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Analgesic and antispasmodic activities of 2-(2-nitro-phenyl)-1*H*-benzimidazole 5-carboxylic acid: evidence for the importance of the 2-(*o*-substituted phenyl) group

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Benzimidazole 5-carboxylic acid derivatives were investigated for analgesic activity in this study. Of the benzimidazole compounds tested, 2-(2-nitro-phenyl)-1*H*-benzimidazole 5-carboxylic acid showed remarkable naloxone sensitive analgesic activity in the tail clamp but not in the tail immersion analgesia tests. This centrally active compound showed antispasmodic activity only on KCl induced contractions of isolated rat ileum and not on acetylcholine induced contractions. Acute toxicity of the compounds were >100 mg/kg i.p mice. It was concluded that substitution of the 2(*o*-phenyl) by nitro- but not by chloro- or methoxy groups is important for naloxone sensititive analgesic activity of benzimidazole compounds and it was hypothetized that new imidazole compounds having a 2-(*o*-substituted phenyl) moiety needs to be investigated.

1. Introduction

Benzimidazole is a widely investigated heterocyclic ring in compounds with several pharmacological activities, including antiviral [1, 2], antibacterial [3], antifungal [4], anthelminthic, antiparasitic [5, 6] antiulcer [7], antihistaminic [8], angiotensin II antagonist [9], antiaggregant [10], vasodilator [11] antiarrhythmic [12], 5-HT antagonist [13], antipsychotic [14], anticonvulsant [15] anticancer [16, 17] and analgesic [18–20] activities.

The aim of this study was to investigate, as part of a screening program the analgesic activity of benzimidazole-5-carboxylic acids which had been synthesized previously [21]. To the best of our knowledge, there have been no reportes of studies either of analgesia or on in vitro isolated intestine.

2. Investigations, results and discussion

Among the benzimidazole 5-carboxylic acid derivatives tested 2-(2-nitro-phenyl)-1*H*-benzimidazole 5-carboxylic acid showed the highest analgesic activity in the tail clamp test (Table 1 and 2). Although the mean value for 2-(2-hydroxyphenyl)-1*H*-benzimidazol 5-carboxylic acid might indicate analgesic activity, its standard deviation was high enough to overlap with the naloxone group.

Table 1: Analgesic activity of 2-(2-substituted phenyl)-1*H* benzimidazole-5-carboxylic acid compounds on mechanical algesic stimulus (tail-clamp test)

Substituted compound	Mean ± StDev
2-(2-Chlorophenyl)-1*H*-benzimidazole-5-carboxylic acid	12.34 ± 47.01
2-(2-Methylphenyl)-1*H*-benzimidazole-5-carboxylic acid	32.00 ± 58.92
2-(2-Methoxyphenyl)-1*H*-benzimidazole-5-carboxylic acid	17.80 ± 8.33
2-(2-Hydroxyphenyl)-1*H*-benzimidazole-5-carboxylic acid	44.94 ± 28.70
2-(2-Nitrophenyl)-1*H*-benzimidazole-5-carboxylic acid	100.00 ± 0 (*)
Naloxone + 2-(2-nitrophenyl)-1*H*-benzimidazole-5-carboxylic acid	−8.18 ± 11.86
Morphine	72.0 ± 38 (*)
Naloxone + morphine	12.0 ± 12

$p < 0.05$

Table 2: Analgesic activity of 2-(2-substituted phenyl)-1*H* benzimidazole-5-carboxylic acid compounds on thermal algesic stimulus (tail-immersion test)

Compound	Mean ± StDev
2-(2-Chlorophenyl)-1*H*-benzimidazole-5-carboxylic acid	−05.95 ± 12.21
2-(2-Methylphenyl)-1*H*-benzimidazole-5-carboxylic acid	−17.78 ± 31.40
2-(2-Methoxyphenyl)-1*H*-benzimidazole-5-carboxylic acid	−04.84 ± 22.89
2-(2-Hydroxyphenyl)-1*H*-benzimidazole-5-carboxylic acid	01.86 ± 05.16
2-(2-Nitrophenyl)-1*H*-benzimidazole-5-carboxylic acid	14.23 ± 09.40
Naloxone + 2-(2-nitrophenyl)-1*H*-benzimidazole-5-carboxylic acid	−38.19 ± 67.88
Morphine	69.30 ± 32 (*)
Naloxone + morphine	12.50 ± 16.2

$p < 0.05$

Since mice of either sex were used in our investigations, this high standard deviation may be due to sex specific activity [22] resulting in statistically non-significant activity, but this awaits further investigations specifically with this benzimidazole compound.

