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Original article

Synthesis, pharmacological activity and hydrolytic behavior of ethylenediamine and benzathine conjugates of ibuprofen

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Abstract

For reducing the gastrointestinal toxicity associated with ibuprofen, its carboxylic group was masked by synthesizing its amide conjugates with ethylenediamine and benzathine (**4a**, **4b**, respectively) by carbodiimide assisted coupling method. In vitro hydrolysis of conjugates showed that they were stable in HCl buffer (pH 1.2) indicating that the prodrugs did not break in stomach and there was no release of ibuprofen at gastric pH, whereas in phosphate buffer (pH 7.4) they undergo significant hydrolysis and thus release ibuprofen in adequate amounts following first order kinetics. The ibuprofen conjugates **4a**, **4b** were retaining anti-inflammatory activity intact and exhibited better analgesic activity along with much reduced ulcerogenicity. These findings suggested that both the conjugates are better in action as compared to parent drug and are advantageous in having less gastrointestinal side effects. Compound **4b** however showed better analgesic activity and longer action ($t_{1/2}$) than **4a**, and hence it could be considered as a better candidate for prodrug among the two.

Keywords: NSAIDs; Ibuprofen amides; Diamine; In vitro hydrolysis; Analgesic; Ulcerogenicity

1. Introduction

Gastrointestinal (GI) side effects constitute the most frequent of all the adverse reactions of nonsteroidal anti-inflammatory drugs (NSAIDs) [1]. Even though ibuprofen is very potent and widely used among other clinically used NSAIDs, literature is abundant with its gastric and other side effects because of the presence of free carboxylic group. These reactions range in both severity and frequency leading to GI bleeding, ulceration and hemorrhage [2,3]. The major factor in the development of GI ulceration and hemorrhage induced by NSAIDs is the inhibition of prostaglandin synthesis, as the endogenous prostaglandins are known to have cytoprotective action on the gastric mucosa [4]. It has also been accepted that GI lesions produced by NSAIDs are the result of two different mechanisms: a direct contact effect and a generalized systemic effect, which may be manifested after absorption following intravenous dosing [5]. This type of damage could be prevented if the carboxylic acid functionality be masked and therefore the use of prodrug has been postulated as an approach to decrease the GI toxicity due to the direct contact effect. Some amide conjugates and a few ester derivatives of ibuprofen have been reported with reduced ulcerogenic tendency [6–11], but the search for a better prodrug with reduced side effects still continues.

The purpose of this investigation was to synthesize the diamide conjugates of ibuprofen with ethylenediamine and benzathine (*N*,*N*′-dibenzylethylenediamine) through *N*,*N*′-1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) or *N*,*N*′-dicyclohexyl carbodiimide (DCC) assisted coupling method and characterize by physicochemical, spectral (UV, IR, NMR and MS) and elemental analyses in order to establish their assigned structures. Furthermore, this report also deals with preliminary pharmacological screening of the above said conjugates along with their in vitro hydrolysis studies for determination of half life ($t_{1/2}$) as well as to evaluate

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effectivity of the diamines employed herein as linker in the prodrug approach. The rationale behind the use of ethylenediamine and benzathine to mask the free carboxylic group of the drug temporarily could be substantiated from the recent report of the effectivity of two carbon chain, i.e. $-(CH_2)_2-$ as a linker in case of polymer-drug conjugates for developing novel drug delivery system because of its structural spacing [12]. Benzathine possessing limited water solubility has been used effectively as depot form for providing effective blood level over longer period of time, as demonstrated by the wide acceptability of long acting benzathine penicillin till date.

2. Chemistry

The synthesis of the title compounds is illustrated in Fig. 1. The ethylenediamine conjugate of ibuprofen **4a** and benzathine conjugate of ibuprofen **4b** were synthesized by carbodiimide assisted coupling method. This involves condensation of the racemic carboxylic acid **1** and diamines **2a**, **2b** in the presence of an activating agent such as EDAC **3a** or DCC **3b** to produce the amide conjugates of the acid **4a**, **4b** from their respective diamines, through formation of the key intermediate *O*-acylisourea [13]. The structures of the synthesized compounds were established by their spectral (UV, IR, NMR and MS) data and elemental analysis. However, the conjugates appeared to be an enantiomeric mixture, since the parent drug employed herein was an enantiomeric mixture.

3. Biological investigations

The anti-inflammatory activity of the conjugates **4a**, **4b** was carried out on Wistar rats by following the method of Winter et al. [14]; analgesic activity was carried out on Swiss albino mice by following the method of Seigmund et al. [15] and ulcerogenic studies were carried out by the method of Cioli et al. [16] on Wistar rats. In vitro hydrolysis studies of conjugates were carried out by UV method at pH 1.2 and 7.4.

4. Results and discussion

4.1. Hydrolysis studies

Hydrolysis studies were carried out in aqueous buffer so as to study whether the prodrugs hydrolyze in aqueous medium and to what extent, or not, suggesting the fate of the prodrugs in the system. Hydrolysis kinetics of the synthesized diamide conjugates **4a** and **4b** were studied in aqueous buffer solution at pH 7.4. Under the experimental conditions the target compounds hydrolyzed to release the parent drug as evident by UV analysis. At constant pH and temperature the reaction displayed strict first order kinetics as the k_{obs} was found to be fairly constant. The data are given in Table 1.

