genetic deletion of the faah gene in mice elevates brain anandamide levels and amplifies the antinociceptive effects of this compound. Likewise, pharmacological blockade of FAAH activity reduces nocifensive behavior in animal models of acute and inflammatory pain. In the present study, we investigated the effects of the selective FAAH inhibitor URB597 (KDS-4103, cyclohexylcarbamic acid 3’-carbamoylbiphenyl-3-yl ester) in the mouse chronic constriction injury (CCI) model of neuropathic pain. Oral administration of URB597 (1–50 mg/kg, once daily) for 4 days produced a dose-dependent reduction in nocifensive responses to thermal and mechanical stimuli, which was prevented by a single i.p. administration of the cannabinoid CB1 receptor antagonist rimonabant (1 mg/kg). The antihyperalgesic effects of URB597 were accompanied by a reduction in plasma extravasation induced by CCI, which was prevented by rimonabant (1 mg/kg i.p.) and attenuated by the CB2 antagonist SR144528 (1 mg/kg i.p.). Oral dosing with URB597 achieved significant, albeit transient, drug levels in plasma, inhibited brain FAAH activity, and elevated spinal cord anandamide content. The results provide new evidence for a role of the endocannabinoid system in pain modulation and reinforce the proposed role of FAAH as a target for analgesic drug development.

The endogenous cannabinoid ligand anandamide (Devane et al., 1992; Di Marzo et al., 1994) and the analgesic and anti-inflammatory factor palmitoylethanolamide (PEA) (Calignano et al., 1998) are members of the fatty acid ethanolamide (FAE) family of lipid mediators. These compounds are found in most mammalian tissues, where they are thought to be stored as the phospholipid precursor N-acylphosphatidylethanolamine and to be produced in a stimulus-dependent manner by activation of phospholipase D or phospholipase C activities (Okamoto et al., 2004; Liu et al., 2006).

After release from cells, anandamide may be eliminated via a two-step process consisting of high-affinity transport into cells (Di Marzo et al., 1994; Beltramo et al., 1997) followed by intracellular degradation, catalyzed by fatty acid amide hydrolase (FAAH) (McKinney and Cravatt, 2005). On the other hand, saturated and monounsaturated FAEs, such as PEA, are poor substrates for the anandamide transport system, and their deactivation may be primarily mediated by intracellular hydrolysis catalyzed by FAAH and/or a distinct N-acylethanolamine acid amidase (Sun et al., 2005).

Mutant mice lacking the gene encoding for FAAH (faah) display reduced FAE hydrolysis and elevated brain levels of...
these lipid amides (McKinney and Cravatt, 2005). Furthermore, FAAH-null mice show signs of enhanced anandamide signaling at cannabinoid CB₁ receptors (e.g., decreased pain sensation) and increased sensitivity to exogenous anandamide, although their overall behavioral phenotype is similar to that of wild-type mice (McKinney and Cravatt, 2005). This finding suggests that drugs targeting FAAH might heighten the tonic actions of anandamide while avoiding unwanted psychotropic effects due to direct activation of CB₁ receptors.

We have recently identified a class of highly selective O-arylcarbamate inhibitors of FAAH activity (Kathuria et al., 2003; Mor et al., 2004). Systemic administration of a lead compound in this class, URB597 (KDS-4103, cyclohexylcarbamic acid 3′-carbamoylphenyl-3′-yl ester), produces profound inhibition of brain FAAH hydrolysis in rats and mice, which is accompanied by elevations of brain FAAH content and potentiation of the actions of anandamide (Kathuria et al., 2003; Fegley et al., 2005). Furthermore, URB597 exerts anxiolytic-like (Kathuria et al., 2003; Patel and Hillard, 2006), antidepressant-like (Gobbi et al., 2005), anti hypertensive (Bátkai et al., 2001), and analgesic (Jayamanne et al., 2006; Jhaveri et al., 2006) effects in rodents. In particular, URB597 reduces pain behaviors in the hot-plate model of thermal nociception (Jayamanne et al., 2003) and the adjuvant model of inflammatory pain (Jayamanne et al., 2006). However, the effects of URB597 in neuropathic pain, a condition that affects more than 2 million patients in the United States alone, have not been established. In a recent study, intrathecal administration of URB597 reduced the responses of spinal wide dynamic range neurons in spinal nerve-ligated neuropathic rats (Jhaveri et al., 2006). In contrast, a single systemic injection of URB597 did not reduce mechanical allodynia in neuropathic rats with partially ligated sciatic nerves (Jayamanne et al., 2006). These contradictory findings prompted us to ask whether repeated treatment with URB597 could effectively reduce pain behaviors in chronic nerve constriction-injured (CCI) mice, a widely used model of neuropathic pain (Bennett and Xie, 1988).