Analgesic activity in the tail clamp test was antagonized by naloxone, indicating the involvement of opioid receptors (Table 1). On the other hand none of the compounds exhibited analgesic activity in the tail-immersion test (Table 2). Since the tail-immersion test which uses for thermal algesic stimulus at 52,5 °C is reported to discriminate between opioid receptor subtypes [23], the lack of analgesic activity in this test suggests opioid receptor subtype selective analgesic activity. To the best of our knowledge, 2-(2-nitro-phenyl)-1*H*-benzimidazole 5-carboxylic acid has not been shown to possess opioidergic activity prior to our experiments. Subtype selectivity was not the aim of this study and delta-and/or kappa opioid receptor selectivity of this active compound needs to be clarified by further investigations.

Centrally active analgesic compounds such as opioid agonists are known to inhibit gastrointestinal functions [24, 25]. Since 2-(2-nitro-phenyl)-1*H*-benzimidazole 5-carboxylic acid was observed to possess the highest *in vivo* analgesic activity, only this compound was investigated at 10^{-6}, 10^{-5} and 10^{-4} M on isolated rat ileum. The centrally active compound inhibited KCl-induced isolated rat ileum contractions in a dose dependent manner but it was inactive on ACh induced contractions. Naloxone was ineffective in inhibitory action on KCl induced contractions (Figs. 1 and 2) indicating a non-opioid mechanism of action. The statistically inactive effects on cholinergic contractions and the opioid antagonist-resistant inhibitory action of the compound on isolated ileum may be interpreted as further evidence for its opioid receptor subtype selective activity, because delta opioid agonists are known to inhibit intestinal activity unrelated to delta-opioid receptor activation [26]. It is known that KCl-induced but not acetylcholine-induced contractions of smooth muscles are mainly dependent on extracellular calcium ion influx [27]. The inhibitory mechanism of action of the active test compound on isolated rat intestine may involve voltage dependent ionic currents, but this awaits further investigation with these compounds.

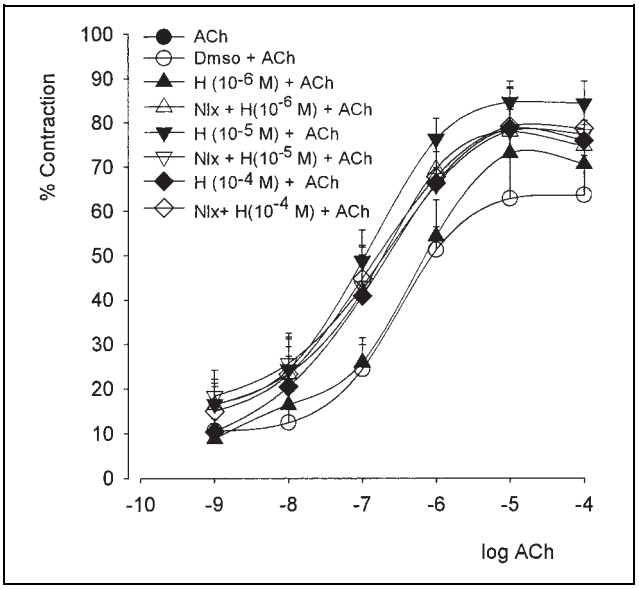

Fig. 1: Effect of three different doses of 2-(2-nitro-phenyl)-1*H*-benzimidazole-5-carboxylic acid (H) on acetylcholine (Ach) induced (2, 4, 8, 16, 32 mM) contractions of isolated rat ileum

