Pharmacological Characterization of the Nociceptin/Orphanin FQ Receptor Antagonist SB-612111 [(−)-cis-1-Methyl-7-[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-ol]: In Vivo Studies

Anna Rizzi, Elaine C. Gavioli, Giuliano Marzola, Barbara Spagnolo, Silvia Zucchini, Roberto Ciccocioppo, Claudio Trapella, Domenico Regoli, and Girolamo Calò
Department of Experimental and Clinical Medicine, Section of Pharmacology and Istituto Nazionale di Neuroscience (A.R., E.C.G., G.M., B.S., S.Z., D.R., G.C.) and Department of Pharmaceutical Science and Biotechnology Center (C.T.) University of Ferrara, Ferrara, Italy; and Department of Experimental Medicine and Public Health (R.C.), University of Camerino, Camerino, Italy

Received November 6, 2006; accepted February 1, 2007

ABSTRACT

The excellent pharmacological profile displayed by the selective nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor antagonist SB-612111 [(−)-cis-1-methyl-7-[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-ol] in vitro prompted us to investigate the actions of this compound in vivo. In the mouse tail withdrawal assay, SB-612111 given i.p. up to 3 mg/kg did not modify per se tail withdrawal latencies but was able to prevent the pronociceptive and the antinociceptive action of 1 nmol of N/OFQ given i.c.v. and i.t., respectively. In food intake studies performed in sated mice, SB-612111 (1 mg/kg i.p.) had no effect on food consumption but fully prevented the orexigenic effect of 1 nmol of N/OFQ i.c.v. In 17-h food-deprived mice, the opioid receptor antagonist naltrexone (1 mg/kg i.p.) had no effect on food consumption but fully prevented the orexigenic effect of 1 nmol of N/OFQ i.c.v. In 17-h food-deprived mice, the opioid receptor antagonist naltrexone (1 mg/kg i.p.) had no effect on food consumption but fully prevented the orexigenic effect of 1 nmol of N/OFQ i.c.v. In 17-h food-deprived mice, the opioid receptor antagonist naltrexone (1 mg/kg i.p.), produced a statistically significant reduction of food intake. In the mouse forced swimming and tail suspension tests, SB-612111 (1–10 mg/kg) reduced immobility time. The antidepressant-like effect elicited by SB-612111 in the forced swimming test was reversed by the i.c.v. injection of 1 nmol of N/OFQ and no longer evident in mice knockout for the NOP receptor gene. In conclusion, the present findings demonstrate that SB-612111 behaves in vivo as a potent and selective NOP antagonist and suggest that the N/OFQ-NOP receptor endogenous system plays an important role in regulating mood-related behaviors. The use of SB-612111 in future pathophysiological studies will certainly contribute to define the therapeutic potential of selective NOP receptor antagonists in different disease areas.

Nociceptin/orphanin FQ (N/OFQ) (Meunier et al., 1995; Reinscheid et al., 1995) selectively activates a G-protein-coupled receptor now referred to as N/OFQ peptide (NOP) receptor (Cox et al., 2000). Via NOP receptor activation, N/OFQ modulates several biological functions including pain transmission, response to stress and anxiety, learning and memory, locomotor activity, food intake, and drug abuse. N/OFQ may also intervene in the regulation of the functions of the cardiovascular, gastrointestinal, renal, genitourinary, and respiratory systems (Calò et al., 2000b).

Early studies in this field were performed simply measuring the pharmacological effects evoked by the administration of exogenous N/OFQ at different sites (i.v., i.t., i.c.v.) (Calò et al., 2000b). The investigation of the roles played by the endogenous N/OFQ-NOP receptor system in various biological functions started when mice knockout for the NOP receptor gene (NOP−/−) (Nishi et al., 1997) and NOP-selective antag-
onists such as the peptides (Nphe)N/OFQ(1–13)-NH₂ (Calò et al., 2000a) and later UFP-101 (Calò et al., 2002) and the nonpeptide J-113397 (Ozaki et al., 2000) became available. Several studies performed using these pharmacological tools shed light on the role of endogenous NOP signaling in regulating various functions including pain transmission (for review, see Zeilhofer and Calò, 2003), locomotor activity (Marti et al., 2004, 2005), and emotional behaviors (for review, see Gavioli and Calò, 2006). However, interpretation of data generated on knockouts suffers the limitation that compensatory mechanisms occurring during development might affect mutants phenotype; data obtained with peptido antagonists are strongly limited by their poor pharmacokinetic profile, especially for chronic studies, whereas interpretation of the results obtained with J-113397 could be hampered by its limited in vivo selectivity of action as recently underlined by Koizumi et al. (2004), who reported that J-113397 (10 mg/kg) induced identical increases in mesolimbic dopamine release in NOP⁻/⁻ and NOP⁺/⁺ mice and that the rewarding effect of 30 mg/kg J-113397 was maintained in NOP receptor knockout mice. Moreover, the effects elicited by several chemically unrelated molecules must be assessed and characterized in detail before classifying a given action as characteristic of a class of drugs.