To examine the degradation of diamide conjugates in pH as that in stomach, pH 1.2 was selected. An assay time of 2 h was selected, after which time stomach emptying would normally be effectively complete. The diamide conjugates did not hydrolyze to release the parent compound suggesting that they are stable at the gastric pH. At pH 7.4 the diamide conjugates **4a** and **4b** hydrolyzed to parent compound indicating that the prodrugs will undergo hydrolysis in the system easily [17].

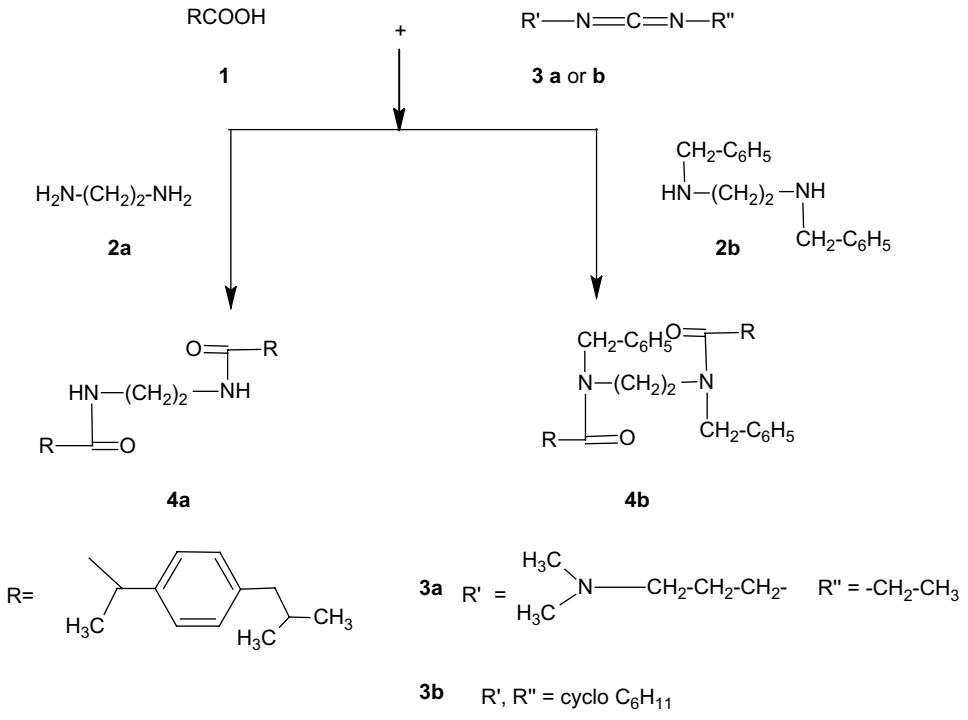

Fig. 1.

Table 1
Hydrolysis of ethylenediamine and benzathine conjugates of ibuprofen

Compound	k_{obs}[a] ± SD	$t_{1/2}$ (min)	
	Phosphate buffer (pH 7.4)	Hydrochloric acid buffer (pH 1.2)	Phosphate buffer (pH 7.4)
4a	0.01 ± 0.007	–	69.5
4b	0.008 ± 0.005	–	82.5

[a] Mean of three sets of experiments.

4.2. Anti-inflammatory activity

The inhibition of swelling in carrageenan-induced edema in rat paw brought about by oral administration of the drugs is shown in Table 2. The percentages of swelling inhibition were calculated using the equation

$$\text{Inhibition \%} = \left\{\left[(V_t - V_0)_{\text{control}} - (V_t - V_0)_{\text{treated}}\right] / (V_t - V_0)_{\text{control}}\right\} \times 100$$

wherein, V_0 and V_t relate to the average volume in the hind paw of the rats ($n = 6$) before any treatment and after anti-inflammatory agent treatment, respectively.

The diamide conjugates **4a** and **4b** showed better activity compared to the free parent drug. The maximum anti-inflammatory activity of diamide conjugates **4a** and **4b** was observed at 3 h and remained practically constant upto 6–8 h. The anti-inflammatory activity of free ibuprofen decreased with time. Statistical significance testing using one way analysis of variance showed that the anti-inflammatory activities of the parent drug and its diamide conjugate were effective in comparison with the control group. However, differences in the potency of anti-inflammatory activity of the diamide conjugates compared to the free ibuprofen were observed over a long period. Thus diamide conjugates were proved to be suitable promoieties for ibuprofen prodrugs.

