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Note

1-ethylazacycloalkan-2-one indomethacin esters as new oral prodrugs: chemical stability, enzymatic hydrolysis, anti-inflammatory activity and gastrointestinal toxicity

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Abstract

In the present study two 1-ethylazacycloalkan-2-one indomethacin esters were evaluated for the potential use as prodrugs for oral delivery. These derivatives were assayed to determine 'in vitro' their stability in pH 7.4 phosphate buffer and in simulated gastric fluid and their susceptibility in undergoing enzymatic cleavage in rat plasma. Besides their anti-inflammatory and analgesic activity and gastrointestinal toxicity after oral administration in rodents were evaluated. The prodrugs were found to be stable both in pH 7.4 phosphate buffer and in simulated gastric fluid, and readily hydrolyzed by rat plasma esterase activity. When tested in the carrageenan-induced edema in the rat paw, both esters showed an anti-inflammatory activity, following chronic administration, similar to that of indomethacin, although at higher doses; interestingly, they were significantly less irritating to the gastric mucosa than the parent drug. Furthermore, in the mouse acetic acid-induced writhing assay, the prodrugs exhibited, following acute administration, a good analgesic activity. In conclusion, the present evaluation indicates that the two tested esters represent potentially useful indomethacin prodrugs for oral administration since they: (1) are stable in aqueous solution and in simulated gastric fluid; (2) are readily hydrolyzed in rat plasma; (3) retain the anti-inflammatory and analgesic activity of the parent drug; and (4) notably inhibit the gastrointestinal irritation induced by indomethacin.

Keywords: Indomethacin; Prodrugs; Hydrolysis; Anti-inflammatory activity; Ulcerogenicity; Rodents

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1. Introduction

Indomethacin is a non-steroidal, anti-inflammatory agent with antipyretic and analgesic properties (Fig. 1). It is used effectively in the management of patients with acute and chronic painful disorders of the locomotor system. Unfortunately, clinical use of indomethacin, as well as that of other nonsteroidal antiinflammatory agents (NSAIDs), is strongly limited by its gastrointestinal (GI) side effects (Champion et al., 1997), which range in both severity and frequency from relatively mild to the more serious and potentially life-threatening states, such as GI ulceration and hemorrhage. The GI side effects are generally believed to be caused by two different mechanisms (Allan and Fletcher, 1990; Guslandi, 1995). The first mechanism is based on a direct contact effect (related to both local irritation produced by the acidic groups of the NSAIDs and local inhibition of cytoprotective prostaglandin synthesis in the gastric mucosa) and on an indirect effect (attributed to a combination of an ion-trapping mechanism of NSAIDs in mucosal cells and hydrogen ion back diffusion from the lumen to the mucosa). The second mechanism involves a generalized systemic effect, occurring after absorption and manifesting itself also following intravenous administration.

The development of prodrugs to mask the acidic group of NSAIDs temporarily has been recently regarded as a promising approach to reduce their GI toxicity (Lancaster, 1995; Whitehouse and Rainsford, 1980). Besides significantly decreasing the GI irritation and retaining the antiinflammatory and analgesic action of the parent drug, NSAID prodrugs, potentially useful for oral administration, should exhibit: (1) a good stability in aqueous solution and in the GI fluid, to temporarily mask the acidic group before absorption by the oral route; (2) suitable water solubility and lipophilicity, to ensure absorption by GI mucosa; and (3) ready susceptibility to plasma hydrolysis, to release the parent drug after GI absorption; moreover, their promoiety groups should possess low systemic toxicity.

With this aim different promoieties have been taken into consideration to design new efficacious NSAID prodrugs (Bansal et al., 1994; Carty et al., 1993; Mishima et al., 1990; Ogiso et al., 1994; Olkkola et al., 1994; Tammara et al., 1993). In the present study the potential use of two 1-ethylazacycloalkan-2-one indomethacin esters as prodrugs for oral delivery was evaluated. For this purpose these compounds were assayed to determine, in vitro, their stability in simulated gastric fluid and their susceptibility in undergoing enzymatic cleavage in plasma. Besides their antiinflammatory and analgesic activity and gastrointestinal toxicity in rodents was evaluated in vivo. The rationale of this work was that a series of 1-ethylazacycloalkan-2-one indomethacin esters was previously synthetized as prodrugs for improved delivery through human skin (Bonina et al., 1991) and proved fairly stable in aqueous media and readily hydrolyzed by porcine esterases (a suitable model of skin enzymatic activity). Furthermore, the two derivatives tested in our study exhibit some favorable requirements needed for prodrug