The recent availability of the novel potent and selective NOP receptor nonpeptide antagonist SB-612111 (Zaratin et al., 2004) and its excellent pharmacological profile displayed in a rather large battery of in vitro assays (see companion paper Spagnolo et al., 2007) prompted us to investigate the in vivo actions of this compound. Thus, in the present study, the in vivo pharmacological profile of SB-612111 was investigated in mice by assessing the effects of the compound against some well established actions of N/OFQ including supraspinal pronociceptive and spinal antinociceptive effects (Zeilhofer and Calò, 2003) as well as on N/OFQ-induced stimulation of feeding (Olszewski and Levine, 2004). In addition, the effects of SB-612111 were also evaluated in the mouse forced swimming and tail suspension tests to determine whether the compound elicits, similar to other NOP-selective antagonists, antidepressant-like effects (Gavioli and Calò, 2006).

### Materials and Methods

#### Animals

Male Swiss albino and CD1/C57BL6J-129 NOP⁺/⁺ and NOP⁻/⁻ mice weighing 25 to 30 g were used. These later animals were obtained by backcrossing hybrid C57BL6/J-129 (Nishi et al., 1997) with CD-1 mice for nine generations. NOP⁺/⁺ and NOP⁻/⁻ mice were genotyped by polymerase chain reaction. Animals were handled according to guidelines published in the European Communities Council directives (86/609/EEC) and Italian national regulations (D.L. 116/92). They were housed in 425-×-266-×-155-mm cages (Techniplast, Milan, Italy), 15 animals/cage, under standard conditions (22°C, 55% humidity, 12-h light/dark cycle, lights on at 7:00 AM) with food (MIL, standard diet; Morini, Reggio Emilia, Italy) and water ad libitum for at least 5 days before experiments began. Each mouse was used only once. Intracerebroventricular (2 µl/mouse) or i.t. (5 µl/mouse) injections were given according to the procedure described by Laursen and Belknap (1986) and Hylden and Wieloch (1980), respectively.

#### Tail Withdrawal Assay

All experiments were started at 10:00 AM and performed according to the procedure described previously in detail (Calò et al., 1998). In brief, the animals were placed in a holder, and the distal half of the tail was immersed in water at 48°C. Withdrawal latency time was measured by an experienced observer blind to drug treatment. A cut-off time of 20 s was chosen to avoid tissue damage. For each experiment, four mice were randomly assigned to each experimental group, and the experiment was repeated at least four times; therefore, each experimental point is the mean of the results obtained in ≥16 mice. Tail-withdrawal latency was determined immediately before and 5, 15, 30, and 60 min after i.c.v. (2 µl) or i.t. (5 µl) injection of saline (control) or N/OFQ (1 nmol). SB-612111 (0.1–3 mg/kg) was given i.p. 30 min before N/OFQ administration. Increased and decreased tail withdrawal latencies compared with baseline indicated antinociceptive and pronociceptive effects, respectively. Raw data from tail withdrawal experiments were converted to the area under the curve (period analyzed, 15 and 30 min for i.c.v. and i.t. studies, respectively) × withdrawal latency curve (AUC). AUC data were used for statistical analysis.

