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Oligoethylene ester derivatives of ketoprofen, naproxen and diclofenac as oral prodrugs: a pharmacological evaluation

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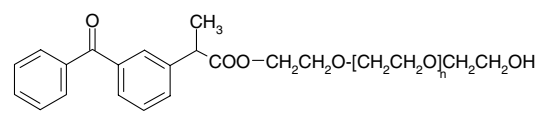
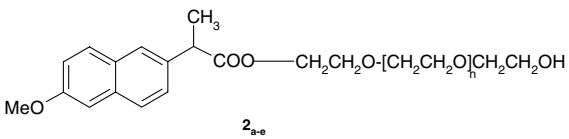
Polyoxyethylene esters of ketoprofen (**1a**–**e**), naproxen (**2a**–**e**) and diclofenac (**3a**–**e**) were tested *in vitro* to determine their stability in pH 7.4 phosphate buffer and in simulated gastric fluid (pH 2.0 buffer) and their susceptibility in undergoing enzymatic cleavage in human plasma. Furthermore their *in vivo* antiinflammatory and analgesic activity and GI toxicity were evaluated in rodents. All the prodrugs showed a good stability both in pH 7.4 phosphate buffer and in pH 2.0 buffer. They were readily hydrolyzed by human plasma and, for each group of prodrugs, no significant difference in hydrolysis rate was observed as the length of the oligoethylene chain increased. Esters **1a**–**e, 2a**–**e** and **3a**–**e** showed an anti-inflammatory activity (expressed as inhibition percent of carrageenan-induced edema in the rat) similar to that of their respective parent drug although at higher doses. The results obtained in the writhing test in mice demonstrated that all the prodrugs tested exhibited, following acute administration, a good analgesic effect. Furthermore these esters were significantly less irritating to the gastric mucosa, although administered at doses higher than the respective parent drug.

1. Introduction

Nonsteroidal antiinflammatory drugs (NSAIDs) are widely used for the treatment of pain, fever, and inflammation [1–3]. The pharmacological activity of NSAIDs is related to the suppression of prostaglandin H_2 (PGH_2) biosynthesis from arachidonic acid by inhibiting the activity of the enzyme cyclooxygenase (COX). It is well known that the chronic use of NSAIDs may elicit appreciable gastrointestinal (GI) irritation, bleeding and ulceration [2, 3], with side effects including nausea, vomiting, abdominal pain, dispepsia and diarrhea [4–6]. GI side effects of orally administered NSAIDs are usually related to two mechanisms: a topical irritancy caused by the carboxylic acid moiety present in most NSAIDs and a decreased synthesis of cytoprotective prostaglandins [7–9]. Synthetic approaches based upon NSAID chemical modification have been taken with the aim of improving the NSAID safety profile. In particular, in the prodrug approach the free carboxylic group of NSAIDs is temporarily masked, thus to prohibit its direct action on the gastric mucosa [10]. 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, (2) suitable water solubility and lipophilicity, to ensure absorption through 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 [11–14].



$\mathbf{1}_{a\text{-}e}$



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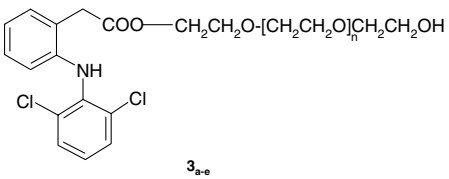

$\mathbf{3}_{a\text{-}e}$

$1_a, 2_a, 3_a$: n = 0
$1_b, 2_b, 3_b$: n = 1
$1_c, 2_c, 3_c$: n = 2
$1_d, 2_d, 3_d$: n = 3
$1_e, 2_e, 3_e$: n = 4

Among the most therapeutically efficacious NSAIDs, ketoprofen, naproxen and diclofenac appear to be very interesting. In a recent study [15] we have developed a new series of ester derivatives of these NSAIDs conjugated with polyoxyethylene glycols. These esters (**1a**–**e**, **2a**–**e** and **3a**–**e**), possessing an improved delivery through human skin, appeared to be endowed with some physicochemical characteristics (fair stability in aqueous media, ready susceptibility to porcine esterase hydrolysis, good water solubility and lipophilicity) which may be favorable for an efficacious employment as oral prodrugs. For this reason, in the present paper we have investigated if conjugation with polyoxyethylene glycols may be a useful strategy to obtain new efficient and more safe oral prodrugs of ketoprofen, naproxen and diclofenac. Thus, esters **1a**–**e**, **2a**–**e** and **3a**–**e** were tested *in vitro* for their stability in simulated gastric fluid and their susceptibility in undergoing enzymatic cleavage in human plasma. Furthermore their *in vivo* antiinflammatory and analgesic activity and GI toxicity were evaluated in rodents.