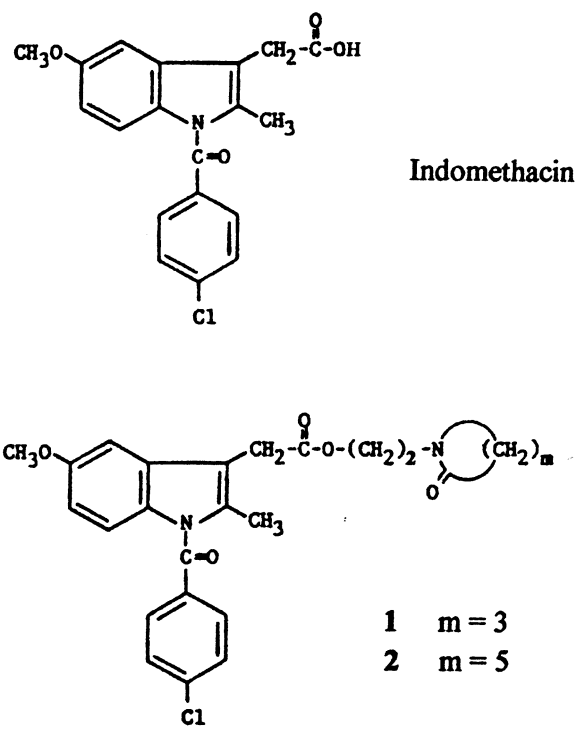

Fig. 1. Structural formulae of indomethacin and its derivatives.

oral delivery. As reported in Table 1, these compounds show good physicochemical properties (water solubility and lipophilicity) to ensure oral absorption (Bonina et al., 1991). Furthermore 1-ethylazacycloalkan-2-ones were chosen as promoieties because they are known to be free of systemic toxicity and side effects; in fact LD_{50} values of 6.5 and 2.2 g/kg are reported for oral administration, in the rat, respectively of 2-pyrrolidone (the promoiety of ester **1**) and caprolactame (the promoiety of ester **2**) (Sax, 1984).

2. Materials and methods

2.1. Drugs

Indomethacin was purchased from Sigma (Milan, Italy); esters **1** and **2** were synthetized according to the method previously described by Bonina et al. (1991). Acetonitrile, acetic acid, methanol and hydrogen chloride were obtained from Sigma (Milan, Italy).

2.2. Chemical and enzymatic hydrolysis

The chemical stability of esters **1** and **2** dissolved in isotonic phosphate buffer, pH 7.4 or in a pH 2 buffer was determined at 32°C, by following their disappearance with the HPLC method described below.

The enzymatic hydrolysis rate of esters **1** and **2** was determined by monitoring their disappearance following dissolution in rat plasma. Briefly, rat plasma samples (4 ml) were diluted with 1 ml of isotonic phosphate buffer, pH 7.4 (80% plasma) and thermostated at 37 ± 0.2°C during the experiments. The reactions were started by adding the opportune volume of a methanolic stock solution of esters **1** and **2** to 5 ml of prethermostated plasma, so as to obtain a final concentration of 4.5 μg/ml. Aliquots (300 μl) were withdrawn at intervals and deproteinized by mixing with 600 μl of 0.01 N HCl in methanol. After centrifugation at $5000 \times g$ for 5 min, 20 μl of the clear supernatant was analyzed for drug content according to the HPLC method described below.

All experiments were carried out in duplicate and repeated at least three times. Pseudo-first order rate constants for chemical and enzymatic hydrolysis were determined from the slopes of linear plots of the logarithm of residual indomethacin esters against time. The half-time ($t_{1/2}$) was calculated from the following equation:

$$t_{1/2} = (\ln 0.5)/K^1$$

where K^1 is the pseudo-first order rate constant.

The HPLC apparatus employed to determine indomethacin esters consisted of a Varian 5000 system (Varian, Walnut Creek, CA) equipped with a 20 μl loop and a Polychrom 3060 WMS detector (Varian). Integration of the chromatographic peaks was achieved with a 4290 integrator (Varian). Chromatography was performed on a Bondapak C18 column (particle size: 10 μm; 30 cm $\times$ 3.9 mm ID, Waters–Millipore, Milford, MA). The mobile phase was acetonitrile/0.1 M acetic acid (60:40, v/v). The flow-rate was set at 1.8 ml/min. Each sample was filtered prior to injection using a Millex HV13 filter (Waters–Millipore, Milford, MA) and an aliquot (20 μl) was injected into the HPLC apparatus. Detection was effected at 249 nm.