**Materials and Methods**

**Chemicals.** URB597 (KDS-4103) was provided by Kadmus Pharmaceuticals, Inc. (Irvine, California) and the Institute of Medicinal Chemistry, University of Urbino “Carlo Bo” (Urbino, Italy). We purchased fatty acid chlorides from Cambridge Isotope Laboratories (Andover, MA), and [2H₄]-ethanolamine from Cambridge Isotope Laboratories (Andover, MA) and was harvested and washed twice with water to achieve a concentration of 10 mg/ml. Fatty acid chlorides were dissolved in dichloromethane and allowed to stir for 2 h at 20°C until use.

**Animals.** All procedures met the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and those of the Italian Ministry of Health (Decreto Legge 116/92). Male Swiss mice (20–25 g) were obtained from Charles River Laboratories, (Wilmington, MA). All animals were maintained on a 12-h/12-h light/dark cycle with free access to water and chow (RMH 2500; Prolab, Framingham, MA) and were habituated to their surroundings for 2 h before experimentation.

**CCI Model.** Sciatic nerve ligation was performed following the method of Bennett and Xie (1988). Mice were first anesthetized with xylazine (10 mg/kg i.p.) and ketamine (100 mg/kg i.p.), the left thigh was shaved and scrubbed with Betadine, and a small incision (2 cm in length) was made in the middle of the left thigh to expose the sciatic nerve. The nerve was loosely ligated at two distinct sites (spaced at a 2-mm interval) around the entire diameter of the nerve with silk sutures (7-0). The surgical area was dusted with streptomycin powder and closed with a single muscle suture and two skin clips and finally scrubbed with Betadine. In sham-operated animals, the nerve was exposed but not ligated. The animals were placed under a heat lamp until they awakened.

**Behavioral Tests.** Pain withdrawal thresholds to mechanical or thermal stimuli were measured on both the ipsilateral paw (ligated) and contralateral paw (nonligated) 2 h after drug administration for acute administration experiments or 2 h after the last drug administration for chronic administration experiments. Cannabinoid antagonists were administered 30 min before behavioral testing. Mechanical hyperalgesia was assessed by measuring the latency in seconds to withdraw the paw away from a constant mechanical pressure exerted onto its dorsal surface. A 15-g calibrated glass rod (diameter = 10 mm) was aimed at the conical point (diameter = 3 mm) was used to exert the mechanical force. The weight was suspended vertically between two rings attached to a stand and was free to move vertically. A cutoff time of 180 s was used. Thermal hyperalgesia was assessed by the method of Hargreaves et al. (1988) by measuring the latency to withdraw the hind paw from a focused beam of radiant heat (thermal intensity: infrared 3.0) applied to the plantar surface, using a commercial apparatus (Ugo Basile, Varese, Italy). The cutoff time was set at 30 s. Mechanical allodynia was assessed using a Dynamic Plantar Anesthesiometer (Ugo Basile, Italy) by measuring the latency to withdraw the hind paw from a graded force applied to the plantar surface of the paws using a Von Frey filament. The cutoff force was set at 50 g. Locomotor activity was assessed using a fully automated system (Technical and Scientific Equipment, Bad Homburg, Germany). The motility system consists of 2 x 6 infrared light-barriers per cage disposed at right angles on the x-y axes to determine the animal’s center of gravity and its displacement over time. Animals were habituated to test cages for 3 days before trials. Animals were monitored for 96 h immediately after the first drug administration on day 3.