#### Food Intake Assay

One series of experiments was carried out in freely feeding and drinking mice. The experiments took place at 10:00 AM. Mice were individually housed in a cage and saline or N/OFQ (0.1–10 nmol) were injected i.c.v. Food intake was measured at 30 and 60 min following drug injection. SB-612111 at 1 mg/kg was given i.p. 30 min before administering N/OFQ. Food intake elicited by 1 nmol of N/OFQ was also evaluated in sated NOP⁺/⁺ and NOP⁻/⁻ mice. In a separate series of experiments, mice were food deprived for 17 h and then treated i.p. with 1 and 10 mg/kg SB-612111 or s.c. with 1 mg/kg naltrexone, and 30 min later, food was placed in the cage. Food intake was measured at 30, 60, 90, and 120 min after access to food. Food intake stimulated by food deprivation was also measured in NOP⁺/⁺ and NOP⁻/⁻ mice. Food intake data were expressed as grams of food per kilogram of body weight.

#### Forced Swimming Test

The test was carried out according to Porso et al. (1977). In brief, the test consisted of placing mice, individually, in a Plexiglas cylinder (18.5 cm high; 12.5-cm diameter; water, 13.5 cm deep) partially filled with water (25 ± 0.5°C), for two swimming sessions: an initial 15-min training session, which was followed, 24 h later, by a 5-min test session. The time each animal remained immobile (immobility time) during the 5-min test was recorded. Animals were judged to be immobile when they ceased struggling/swimming and remained floating motionless in the water, making only those movements necessary to keep their heads above the water line. At the end of each swimming session, the animal was removed from the cylinder, dried with paper towels, placed in an individual cage for rest and recovery over 15 min, and then returned to its collective home cage.

#### Tail Suspension Test

The test was carried out according to Steru et al. (1985). In brief, mice were isolated acoustically and visually and suspended 50 cm above the floor by an adhesive tape placed 1 cm from the tip of the tail. The total amount of time each animal remained immobile (i.e., did not struggle) during the 5-min session in which the animal remained suspended was recorded (in seconds) as immobility time. To improve response reliability, the mice were submitted to an initial 5-min training session of tail suspension 24 h before the actual test session. The compound SB-612111 (10 mg/kg) was administered i.p. 30 min before the test. According to Steru et al. (1985), a reduction in immobility time suggests an antidepressant-like effect.

#### Open-Field Test

This test was used to evaluate locomotor activity and spontaneous exploratory behavior. The apparatus, made of wood covered with impermeable plastic, had a white floor of 40 × 40 cm (divided by black lines into 16 squares of 10 × 10 cm) and white walls, 20 cm high. Each mouse was placed in the center of the open field, and the numbers of squares crossed and of rearing and grooming...
ing behaviors were registered, through direct visual observation, for 5 min. At the end of each animal observation, the floor of the open field was cleaned with a wet sponge and a dry paper towel. The compound SB-612111 (10 mg/kg) was administered i.p. 30 min before the test.

**Drugs.** N/OFQ was prepared and purified in house as described previously (Guerrini et al., 1997). The compound SB-612111 was synthesized following the experimental conditions and protocols described in detail by Palombi and Ronzoni (2003). Naltrexone was purchased from Sigma (Poole, UK). N/OFQ and naltrexone were solubilized in physiological medium, whereas SB-612111 was dissolved in dimethyl sulfoxide (final concentration not exceeding 0.8%).

**Data Analysis and Terminology.** All data are expressed as means ± S.E.M. of n experiments. Data have been analyzed statistically using the Student’s t test for unpaired data or the one-way ANOVA followed by the Dunnett’s test or the Student-Newman-Keuls test, as specified in table and figure legends. Statistically significant was set at \( p < 0.05 \).

**Results**

**Tail Withdrawal Assay.** In tail withdrawal experiments, mice injected with saline (either i.c.v. or i.t.) displayed tail withdrawal latencies of approximately 5 s that were stable over the time course of the experiment (Fig. 1). The i.p. administration of SB-612111 up to 3 mg/kg did not modify tail withdrawal latencies (AUC\(_{[0–30 \text{ min}]}\) saline, 150 ± 13; 3 mg/kg SB-612111, 141 ± 9). In line with previous studies, 1 nmol of N/OFQ given i.c.v. induced a robust although short-lasting pronociceptive effect (AUC\(_{[0–15 \text{ min}]}\) saline, 80 ± 7; 1 nmol of N/OFQ, 51 ± 6) (Fig. 1A). This action of N/OFQ was prevented by SB-612111 at 1 mg/kg (AUC\(_{[0–15 \text{ min}]}\) saline, 82 ± 9), whereas the lower dose of antagonist (i.e., 0.1 mg/kg) was inactive (AUC\(_{[0–15 \text{ min}]}\), 55 ± 7) (Fig. 1A).