2.3. Pharmacodynamic profile

The experiments were carried out on male Sprague Dawley rats (320–350 g bw) or Swiss mice (20–22 g bw) received from Charles River Italia (Calco, Italy). The animals were maintained under normal controlled lighting and temperature conditions and allowed free access to food and water until used. The animals were fasted with free access to water for 12 h prior to the tests.

2.3.1. Anti-inflammatory activity and gastrointestinal toxicity

The rats were divided by randomization in groups (each of six animals) and given indomethacin (5, 7 mg/kg) or its derivatives (20, 40, 60 mg/kg) daily for 4 consecutive days. The drugs were administered orally, by gastric gavage, as suspensions in 10% arabic gum suspension; control animals received the same amount (10 ml/kg) of the vehicle alone.

Table 1
Chemical and enzymatic hydrolysis, water solubility and lipophilic indeces (Log K) of indomethacin and prodrugs **1** and **2**. Experiments were carried out as reported in Materials and Methods

Drugs	Half-Time			Water solubility[a] (μg/ml)	Log K[a]
	pH 7.4 buffer (h)	pH 2.0 buffer (h)	Rat plasma (min)		
Indomethacin	—	—	—	4.03	0.125
1	482	83.37	5.99	4.51	0.221
2	436	68.08	50.01	5.89	0.392

[a]data reported from Bonina et al. (1991).

The anti-inflammatory activity of esters **1** and **2** was assessed by the carrageenan-induced rat paw edema assay (Winter et al., 1962). One hour following the last drug administration, 0.2 ml of 1% carrageenan suspension in normal saline was injected subcutaneously under the planter surface of the right hind paw. The volume of the paw was measured immediately and 3 h after carrageenan injection, by the displacement technique using a plethysmometer (Basile, Comerio, Varese, Italy). The average foot swelling in each group of drug-treated rats was compared with that of the control group and the degree of anti-inflammatory activity was expressed as percent inhibition calculated according to the formula:

$$\%\text{inhibition} = \left(1 - \frac{Ed_{\text{drug}}}{Ed_{\text{contr}}}\right) \times 100$$

where Ed_{drug} is the edema volume in drug-treated rats and Ed_{contr} the edema volume in control rats.

To evaluate the gastrointestinal toxicity of derivatives **1** and **2**, the rats were sacrified by decapitation 3 h after carrageenan injection and their stomach removed, opened and washed with distilled water. The lesions on the gastric mucosa were counted by visual examination using a 2×2 binocular magnifier and their severity was scored on an arbitrary 0–4 point scale (Cioli et al., 1967); the ulceration index was the sum of their scores.

The results, expressed as mean $\pm$ S.D. of six experiments, were compared by Student's t-test for non-paired data; the difference between two values was considered significant when $p < 0.05$.

2.3.2. Analgesic activity

The analgesic activity of derivatives **1** and **2** was assessed by the acetic acid-writhing assay in the mouse (Koster et al., 1959). The animals were divided by randomization in groups (each of six mice) and given, by oral gavage, indomethacin (5, 7 mg/kg) or its esters (20, 40, 60 mg/kg), suspended in the same vehicle as that described above; control mice received the same amount (0.1 ml/10 g bw) of the drug vehicle alone. After 1 h the writhing syndrome was elicited by the intraperitoneal (i.p.) injection of 0.1 ml/10 g bw of 0.6% acetic acid in 0.9% saline solution and the number of writhes for each mouse was counted for the 20 min period between 5 and 25 min after the acetic acid injection. The average number of writhes in each group of drug-treated mice was compared with that in the control group and the degree of analgesia was expressed as percent inhibition calculated according to the formula:

$$\%\text{inhibition} = \left(1 - \frac{T}{S}\right) \times 100$$

where S is the number of writhes in control animals and T is the number of writhes in drug-treated mice.

The results, expressed as mean $\pm$ S.D. of six experiments, were compared by Student's t-test for non-paired data; the difference between two values was considered significant when $p < 0.05$.

3. Results and discussion

In the first part of our study, we investigated the chemical and enzymatic stability of the in-

Table 2
Anti-inflammatory, ulcerogenic and analgesic activity of indomethacin and prodrugs **1** and **2**. Experiments were carried out as reported in Section 2. Data concerning edema volume, ulcer index and number of writhes are expressed as mean ± S.D. of six experiments; the percent reduction in paw edema and in writhe number is reported in parenthesis