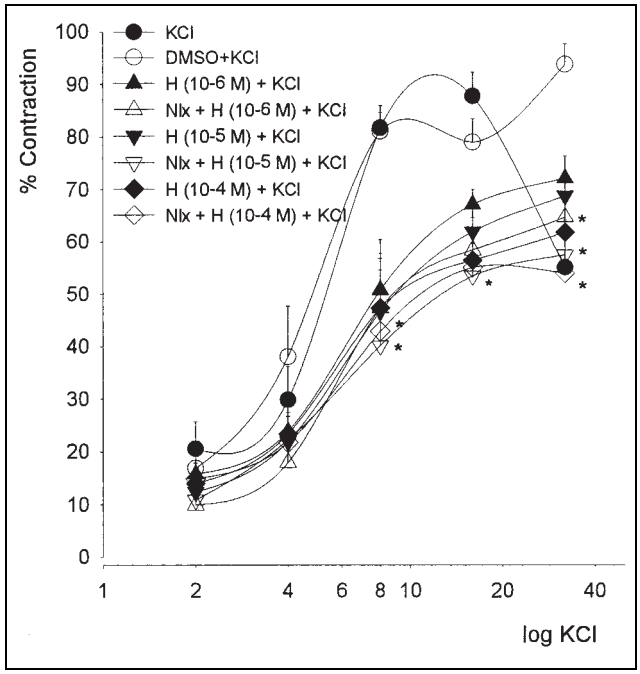

Fig. 2: Effect of three different doses of 2-(2-nitro-phenyl)-1*H*-benzimidazole-5-carboxylic acid (H) on KCl induced (2, 4, 8, 16, 32 mM) contractions of isolated rat ileum. * $p < 0.05$

2-(2-Substituted phenyl)-1*H*-benzimidazole 5-carboxylic acid derivatives have been previously reported to have *in vivo* analgesic activity. It was observed that central analgesic activity increased as the hydroxyl and nitro substitutions were moved from para- to ortho-position on the substituted phenyl group [28] (Fig. 3).

Recent reports emphasise the importance of the aromatic rings for molecules which do not have a benzodiazepine structure [29–31]. On the other hand, data obtained from our experiments demonstrated the importance of the substitution of the phenyl ring for analgesic activity. The highest activity was observed for the nitro-substituted compound. Since the importance of the substitution of the phenyl ring on analgesia and delta opioid receptor selectivity has been reported previously [32, 33], further investigations are needed to clarify its opioid-receptor subtype selectivity.

Based on previously published pharmacological results [30], we propose that the functional groups and their posi-

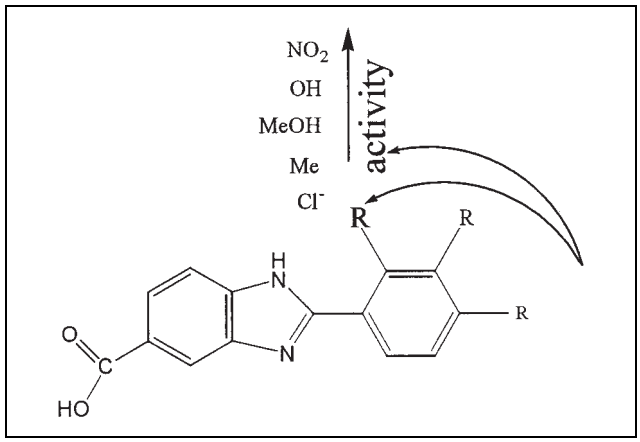

Fig. 3: Relationship between substitution and analgesic activity on 2-(substituted-phenyl) groups

tion (in terms of para-, meta or ortho) on the 2-(*o*-substituted phenyl) ring of the benzimidazole 5-carboxylic acid play a significant role. On the basis of the results of our present investigations, the functional groups on the 2-(*o*-substituted phenyl) structure play a significant role in analgesic activity, nitro- and hydroxyl-being the most potent of those tested (Table 1). As a result of these new proposals, we hypothesize that the role of 2-(*o*-substituted phenyl) structure requires to be verified in terms of analgesia and 4,5-dialkyl-2-(*o*-substituted phenyl) imidazole compounds need to be investigated as potential new opioid analgesic compounds.

3. Experimental

3.1. Animals and chemicals

Adult Swiss albino mice (28–38 g) of either sex were used in this study. They were housed in well ventilated rooms with a room temperature of 18–25 °C. All mice were fed with a standard diet (Esyem A. S., Eskisehir) and water *ad libitum*. The chemicals used as test materials [2-(2-chlorophenyl)-1*H*-benzimidazole 5-carboxylic acid; 2-(2-methylphenyl)-1*H*-benzimidazole 5-carboxylic acid; 2-(2-methoxyphenyl)-1*H*-benzimidazole 5-carboxylic acid; 2-(2-hydroxyphenyl)-1*H*-benzimidazole 5-carboxylic acid and 2-(2-nitrophenyl)-1*H*-benzimidazole 5-carboxylic acid] were obtained from the Department of Medicinal Chemistry, Faculty of Pharmacy, Anadolu University, and had been synthesized previously [21].

3.2. Behavioural observation of the animals

After the injection of saline, sunflower oil and test substances (100 mg kg^{-1} i.p), all the animals were placed separately in transparent containers and observed for 30 min until the animals were subjected to tail-clip or water immersion tests.