When the same dose of N/OFQ was administered i.t., a statistically significant antinociceptive effect was recorded (AUC\(_{[0–20 \text{ min}]}\) saline, 168 ± 15; 1 nmol of N/OFQ, 326 ± 25) (Fig. 1B). This effect was antagonized by SB-612111 in a dose-dependent manner (AUC\(_{[0–20 \text{ min}]}\) saline, 240 ± 19; 1 mg/kg, 199 ± 14) (Fig. 1B).

**Food Intake Studies.** Under the present experimental conditions, mice treated with saline ate 1.63 ± 0.61 and 4.50 ± 0.96 g/kg at 30 and 60 min, respectively. The i.c.v. injection of N/OFQ induced a dose-dependent hyperphagic effect. The peptide was inactive at 0.1 nmol, significantly stimulated food intake only at 60 min at 0.3 nmol, and at 1 nmol produced a statistically significant orexigenic effect both at 30 and 60 min (Fig. 2A). Increasing the dose of peptide to 10 nmol resulted into a loss of effect, thus making the dose-response curve to N/OFQ bell-shaped. It is worthy of mention that the administration of such a high dose of peptide was associated with evident sedative effects (i.e., decrease in locomotor activity, reduction of muscle tone, ataxia, and impairment of the righting reflex) as previously reported by us and other groups (Calo et al., 2000b).

The dose of N/OFQ producing the maximal orexigenic effect, i.e., 1 nmol, was selected for further receptor antagonist and knockout studies. In Swiss sated mice, SB-612111 (1 mg/kg i.p. 30 min pretreatment) given alone had no effect on food intake but fully prevented the orexigenic effect of N/OFQ (Fig. 2B).

N/OFQ given i.c.v. at 1 nmol significantly stimulated food intake in wild-type mice, whereas it was completely inactive in NOP\(^{-/-}\) animals (Fig. 3). It is worthy of mention that the orexigenic effect of N/OFQ is much more pronounced in CD1/C57BL6/J-129 (approximately 430% of controls) than in Swiss mice (approximately 220% of controls).

A separate series of experiments were performed in food-deprived mice. Food deprivation (17 h) was followed by a remarkable food intake occurring within the first couple of hours of access (see Fig. 4). SB-612111 (1 and 10 mg/kg) did not modify food intake induced by food deprivation, whereas the classical opioid receptor antagonist naltrexone (1 mg/kg) reduced it. One-way ANOVA followed by the Dunnett’s test revealed that the effect of naltrexone was statistically significant at 30 and 60 min after access to food (Fig. 4). Finally, under the present experimental conditions, no differences were recorded in food intake stimulated by 17-h food deprivation between NOP\(^{+/+}\) and NOP\(^{-/-}\) mice (data not shown).

**Forced Swimming and Tail Suspension Tests.** As shown in Fig. 5A, SB-612111 (1–10 mg/kg i.p. injected 30 min before the test) reduced immobility time of mice subjected to the forced swimming test in a dose-dependent manner, evoking statistically significant effects at the highest dose tested (i.e., 10 mg/kg). To investigate the receptor mechanism involved in this SB-612111 action, the effect of N/OFQ injected i.c.v. in mice pretreated with the NOP antagonist was assessed. As shown in Fig. 5B, 1 nmol of N/OFQ was inactive per se but reversed the reduction of immobility time elicited by SB-612111.

The antidepressant-like effects promoted by SB-612111 administration in the forced swimming test were further investigated in NOP\(^{+/+}\) and NOP\(^{-/-}\) mice. As shown in Fig. 6 and in line with previous findings (Gavioli et al., 2003),
animals displayed a reduced immobility time compared with NOP+/− mice, and this difference was statistically significant (NOP+/+, 163 ± 13 s; NOP−/−, 104 ± 13 s; p < 0.05, according to the Student’s t test for unpaired data). The administration of SB-612111 (10 mg/kg) reduced the immobility time in NOP+/+ mice while being inactive in animal knockout for the NOP receptor gene (Fig. 6). Swiss mice subjected to the tail suspension test displayed an immobility time of 129 ± 15 s (n = 8). SB-612111 (10 mg/kg) reduced immobility time of mice subjected to this test in a statistically significant manner (86 ± 11 s, n = 9, p < 0.05 versus control, according to the Student’s t test for unpaired data).