Plasma extravasation was assessed by the method of Joris et al. (1990). In brief, on day 7 after CCI, Evans’ blue dye was injected i.v. (75 mg/kg), and 30 min later, the mice were sacrificed, and the paws were excised. Plantar skin biopsies (6-mm-diameter punches) were taken from the hind paws, and the dye was extracted with 1 ml of formamide for 72 h. Evans’ blue dye concentrations were determined by measuring optical density at λ = 550 nm.

**FAAH Activity** was measured in homogenates under conditions that were linear with respect to protein concentration and time as described previously (Fegley et al., 2005). In brief, homogenates were incubated with anandamide[ethanolamine-3H] (60 Ci/mmol; Ameri-
can Radiolabeled Chemicals, St. Louis, MO) at 37°C for 30 min in 0.5 ml of Tris buffer (50 mM, pH 7.5) containing fatty acid-free bovine serum albumin (0.05%). Radioactivity was measured in the aqueous phase after chloroform extraction.

**Lipid Extractions.** Frozen tissue samples were weighed and homogenized. Tissue homogenates were spiked with [3H]oleyl-ethanolamide (OEA), [3H]PEA, [3H]anandamide, and [3H]2-AG and were subjected to methanol-chloroform (1:2, v/v) extraction. After centrifugation, the organic layer was carefully removed and transferred to another vial. The aqueous layer was reextracted with additional methanol-chloroform-water (1:2:1, v/v/v), and the organic layers from the first and second extractions were combined and concentrated under N2 and fractionated by open-bed silica gel G column chromatography. In brief, the lipid extracts were reconstituted in chloroform and loaded onto small glass columns packed with silica gel G (60–230–400 mesh ASTM; Whatman, Clifton, NJ). Analytes were eluted with 9:1 (v/v) chloroform-methanol. Eluates were dried under N2 and reconstituted in 0.1 ml of chloroform-methanol (1:4, v/v) for LC/MS analyses. LC/MS analysis of FAE and 2-AG was performed using an 1100-LC system coupled to a 1946A-MS detector (Agilent Technologies, Palo Alto, CA) equipped with an electrospray ionization interface. An XDB Eclipse C18 column (2 × 50 mm, 5 μm; Phenomenex, Torrance, CA) and a gradient consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The flow rate was 0.3 mm/min, column temperature was kept at 40°C. Mass spectrometric detection was in the positive ionization mode, capillary voltage was set at 3 kV, and fragmentor voltage was at 40 V and capillary voltage of 3.2 kV. The source and desolvation temperature settings were 130 and 500°C, respectively. The results indicate that URB597 is systemically absorbed and inhibits brain FAAH activity after oral administration.

**Oral URB597 Reduces Mechanical Hyperalgesia.** We next asked whether oral URB597 inhibits pain behavior in neuropathic mice. We produced peripheral neuropathy by loosely ligating the left sciatic nerve, a surgical procedure that results in the development of mechanical and thermal hyperalgesia (Bennett and Xie, 1988), as well as plasma extravasation in the operated limb. Three days after surgery,
when pain behavior is maximal in mice (day 0, presurgery: 54.5 ± 2.1, day 3: 23.9 ± 3.1, day 7: 21.3 ± 1.4; results are paw withdrawal latencies(s) for a mechanical stimulus), we initiated a 4-day treatment regimen with either vehicle or URB597 (10 mg/kg p.o.) administered once daily. On the fourth day of treatment, 7 days after surgery, paw withdrawal latencies were significantly decreased in ligated mice (Fig. 2A), but not in sham-operated animals (Fig. 2B). Administration of URB597 (10 mg/kg p.o.) significantly reduced mechanical hyperalgesia in the operated paw (Fig. 2A) without affecting withdrawal latencies in the nonoperated (contralateral) limb (Fig. 2A). Administration of a single acute dose of URB597 (10 mg/kg p.o.), 7 days after surgery and 2 h before pain testing, produced only a limited effect (Fig. 2C). The antihyperalgesic effects of repeated URB597 dosing were dose-dependent (Fig. 3A) and comparable in magnitude with those elicited by the clinically used analgesic gabapentin (50 mg/kg i.p., once daily for 4 days) (Fig. 3B). Moreover, these effects were not accompanied by any significant change in locomotor activity when measured for 24 h after the last dose on day 4 (vehicle, 18,256 ± 1095; URB, 17,241 ± 1705; results are expressed as total beam breaks).