Drugs	Dose		Edema volume	Ulcers	Writhing
	mg/kg	mmol/kg	ml (% inhibition)	Ulcer index	No. writhes (% inhibition)
Vehicle	—	—	1.310 ± 0.212 (-–)	0	52.25 ± 4.63 (—)
Indomethacin	5	0.014	0.389 ± 0.071* (70.28)	1.76 ± 0.13	21.73 ± 1.78* (58.41)
	7	0.019	0.228 ± 0.036* (82.55)	3.21 ± 0.21	15.99 ± 1.22* (69.38)
1	20	0.064	0.454 ± 0.086* (65.35)	0**	28.40 ± 2.35* (45.63)
	40	0.085	0.386 ± .0.074* (70.56)	0**	26.42 ± 2.18* (49.44)
	60	0.128	0.296 ± .0.042* (77.38)	0**	24.00 ± 2.76* (54.06)
2	20	0.040	0.527 ± 0.128* (59.74)	0**	24.90 ± 2.61* (52.35)
	40	0.080	0.477 ± 0.903* (63.58)	0**	21.80 ± 2.48* (58.27)
	60	0.120	0.363 ± 0.644* (72.28)	0**	18.33 ± 2.59* (64.91)

* $P<0.05$ versus vehicle. ** $P<0.05$ versus indomethacin 5 mg/kg and indomethacin 7 mg/kg.

domethacin esters. As reported in Table 1, compounds **1** and **2** show a notable chemical stability in phosphate buffer at pH 7.4. Also when their stability is tested using buffer at pH 2 to simulate gastric fluid, esters **1** and **2** exhibit good stability. Conversely, both compounds are readily hydrolized by rat plasma esterase activity, regenerating the parent drug. Compared to ester **2**, ester **1** is hydrolyzed more rapidly in rat plasma; this observation could be attributed to the structural difference enabling a better fit of **1** to the active site of the hydrolytic enzyme.

Thus, since esters **1** and **2** have appeared to possess the chemical requirements (good stability in aqueous solution and GI fluid and ready susceptibility to plasma hydrolysis) to be regarded as indomethacin prodrugs potentially useful for oral administration, we have characterized their pharmacodynamic profile.

Table 2 shows the anti-inflammatory activity of indomethacin and of its esters after multiple oral administration in the carrageenan-induced paw edema test. These results indicate that indomethacin esters **1** and **2** maintain the anti-inflammatory activity of the parent drug, inhibiting edema formation in a dose-dependent, significant manner.

The analgesic activity of indomethacin and its esters, evaluated in the acetic acid-writhing assay, is reported in Table 2. In mice receiving an acute oral administration of derivatives **1** and **2**, an evident and dose-dependent analgesic effect was observed. The present data appear of particular interest, since other new synthesized indomethacin prodrugs, devoid of gastrolesive effects, have shown to retain the anti-inflammatory activity, but not the analgesic activity, of the parent drug (Venuti et al., 1989).

Also if both prodrugs retain the pharmacological properties of indomethacin, their anti-inflammatory and analgesic activity appears significantly lower than that of the parent drug; in fact, esters **1** and **2** elicited a pharmacological effect similar to that induced by indomethacin, but at higher doses (Table 2). This pharmacodynamic pattern appears consistent with that determined following both single and multiple oral doses of other NSAID prodrugs (Carty et al., 1993; De Capraris et al., 1994). One tempting explanation may be that the absorption of esters **1** and **2** in the GI tract is slower and/or incomplete, in comparison with that of indomethacin, very likely because of their higher molecular weight. However, also if supported by other data reported in literature (Bonina et al., 1991, 1996; Ogiso et al., 1994; Olkkola et al., 1994; Ranucci et al., 1994), such a hypothesis remains merely speculative at the present time.

As demonstrated by ulcer indeces reported in Table 2, both derivatives **1** and **2** are significantly less irritating to the gastric mucosa than the parent drug, at all doses tested (which are, on a molar ratio, higher than those employed for indomethacin). As the prodrugs remain unchanged for several hours in simulated gastric fluid, it can be assumed that they are absorbed intact, hence eliminating the local irritation produced by the free carboxilic group. Furthermore, one should take into account that other NSAID prodrugs (such as indomethacin farnesil and ampiroxicam) have been shown to be less potent than the respective parent drugs in inhibiting prostaglandin generation (Arakawa et al., 1995; Carty et al., 1993).

In conclusion, the present in vitro and in vivo evaluation indicates that 1-ethylazacycloalkan-2-one esters **1** and **2** represent potentially useful indomethacin prodrugs for oral administration. In fact, they are stable in aqueous solution and in simulated gastric fluid, are readily hydrolyzed in rat plasma, retain the antiinflammatory and analgesic action of the parent drug and notably inhibit the GI irritation produced by indomethacin. Further studies are needed to investigate the pharmacokinetic profile of these compounds and so to explain the pharmacological findings obtained in this work.

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