Open-Field Test. As summarized in Table 1, no alterations in spontaneous locomotor activity as well as rearing and grooming behaviors were observed in mice treated with the dose of 10 mg/kg i.p. SB-612111 in the open-field test compared with saline-treated animals.

Discussion

Present data demonstrate that SB-612111 antagonizes supraspinal pronociceptive and orexigenic action of N/OFQ and reduces the spinal antinociceptive effects of the peptide; moreover, similar to other NOP-selective antagonists, SB-612111 evokes antidepressant-like effects in mice without causing modification of the animal gross behavior. Overall, these findings demonstrate that SB-612111 behaves in vivo as a highly potent and selective antagonist for the NOP receptor.

N/OFQ has been shown by us and several other groups to elicit in rodents pronociceptive and antinociceptive effects following supraspinal and spinal injection, respectively (Zeilhofer and Calo, 2003). Results obtained in the present experiments using N/OFQ 1 nmol are therefore in line with data from the literature. These actions of N/OFQ were dose de-
J-113397 does not produce per se any effect under similar response. This is corroborated by the following findings. Acute stimulus employed for evoking the nociceptive receptor system is not activated by the mild and withdrawal latencies, indicating that the endogenous NOP receptor activation. The systemic administration of SB-616211 at 1 mg/kg, the dose able to prevent the orexigenic action of exogenous N/OFQ, and at 10-fold higher doses (i.e., 10 nmol i.c.v. of N/OFQ was prevented by SB-612111 at 1 mg/kg, the dose able to prevent the orexigenic action of N/OFQ are exclusively due to NOP receptor antagonists (e.g., the nonpeptide J-113397 (Ozaki et al., 2000), and NOP receptor signaling is activated by prolonged but not acute nociceptive inputs, and this has consequences on the actions of NOP antagonists on pain transmission. For detailed discussion of this topic and of the neurobiological mechanisms involved in actions of N/OFQ at spinal and supraspinal levels, see Zeihofer and Calò (2003).

Several studies performed in rats have shown that the i.c.v. administration of N/OFQ or NOP receptor agonists evokes robust orexigenic effects (Olszewski and Levine, 2004). In the present article, we showed for the first time that N/OFQ induces a robust hyperphagic effect also in sated mice. The dose-response curve to N/OFQ was found to be bell-shaped, with the maximal effect elicited by the peptide at 1 nmol. The reduced hyperphagic effect of 10 nmol of N/OFQ can be probably ascribed to the inhibitory action on motor activity elicited by high doses of the peptide. Both pharmacological and knockout findings converge, indicating that the orexigenic effects induced by N/OFQ are exclusively due to NOP receptor activation. In fact, the orexigenic action of 1 nmol of N/OFQ was prevented by SB-612111 at 1 mg/kg and no longer evident in NOP mice. These results are in line with those obtained in rats with both NOP-selective agonists and antagonists (Economou et al., 2006).

Polidori et al. (2000) showed that the i.c.v. injection of selective NOP receptor peptide antagonist [Nphe1]N/OFQ(1–13)-NH2 not only prevented the orexigenic action of N/OFQ but also significantly reduced food intake induced by food deprivation in rats. This suggests that brain N/OFQergic signaling may be activated during fasting and contributes to food intake induced by food deprivation. These findings prompted us to evaluate the effect of SB-612111 on food intake stimulated by fasting. However, SB-612111 tested in mice that were food deprived for 17 h did not modify the food intake of the animals. This negative result is unlikely to depend on the dose of antagonist employed because we tested SB-612111 at 1 mg/kg, the dose able to prevent the orexigenic action of exogenous N/OFQ, and at 10-fold higher doses (i.e., experimental conditions (Ozaki et al., 2000), and NOP mice do not show any phenotype in the tail-flick assay (Nishi et al., 1997). However, this does not exclude that N/OFQergic signaling might be activated and modulate pain transmission under other conditions. As a matter of fact, in the mouse formalin test, where the nociceptive response is evoked by a tonic stimulus, J-113397 produces pronociceptive effects, and NOP (as well as ppN/OFQ(1–13)) mice show a pronociceptive phenotype (Depner et al., 2003; Rizzi et al., 2006). Collectively, these findings indicate that endogenous N/OFQ-NOP receptor signaling is activated by prolonged but not acute nociceptive inputs, and this has consequences on the actions of NOP antagonists on pain transmission. For detailed discussion of this topic and of the neurobiological mechanisms involved in actions of N/OFQ at spinal and supraspinal levels, see Zeihofer and Calò (2003).
In Vivo Studies on the NOP Antagonist SB-612111