To explore the contribution of cannabinoid receptors to URB597-mediated antihyperalgesia, on day 7 after nerve ligation, we administered the CB2-selective antagonist rimonabant (SR141716) or the CB2α-selective antagonist SR144528 to CCI mice 30 min before pain assessment. Confirming a role for CB2 receptors, rimonabant (1 mg/kg i.p.) (Fig. 4A) completely prevented the antihyperalgesic actions of URB597 (10 mg/kg p.o.), whereas SR144528 (1 mg/kg i.p.) had no such effect (Fig. 4B). These findings suggest that multiple oral dosing with URB597 reduces mechanical hyperalgesia in neuropathic mice through a CB2-dependent mechanism.

**Oral URB597 Reduces Thermal Hyperalgesia and Mechanical Allodynia.** Treatment with URB597 (10 mg/kg p.o., once daily for 4 days) reduced thermal hyperalgesia (Fig. 5A) and mechanical allodynia in CCI mice (Fig. 5B). In both tests, the analgesic effects of URB597 were prevented by rimonabant (1 mg/kg i.p., 30 min before pain assessment) (Fig. 5, A and B) and attenuated by SR144528 (1 mg/kg i.p., 30 min before pain assessment) (Fig. 5, A and B). In agreement with our previous findings (Fig. 2B), URB597 did not change nocifensive responses to thermal stimuli (Fig. 5A) or mechanical pressure (Fig. 5B) applied to nonoperated paws.

**Oral URB597 Reduces Plasma Extravasation.** The predominant mechanism by which CB2 receptor activation produces analgesia involves the suppression of nociceptive neuron activity (Walker and Hohmann, 2005). However, CB2 agonists may also exert local anti-inflammatory effects that might reduce pain sensation (Marchalant et al., 2007). To investigate whether URB597 affects the neurogenic inflammatory response produced by sciatic nerve ligation, we examined whether this drug influences plasma extravasation in the paws of CCI mice. On day 7 after surgery, vehicle-treated mice (once daily for 4 days p.o.) displayed a significant increase in Evans’ blue dye permeability in paw tissue compared with control, nonligated animals (Fig. 6). Oral administration of URB597 (10 mg/kg) for 4 days markedly reduced this response (Fig. 6), without changing Evans’ blue dye permeability in nonligated paws (Fig. 6). These anti-inflammatory effects of URB597 were completely prevented by rimonabant (1 mg/kg i.p., 30 min before pain assessment) and significantly reduced by SR144528 (1 mg/kg i.p.).

**Oral URB597 Increases Spinal Cord FAE Levels.** Inhibition of FAAH by URB597 has been shown to increase the levels of anandamide and other noncannabinoid analgesic FAEs, such as PEA, in regions of the brain that process nociceptive stimuli (Fegley et al., 2005; Gobbi et al., 2005; Bortolato et al., 2007). To examine whether similar changes occur in the spinal cord, we quantified FAE levels in lumbar spinal cord segments (L1–L5) of CCI mice treated with either

![Fig. 2. Oral URB597 reduces mechanical hyperalgesia. A and B, effects of vehicle (V, □) or URB597 (URB, ■, 10 mg/kg p.o.) on mechanical paw withdrawal latencies before surgery (BL, baseline, □) after (B) repeated dosing or (C) a single drug administration or after 1 week (□) and (■) of CCI in the (A) ligated (IL, ipsilateral) or nonligated (CL, contralateral) paws of CCI mice (n = 12) or (B) sham-operated mice after repeated dosing. * P < 0.05; ** P < 0.01 versus CCI vehicle; ††, P < 0.01 versus BL.](image)

![Fig. 3. Oral URB597 dose dependently reduces mechanical hyperalgesia with an efficacy comparable with that of gabapentin. A, effects of vehicle (V, □) or URB597 (URB, ■, 1–50 mg/kg p.o.) on mechanical paw withdrawal latencies on day 0 before surgery (BL, baseline, □) or after 1 week (□ and ■) of CCI in mice. B, effects of vehicle (V, □), URB597 (URB, 50 mg/kg p.o.), or gabapentin (GP, 50 mg/kg p.o.) on mechanical paw withdrawal latencies before surgery (BL, baseline, □) or after 1 week (□ and ■) of CCI in mice (n = 12). *, P < 0.05; **, P < 0.01 versus CCI vehicle; ††, P < 0.01 versus BL.](image)