2. Investigations, results and discussion

2.1. Chemical and enzymatic hydrolysis

As reported in Table 1, esters **1a**–**e**, **2a**–**e** and **3a**–**e** showed a notable chemical stability in both buffers pH 7.4 and pH 2.0. For each group of prodrugs no significant differences in the chemical hydrolysis rate of esters **1a**–**e**, **2a**–**e** and **3a**–**e** was observed as the length of the oligoethylene chain increased. Furthermore, as reported in Table 1, the hydrolysis rate of the esters in phosphate buf-

Table 1: Chemical and enzymatic hydrolysis of prodrugs 1a–e, 2a–e and 3a–e

Compd.	$t_{1/2}$ (h)		
	Buffer pH 7.4	Buffer pH 2.0	Human plasma
1a	557	561	3.3
1b	485	467	3.6
1c	476	498	3.2
1d	591	615	2.9
1e	495	487	2.7
2a	537	548	3.5
2b	463	453	3.4
2c	491	501	3.8
2d	425	436	2.9
2e	513	518	3.1
3a	590	602	3.2
3b	547	544	2.8
3c	455	464	3.1
3d	495	488	2.9
3e	402	397	3.0

fer 7.4 was very close to that observed at pH 2.0. Since an essential prerequisite for the successful use of a prodrug is its ability to readily release the parent drug after oral administration, we assessed the enzymatic cleavage of esters **1a**–**e**, **2a**–**e** and **3a**–**e** using human plasma. In each group of prodrugs, all the esters were readily hydrolyzed by human plasma (Table 1), and no significant difference in hydrolysis rate was observed as the length of the oligoethylene chain increased.

2.2. Anti-inflammatory and analgesic activity

The percent inhibition values of carragenaan-induced edema formation by ketoprofen, naproxen and diclofenac derivatives (**1a**–**e**, **2a**–**e** and **3a**–**e**) are shown in Tables 2–4. From the results obtained esters **1a**–**e**, **2a**–**e** and **3a**–**e** appear able to elicit an appreciable anti-inflammatory activity. The analgesic activity of esters **1a**–**e**, **2a**–**e** and **3a**–**e** and their respective parent drugs, evaluated in the acetic acid-writhing assay, is reported in Tables 2–4, too. In mice receiving an acute oral administration of derivatives **1a**–**e**, **2a**–**e** and **3a**–**e** an evident dose-dependent analgesic effect was observed. However, also if the prodrugs tested retain the pharmacological properties of their respective parent drugs, their anti-inflammatory and analgesic activity appears less evident than that of the correspondent parent drugs, which elicit their pharmacological activity at much lower doses. Consistently with these data, we have previously observed that oligoethylene esters of indomethacin elicit a significant antiinflammatory and analgesic effect at a dosage level higher than that employed for the prodrug [13]. One could suggest that, due to their higher molecular weight, the GI absorption of these esters might be slower and/or incomplete in comparison with that of the parent drug; this hypothesis is confirmed by other data reported in literature [16, 17].

2.3. Ulcerogenic activity

The ulcer indexes obtained after oral administration of esters **1a**–**e**, **2a**–**e** and **3a**–**e** and of their respective parent drugs are reported in Tables 2–4. It is evident that all esters tested are significantly less irritating to the gastric mucosa than the correspondent parent drugs, at all doses tested. Since they are stable in simulated gastric fluid, the esters **1a**–**e**, **2a**–**e** and **3a**–**e** may be absorbed, very likely, intact, so preventing the local irritation produced by the free carboxylic group and the consequent gastrolesive action.

In conclusion, the present *in vitro* and *in vivo* evaluation indicates that oligoethylene esters (**1a**–**e**, **2a**–**e** and **3a**–**e**) of ketoprofen, naproxen and diclofenac are potentially efficacious prodrugs for oral administration. In fact, they are stable in aqueous solution and in simulated gastric fluid and are readily hydrolyzed in human plasma; interestingly,

Table 2: Anti-inflammatory, analgesic and ulcerogenic activity of ketoprofen and prodrugs 1a–e