10 mg/kg) obtaining similar negative results. On the other hand, our experimental conditions were suitable for detecting the effect of drugs inhibiting food intake induced by food deprivation since we obtained results superimposable to those present in literature (for examples, see Taber et al., 1998; Hadjimarkou et al., 2004), with the universal opioid receptor antagonist naltrexone. Thus, the data obtained with SB-612111 suggest that in mice, unlike in rats (Polidori et al., 2000), the N/OFQ-NOP receptor system does not play a major role in controlling food intake induced by food deprivation. This conclusion is supported by the results obtained evaluating the food intake induced by 17-h food deprivation in NOP−/− and NOP+/− mice; in fact, no differences between the two groups of animals were evident in this experiment. For a detailed discussion of the nature of the hyperphagic effects of N/OFQ and their relationship with other systems regulating food intake, see Olszewski and Levine (2004).

Recent pharmacological and knockout evidence demonstrated that blocking N/OFQ signaling evoked antidepressant-like effects in rodents (Gavioli and Calò, 2006). In fact, chemically unrelated molecules such as J-113397, [Nphe1]N/OFQ(1–13)-NH2, and UFP-101 were able to reduce immobility time in the mouse and rat forced swimming and the mouse tail suspension tests, and NOP−/− mice displayed an antidepressant-like phenotype in these assays (Redrobe et al., 2002; Gavioli et al., 2003, 2004). In the mouse tail suspension test, SB-612111 mimicked the effect of NOP-selective antagonists, reducing the immobility time in a dose-dependent manner and producing statistically significant effects at 10 mg/kg. The same dose of antagonist was also active in the mouse tail suspension assay. Moreover, in the mouse forced swimming assay, the effect of SB-612111 was reversed by the coadministration of N/OFQ, which was per se inactive, suggesting the selective involvement of the NOP receptor in this action of the antagonist. Moreover, the antidepressant-like effects of SB-612111 were no longer evident in NOP−/− mice, although these results are biased by the reduced immobility time displayed by the mutant mice compared with the wild-type animals (Gavioli et al., 2003). False-positive results may be obtained in these behavioral assays with drugs that stimulate locomotion (Bourin et al., 2001). This is unlikely to apply to SB-612111 (and to the other NOP-selective antagonists) (Gavioli and Calò, 2006) because no alteration of this parameter (as well as gross behavior) was recorded in the open-field test in response to 10 mg/kg SB-612111. For a detailed discussion of this topic and of the possible mechanisms underlying the antidepressant-like effect of NOP antagonists, see Gavioli and Calò (2006).

Thus, results obtained with the novel NOP-selective antagonist SB-612111 confirm previous findings (Gavioli and Calò, 2006), indicating that the block of N/OFQ-NOP receptor signaling in the brain produces antidepressant-like effects in rodents and supporting the proposal of NOP receptors as candidate target for the development of innovative antidepressant drugs.

In conclusion, present data demonstrated that SB-612111 is a highly potent and selective nonpeptide antagonist at the NOP receptors in mice. This pharmacological profile associated with the favorable pharmacokinetic properties deriving from its nonpeptide nature, makes SB-612111 an ideal pharmacological tool for further studies investigating the different biological functions regulated by the N/OFQ-NOP receptor system and for firmly defining the therapeutic potential of NOP receptor antagonists.

References


Address correspondence to: Dr. Girolamo Calò, Department of Experimental and Clinical Medicine, Section of Pharmacology, via Fossati di Mortara 19, 44100 Ferrara, Italy. E-mail: g.calò@unife.it