3.3. Acute toxicity tests

Acute toxicity tests were performed as described previously (34). No mortality was observed after the injections of test substances (100 mg kg^{-1} i.p), which showed the LD_{50} values for the compounds were >100 mg kg^{-1} i.p for mice.

3.4. Tail-clip tests

Experiments were performed on freely moving Swiss albino mice, 28–38 g. Analgesic (antinociceptive) activities of morphine and the test compounds were measured by the application of a mechanical tail-clip as described previously elsewhere [35]. A control response (2–4 s) was determined with 0.1 mL sunflower oil and 0.9% physiological saline solution, since the test compounds and standard pure chemicals (morphine sulfate and naloxone hydrochloride) were diluted in sunflower oil and in saline solution respectively. Test latencies were assessed 30 min after the administration of drugs to the mice for all test substances and control groups. Naloxone, a specific antagonist for opioid receptors, was given 15 min prior to administration of drugs and test substances (5 mg kg^{-1} i.p.). Morphine sulphate was used as a standard opioid agonist (10 mg kg^{-1} i.p). To avoid any damage to the tail structures of the mice, a maximum latency of 15 sec was imposed for tail-clip if no response occurred within that time. % Analgesia was calculated by the following formula:

$$\%\ \text{Analgesia} = \left[\frac{(\text{postdrug latency}) - (\text{predrug latency})}{(\text{cutoff time} - \text{predrug latency})}\right] \times 100$$

3.5. Tail immersion tests

Experiments were performed on Swiss albino mice, 28–38 g. The details of the tail immersion test procedure used were essentially similar to those published previously elsewhere [23]. Through the use of a circulating water heater (Heto, Allerod, Denmark) a constant temperature of 52.5 ± 0.2 °C was maintained in a water bath, in which the terminal 3 cm of the animal's tail was immersed. The nociceptive end-point was characterized by a jerk of the tail. While nociception measurements were being made, the animals were briefly immobilized by gently wrapping them. A control response was determined with 0.1 mL sunflower oil and 0.9% physiological saline solution, since the test compounds and standard pure chemicals (morphine sulfate and naloxone hydrochloride) were diluted in sunflower oil and in saline solution respectively. Test latencies were assessed 30 min, after the administration of drugs to mice for all test substances and control groups. Naloxone, a specific antagonist for opioid receptors, was given 15 min prior to administration of drugs and test substances (5 mg kg^{-1} i.p.). Morphine sulphate was used as a standard opioid agonist (10 mg kg^{-1} i.p). To avoid any damage to the tail structures of the mice, a maximum latency of 15 s was imposed if no response occurred within that time. % Analgesia (% MPE) was expressed according to the formula given above.

3.6. Isolated organ bath experiments

Rats were killed by stunning and decapitation. The ileum was then excised from each animal and kept in Krebs' solution with the following composition (in mM): NaCl, 118.4; KCl, 4.7; $CaCl_2 \cdot 2\,H_2O$, 1.9; $NaHCO_3$, 25.0; $MgSO_4 \cdot 7\,H_2O$, 1.2; KH_2PO_4, 1.2 and glucose 11.1. The ileum was cleaned of adhering fat and connective tissue and cut into segments about 1.5 cm long. Isolated tissues were suspended in isolated organ baths filled with 10 ml of Krebs' solution (pH 7.4) continuously aerated with a mixture of 5% CO_2 and 95% O_2 at 37 °C. One end of the isolated ileum was connected to a tissue holder and the other to an isotonic transducer (Ugo Basile, No. 7006, Varese Italy) which was connected to a two channel pen recorder (Ugo Basile, No.7070 'Gemini', Varese, Italy). The tissues were equilibrated by incubation in the Krebs' solution for 60 min under a resting tension of 1.0 g. During the incubation period, cumulative concentration-response curves were obtained with acetylcholine chloride for ileum in the absence and presence of the test compounds. After a reproducible concentration-response relationship was obtained by repetition of the same procedure, the test substance (10^{-6}, 10^{-5} and 10^{-4} M) was used. The dose of naloxone hydrochloride was 10^{-6} M for isolated organs. The contact time of the test material was 5 min.

3.7. Statistical evaluation of data

Results are presented as mean ± s.e.m. and statistical significance between groups was analysed by analysis of variance followed by Tukey's HSD multiple comparison test and results were considered as significant where p value was <0.05.

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