Drugs	Dose		Edema volume ml (% inhibition)	Ulcers Ulcer index	Writhing No. Writhes (% inhibition)
	mg/kg	mmol/kg			
Vehicle	–	–	1.720 ± 0.275 (–)	0	51.94 ± 4.77 (–)
Ketoprofen	10	0.039	0.616 ± 0.094* (64.2)	3.72	15.69 ± 1.38* (69.8)
	15	0.058	0.506 ± 0.076* (70.6)	5.21	9.29 ± 0.82* (82.2)
1a	25	0.073	0.651 ± 0.100* (62.2)	0.48**	22.55 ± 2.05* (56.6)
	50	0.146	0.580 ± 0.087* (66.3)	0.93**	16.16 ± 1.48* (68.9)
	75	0.219	0.490 ± 0.072* (71.4)	1.45**	14.96 ± 1.33* (71.2)
1b	25	0.064	0.666 ± 0.099* (61.3)	0.41**	21.66 ± 1.90* (58.3)
	50	0.129	0.578 ± 0.086* (66.4)	0.95**	16.52 ± 1.50* (68.2)
	75	0.194	0.503 ± 0.075* (70.8)	1.21**	13.25 ± 1.19* (74.5)
1c	25	0.058	0.692 ± 0.105* (59.8)	0.35**	20.05 ± 1.76* (61.4)
	50	0.116	0.604 ± 0.093* (64.9)	0.88**	16.21 ± 1.41* (68.8)
	75	0.174	0.513 ± 0.078* (70.2)	1.13**	12.89 ± 1.14* (75.2)
1d	25	0.052	0.720 ± 0.109* (58.7)	0.24**	18.96 ± 1.70* (63.5)
	50	0.105	0.650 ± 0.098* (62.5)	0.76**	15.48 ± 1.40* (70.2)
	75	0.158	0.529 ± 0.079* (69.3)	1.02**	11.48 ± 1.05* (77.9)
1e	25	0.048	0.747 ± 0.110* (56.6)	0.22**	17.77 ± 1.58* (65.8)
	50	0.096	0.704 ± 0.104* (59.1)	0.68**	14.60 ± 1.28* (71.9)
	75	0.144	0.596 ± 0.088* (65.4)	0.93**	11.85 ± 1.03* (77.2)

* $p < 0.01$ versus vehicle; ** $p < 0.05$ versus ketoprofen 10 and 15 mg/kg.
Experiments were carried out as reported in section 3.4. Data are expressed as mean ± S.D. of six experiments; the percent reduction in paw edema and writhe number is reported in parenthesis

Table 3: Anti-inflammatory, analgesic and ulcerogenic activity of naproxen and prodrugs 2a–e

Drugs	Dose		Edema volume ml (% inhibition)	Ulcers Ulcer index	Writhing No. Writhes (% inhibition)
	mg/kg	mmol/kg			
Vehicle	–	–	1.681 ± 0.204 (–)	0	49.28 ± 4.48 (–)
Naproxen	10	0.043	1.031 ± 0.153* (38.7)	2.83	21.49 ± 1.89* (56.4)
	15	0.065	0.949 ± 0.139* (43.6)	4.98	19.08 ± 1.67* (61.3)
2a	25	0.078	1.061 ± 0.161* (36.9)	0.41**	33.22 ± 2.89* (32.6)
	50	0.157	1.007 ± 0.155* (40.1)	1.01**	26.07 ± 2.32* (47.1)
	75	0.235	0.938 ± 0.140* (44.2)	1.36**	22.38 ± 1.96* (54.6)
2b	25	0.068	1.087 ± 0.164* (35.3)	0.35**	31.89 ± 2.77* (35.3)
	50	0.137	1.002 ± 0.148* (40.4)	0.98**	24.45 ± 2.12* (50.4)
	75	0.206	0.914 ± 0.136* (45.6)	1.19**	21.10 ± 1.90* (57.2)
2c	25	0.061	1.107 ± 0.162* (34.1)	0.29**	32.13 ± 2.92* (34.8)
	50	0.123	1.013 ± 0.147* 39.7)	0.74**	24.05 ± 2.21* (51.2)
	75	0.184	0.949 ± 0.144* (43.5)	0.88**	21.39 ± 1.92* (56.6)
2d	25	0.055	1.149 ± 0.172* (32.0)	0.22**	30.51 ± 2.71* (38.1)
	50	0.110	1.049 ± 0.158* (37.6)	0.64**	22.48 ± 1.97* (54.4)
	75	0.166	0.983 ± 0.145* (41.5)	0.79**	20.90 ± 1.86* (57.6)
2e	25	0.050	1.139 ± 0.167* (32.2)	0.20**	28.88 ± 2.59* (41.4)
	50	0.101	1.072 ± 0.159* (36.2)	0.70**	22.08 ± 2.00* (55.2)
	75	0.151	0.995 ± 0.152* (40.8)	0.65**	19.77 ± 1.75* (59.9)

* $p < 0.01$ versus vehicle; ** $p < 0.05$ versus naproxen 10 and 15 mg/kg
Experiments were carried out as reported in section 3.4. Data are expressed as mean ± S.D. of six experiments; the percent reduction in paw edema and writhe number is reported in parenthesis

they retain the antiinflammatory and analgesic action of the respective parent drug, but possess a significantly lower GI toxicity. However, short term animal studies with high doses of NSAIDs may not accurately predict their toxicological profiles following long term use in humans. Further studies, especially concerning the pharmacokinetic characteristics of these esters, are needed.