### Table 1: Pharmacokinetic profile of URB597 after oral administration in mice (n = 3–4)

| Dose (mg/kg p.o.) | AUC (ng·h/ml) | C_{max} (ng/ml) | T_{max} (min) |
|-------------------|--------------|----------------|--------------|
| 10                | 26           | 16             | 15           |
| 50                | 170          | 90             | 15           |
vehicle or URB597 (10 mg/kg, once daily for 4 days). As anticipated, URB597 selectively increased spinal levels of anandamide (Fig. 7A), PEA (Fig. 7B), and OEA (Fig. 7C), without affecting levels of 2-AG (Fig. 7D), an endocannabinoid lipid that is not a substrate for FAAH.

Discussion

The main finding of the present study is that repeated oral administration of URB597 produces significant antihyperalgesic and antiallodynic effects in the mouse CCI model of neuropathic pain (Bennett and Xie, 1988). These effects are accompanied by an increase in spinal cord anandamide levels, are prevented by the CB1 antagonist rimonabant, and are reduced by the CB2 antagonist SR144528 when the stimuli are thermal or tactile, suggesting that they are caused by anandamide-mediated activation of both CB1 and CB2 receptors. It is noteworthy that the analgesic actions of URB597 are associated with a marked reduction in plasma extravasation, a finding that supports a role for anandamide in the modulation of neurogenic inflammation (Richardson et al., 1998).
A large body of evidence indicates that direct-acting cannabinoid agonists reduce nociceptive behaviors in animals and alleviate pain in humans. In animals, systemic or intracerebral administration of cannabinoid agonists exerts profound antinociceptive effects and suppresses activity of CB1-expressing nociceptive neurons in the thalamus, midbrain, and brainstem (Walker and Hohmann, 2005). In addition to these central actions, cannabinoid agonists also prevent formalin-evoked pain responses in mice (Calignano et al., 1998; Jaggar et al., 1998) and capsaicin-evoked pain in monkeys and human volunteers (Ko and Woods, 1999; Rukwied et al., 2003) presumably by interacting with CB1 or CB2 receptors localized on peripheral sensory neuron terminals or resident non-neural cells (Hohmann et al., 1999; Ibrahim et al., 2003).

Three sets of results support the idea that the FAAH inhibitor UR597 produces its analgesic effects by blocking anandamide hydrolysis, thus magnifying the ability of this endocannabinoid ligand to activate CB1 and CB2 receptors. First, CB2 receptor blockade enhances pain behaviors in various pain models (Calignano et al., 1998; Strangman et al., 1998) and abrogates nonopioid stress-induced analgesia, suggesting the existence of an analgesic endocannabinoid tone mediated by anandamide. Second, genetic deletion of the faah gene and pharmacological inhibition of FAAH activity, each of which elevates brain anandamide levels, reduce nociceptive behaviors in mice and rats (Kathuria et al., 2003; McKinney and Cravatt, 2005). Third, genetic linkage studies have identified FAAH haplotypes in humans, which are linked to variations in pain sensitivity (Kim et al., 2006).

In the present study, we show that UR597 reduces hyperalgesia and allodynia associated with CCI and increases spinal cord levels of anandamide. UR597 does not directly interact with cannabinoid receptors (Kathuria et al., 2003; Piomelli et al., 2006), yet its analgesic effects are blocked by the CB1 antagonist rimonabant. This suggests that one mechanism by which UR597 produces analgesia is elevating anandamide levels at CB1 receptors. In addition, recent studies in neuropathic rats have identified analgesic effects mediated by CB2 receptors (Ibrahim et al., 2003; Scott et al., 2004; Whiteside et al., 2005), raising the possibility that UR597 might modulate pain through both cannabinoid receptor subtypes. Supporting this hypothesis, we found that the CB2 antagonist SR144528 reduced UR597-mediated reductions in plasma extravasation, neuropathic thermal hyperalgesia, and allodynia. In contrast, SR144528 did not affect UR597-mediated analgesia when the pain stimulus was mechanical. These differences may be partly explained by the predominant localization of CB1 receptors to neurons, which contrasts the more predominant expression of CB2 receptors in immune cells and microglia, where they are thought to regulate neuroinflammatory processes (Cabral and Marciano-Cabral, 2005).