3. Experimental

3.1. Materials

Ketoprofen (**1**), diclofenac (**2**) and naproxen (**3**) were all obtained from Sigma (St. Louis, MO). Esters **1a–e**, **2a–e** and **3a–e** were synthesized according to the method previously described by Bonina et al. [15]. Acetonitrile and water used in the HPLC procedures were of LC grade and were obtained from Fluka. All other chemicals were of reagent grade.

3.2. Chemical and enzymatic hydrolysis

The chemical stability of esters **1a–e**, **2a–e** and **3a–e** as solutions in isotonic phosphate buffer, pH 7.4, and in a pH 2.0 buffer was determined at 32 °C, by following the disappearance of the esters with the HPLC method described below. Human plasma fractions (4 ml) were diluted with 1 ml of isotonic phosphate buffer, pH 7.4 (80% plasma). Plasma samples were thermostated at 37 ± 0.2 °C during the experiments. The reactions were started by adding 100 µl of a stock solution of esters **1a–e**, **2a–e** and **3a–e** (1.0 mg/ml in methanol) to 5 ml of prethermostated plasma. 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 g for

Table 4: Anti-inflammatory, analgesic and ulcerogenic activity of diclofenac and prodrugs 3a–e

Drugs	Dose		Edema volume ml (% inhibition)	Ulcers Ulcer index	Writhing No. Writhes (% inhibition)
	mg/kg	mmol/kg			
Vehicle	–	–	1.794 ± 0.270 (–)	0	53.64 ± 4.66 (–)
Diclofenac	10	0.033	0.944 ± 0.138* (47.4)	2.16	27.84 ± 2.50* (48.1)
	15	0.050	0.845 ± 0.126* (52.9)	4.28	24.95 ± 2.29* (53.5)
3a	25	0.065	1.007 ± 0.154* (43.9)	0.37**	40.77 ± 3.71* (24.0)
	50	0.130	0.969 ± 0.144* (46.0)	0.98**	32.51 ± 2.86* (39.4)
	75	0.195	0.903 ± 0.136* (49.7)	1.20**	28.92 ± 2.66* (46.1)
3b	25	0.058	0.985 ± 0.147* (45.1)	0.29**	38.20 ± 3.43* (28.8)
	50	0.116	0.937 ± 0.137* (47.8)	0.79**	31.06 ± 2.73* (42.1)
	75	0.175	0.890 ± 0.131* (50.4)	0.94**	27.20 ± 2.50* (49.3)
3c	25	0.052	1.002 ± 0.154* (44.2)	0.31**	36.69 ± 3.33* (31.6)
	50	0.105	0.953 ± 0.140* (46.9)	0.86**	30.85 ± 2.74* (42.5)
	75	0.158	0.914 ± 0.136* (49.1)	1.14**	26.18 ± 2.27* (51.2)
3d	25	0.048	0.966 ± 0.145* (46.2)	0.26**	37.77 ± 3.36* (29.6)
	50	0.096	0.917 ± 0.137* (48.9)	0.68**	30.31 ± 2.72* (43.5)
	75	0.145	0.894 ± 0.126* (50.2)	0.81**	26.88 ± 2.47* (49.9)
3	25	0.044	0.944 ± 0.144* (47.4)	0.19**	35.35 ± 3.18* (34.1)
	50	0.089	0.910 ± 0.133* (49.3)	0.61**	27.74 ± 2.52* (48.3)
	75	0.133	0.874 ± 0.129* (51.3)	0.93**	24.41 ± 2.17* (54.5)

* $p < 0.01$ versus vehicle; ** $p < 0.05$ versus naproxen 10 and 15 mg/kg
Experiments were carried out as reported in section 3.4. Data are expressed as mean ± S.D. of six experiments; the percent reduction in paw edema and writhe number is reported in parenthesis

5 min, 25 µl of the clear supernatant was chromatographed as described below. The hydrolysis rate of esters **1a–e**, **2a–e** and **3a–e** was monitored following the disappearance of the ester in the plasma samples. Pseudo-first order rate constants for chemical and enzymatic hydrolysis were determined from the slopes of linear plots of the logarithm of residual esters against time.