An additional possibility is that anandamide and PEA, a noncannabinoid FAAH that produces broad spectrum analgesia by activating the nuclear receptor peroxisome proliferator-activated receptor-α (LoVerme et al., 2006), cooperate to reduce pain synergistically. Indeed, synergistic interactions between PEA and anandamide have been reported (Calignano et al., 1998; Russo et al., 2007).

The analgesic effects of UR597 reported here, when the drug is administered for 4 days, contrast those of a previous study reporting that a single dose of UR597 does not affect mechanical allodynia in neuropathic rats (Jayamanne et al., 2006). Indeed, experiments in our laboratory have confirmed that acute UR597 administration (10 mg/kg p.o.) has only limited effects in CCI mice (day 0, presurgery: 54.5 ± 2.1, day 3: 23.9 ± 3.1, day 7: 21.3 ± 1.4; results are paw withdrawal latencies (seconds) from a mechanical stimulus). One plausible explanation for this result is that short-term dosing with UR597 might induce neuroplastic changes that are responsible for the enhanced efficacy of the drug. Similar enhancements in efficacy after repeated administrations have been observed with the ability of UR597 to increase serotonergic neuron firing in the dorsal raphe nucleus (Gobbi et al., 2005), as well as with the analgesic effects of cannabinoid agonists (Costa et al., 2004) and gabapentin (Fox et al., 2003). The alternate possibility that repeated dosing with UR597 causes incremental elevations in anandamide levels, for example, through alterations in cellular uptake (Kaczocha et al., 2006), is rendered less likely by our finding that single or repeated administration of UR597 elicits similar changes in spinal cord FAAE levels (unpublished data).

The side effects and abuse potential of agonists that target CB1 receptors are well documented, making these drugs less than ideal for clinical use. Previous experiments have shown that UR597 does not share the pharmacological profile of direct-acting cannabinoid agonists (Piomelli et al., 2006). In particular, FAAH inhibition does not cause hypothermia, catalepsy, or hyperphagia, three typical signs of CB1 receptor activation (Kathuria et al., 2003). Moreover, UR597 does not produce rewarding effects in the rat conditioned place preference test and does not substitute for cannabinoid agonists in a rat drug discrimination test (Gobbi et al., 2005). This lack of overt cannabinoid effects has been attributed to the ability of UR597 to inhibit FAAH activity without directly activating CB1 receptors (Kathuria et al., 2003). The favorable pharmacological properties of UR597 underscore the value of FAAH as a target for innovative analgesic drugs.

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Bai et al. (1998) A peripheral mononeuropathy in rat that produces hyperalgesia and allodynia associated with CCI and increases spinal cord levels of anandamide. UR597 does not directly interact with cannabinoid receptors (Kathuria et al., 2003; Piomelli et al., 2006), yet its analgesic effects are blocked by the CB1 antagonist rimonabant. This suggests that one mechanism by which UR597 produces analgesia is elevating anandamide levels at CB1 receptors. In addition, recent studies in neuropathic rats have identified analgesic effects mediated by CB2 receptors (Ibrahim et al., 2003; Scott et al., 2004; Whiteside et al., 2005), raising the possibility that UR597 might modulate pain through both cannabinoid receptor subtypes. Supporting this hypothesis, we found that the CB2 antagonist SR144528 reduced UR597-mediated reductions in plasma extravasation, neuropathic thermal hyperalgesia, and allodynia. In contrast, SR144528 did not affect UR597-mediated analgesia when the pain stimulus was mechanical. These differences may be partly explained by the predominant localization of CB1 receptors to neurons, which contrasts the more predominant expression of CB2 receptors in immune cells and microglia, where they are thought to regulate neuroinflammatory processes (Cabral and Marciano-Cabral, 2005).

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