3.3. HPLC analysis

Ketoprofen (**1**), naproxen (**2**) and their ester derivatives **1a–e** and **2a–e** were determined by HPLC using a convex gradient starting with acetonitrile/acetic acid 0.1 M 40 : 60, changing to acetonitrile/0.1 M sodium acetate 40 : 60 over 10 min, then changing to acetonitrile/0.1 M sodium acetate 60 : 40 over 10 min and then returning to the initial conditions over 5 min. Diclofenac (**3**) and esters **3a–e** were, also, determined by HPLC using a convex gradient starting with methanol/acetonitrile 60 : 40, changing to methanol/acetonitrile/0.02 M sodium acetate 25 : 20 : 55 over 10 min, then changing to methanol/acetonitrile/0.02 M sodium acetate 10 : 60 : 30 over 10 min and then returning to the initial conditions over 15 min. The flow rate was 1.4 ml/min and the effluent was continuously monitored at 254 nm. The retention times are reported in Table 1.

3.4. Animal studies

3.4.1. Animals

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, Lecco, Italy). The animals were maintained under normal conditions and allowed free access to food and water until used; they were fasted with free access to water for 12 h prior to the tests. The animal experiments were carried out according to the pertinent Italian Guidelines (D.L. 27/01/1992, n. 116); the experimental schedule was approved from the local suitable Bioethical Committee.

3.4.2. Anti-inflammatory activity

The anti-inflammatory activity of derivatives **1a–e**, **2a–e** and **3a–e** was assessed by the carrageenan-induced rat paw edema test. The rats were divided by randomization in groups of six animals. Derivatives **1a–e**, **2a–e** and **3a–e** were administered orally by gastric probe, as suspensions in 10% gum arabic solution, at three different doses (25, 50 and 75 mg/kg). Also, ketoprofen (**1**), diclofenac (**2**) and naproxen (**3**) were administered orally by gastric probe, in the same vehicle used for derivatives **1a–e**, **2a–e** and **3a–e**, at the dose of 10 and 15 mg/kg. The drugs were administered daily for four consecutive days. Control animals received the same amount (10 ml/kg) of the vehicle alone. One hour after the last drug administration, 0.2 ml of a 1% carrageenan solution in normal saline was injected subcutaneously under the plantar surface of the right hind paw. The volume of the paw was measured immediately and after 3 h, using a plethysmometer. The differences in the anti-inflammatory activity (percent inhibition) observed after the administration of derivatives **1a–e**, **2a–e** and **3a–e** and their parent drugs (**1-3**) were expressed as mean ± S.D. of six experiments and analyzed by the Student's t-test for non paired data.

3.4.3. Analgesic activity

The analgesic activity of derivatives **1a–e**, **2a–e** and **3a–e** was assessed by the acetic acid-induced writhing test. The mice were divided by randomization in groups of six animals. Compounds **1–3** (10, 15 mg/kg) or their derivatives **1a–e**, **2a–e** and **3a–e** (25, 50 and 75 mg/kg) were administered orally by gastric probe, in the same vehicle described above, 1 h before the intraperitoneal (ip) injection of 0.25 ml of a 0.5% acetic acid in 0.9% saline solution. Control animals received the same amount (0.1 ml/10 g) of the vehicle alone. The number of writhes for each mouse was counted for 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 the following expression:

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

where S is the number of writhes in control animals and T is the number of writhes in drug-treated animals. The results, expressed as mean ± S.D. of six experiments, were compared by Student's t-test for non paired data.

3.4.4. Gastric ulceration assay

Gastrointestinal toxicity was evaluated in rats, by using groups of six animals. The parent drugs (10, 15 mg/kg) or the prodrugs **1a–e**, **2a–e** and **3ae** (25, 50 and 75 mg/kg) were administered orally, by gastric probe, daily for 4 days, using the same vehicle reported above. The control animals were given only the vehicle (10% gum arabic solution). The rats were sacrificed by decapitation 4 h after the last drug administration 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; the severity of mucosal damage was assessed by a modification of a previously reported arbitrary point scale [18], where no observable damage = 0, punctiform lesions <1 mm = 1, filiform lesions <5 mm = 3, punctiform lesions >1 mm or filiform lesions >5 mm = 4. The ulceration index of each stomach was the sum of its scores.
The data concerning the gastrointestinal toxicity are reported as mean of ulcer indices calculated in six experiments and were analyzed by the Mann-Whitney test.

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