4.3. Analgesic activity

The percent protection in mice brought about by administration of the drug is shown in Table 2. The diamide conjugates showed analgesic activity comparable to ibuprofen. The percent protection was calculated using equation

$$\text{Protection \%} = 100 - [\text{number of writhings in test} / \text{number of writhings in control} \times 100]$$

Table 2
Pharmacological profile of ethylenediamine and benzathine conjugates of ibuprofen

Compound	Oral dose, mg kg^{-1}	Anti-inflammatory activity (% inhibition of edema)[a]		Analgesic activity (% analgesia)[a]	Ulcer index[b]
		3 h	24 h		
Ibuprofen	20	52.15	35.65	23.42	13.51 ± 0.47
ECI	21.15	48.03	38.03	29.63	4.63 ± 0.41
BCI	29.98	53.48	32.37	35.84	Nil

[a] Statistical analysis was performed with ANOVA followed by *t*-test, $P < 0.001$.
[b] Dose: 100 mg kg^{-1} for ulcerogenic activity.

4.4. Ulcerogenic study

The ulcerogenic effect of ibuprofen and diamide conjugates **4a** and **4b** was studied at a dose of 100 mg kg^{-1}. It was observed that the ulcerogenic dose for the conjugates was almost approximately five times the dose of ibuprofen. Less number of ulcers were seen in animals treated with ethylenediamine conjugates of ibuprofen compared with the animals treated with ibuprofen. All the animals treated with benzathine conjugates of ibuprofen did not develop ulcers as they did not hydrolyze in gastric pH. These findings suggested successful masking of the carboxylic function of ibuprofen.

5. Conclusions

The diamide conjugates of ibuprofen employing ethylenediamine and benzathine were successfully synthesized by carbodiimide assisted coupling method and characterized by spectral (UV, IR, NMR and MS) data and elemental analysis. The diamide conjugates released ibuprofen quantitatively at pH 7.4, but were resistant to hydrolysis at pH 1.2 indicating that the conjugates are resistant to acidic condition and both were found to be significantly less ulcerogenic showing enhanced anti-inflammatory and analgesic activities than the parent drug. Hence diamides having two carbon atom chain as a linker could be used as effective prodrug moieties for ibuprofen. Furthermore, the benzathine conjugate of ibuprofen showed longer time of action ($t_{1/2}$) than the corresponding ethylenediamine conjugate, and hence it could be a better prodrug candidate for ibuprofen than the other one.

6. Materials and methods

All reagents were obtained from Loba Chem (India) Ltd. Ibuprofen was procured from Sun Pharmaceuticals. All the solvents used in these studies were dried and distilled before use. Wistar rats of either sex weighing between 150 and 200 g and Swiss albino mice of either sex weighing between 25 and 30 g were procured from Animal House of Dr. D.Y. Patil Institute of Pharmaceutical Sciences and Research, Pune.

6.1. Characterization of compounds

Melting points (mp): Veego VMP-PM digital melting point apparatus, uncorrected. UV spectra: Shimadzu Pharmspec 1700, UV–VIS spectrophotometer. IR spectra: Shimadzu 8400 S FT-IR. ^{1}H NMR spectra: 300 MHz JEOL NMR Spectrophotometer. The EI mass spectra were obtained from Pune University, Maharashtra, India.

6.2. 2-(4-Isobutylphenyl)-N-{2-[2-(4-isobutylphenyl) propionylamino]ethyl}propionamide **4a**

A solution of **3a** (2.67 g, 14 mmol) in 8 ml dimethylformamide was added to cooled stirring solution of **1** (2.88 g, 14 mmol) in 50 ml chloroform at 0–2 °C and kept stirring for 2 h. To the above stirring solution, **2a** (0.46 ml, 7 mmol) was then slowly added maintaining the temperature 0–2 °C and the reaction mixture was then stirred for a further period of 2 h during which it attains room temperature. After completion of reaction (TLC), it was successively washed twice with 5% $NaHCO_3$ (10 ml × 2) followed by water wash (15 ml × 2). The organic layer was separated and dried over anhydrous Na_2SO_4 followed by usual work-up to give a semisolid mass, which on trituration with pet. ether gave off-white solid product in about 61% yield. A portion of this product on recrystallization from acetone and ethanol mixture gave **4a** as white solid melting at 137–139 °C; R_f: 0.40 in benzene:ethyl acetate (2:1). UV ($CHCl_3$): λ_{max} at 244 nm (log ε 3.35). IR (KBr): 3315, 2952, 1645, 1537, 1365, 1232, 848 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.9 (s, 12H, $4CH_3$), 1.42 (d, 6H, $2CH_3$), 1.73 (m, 2H, 2C–H), 2.57 (d, 4H, $2CH_2$), 3.42 (s, 4H, CH_2–CH_2), 3.66 (s, 2H, 2CO–CH), 4.46 (br s, 2H, 2NH, D_2O exch.), 6.9–7.4 (m, 8H, Ar-H) ppm. MS (EI): *m/z* 437 (m + 1), 276, 248, 188, 161 (100%), 91, 58, 28; calc. for $C_{28}H_{40}N_2O_2$: C-77.02%, H-9.23% and N-6.42%, found: C-76.63%, H-9.16% and N-6.35%.

6.3. N-Benzyl-N-(2-{benzyl[2-(4-isobutylphenyl) propionyl]amino}ethyl)-2-(4-isobutylphenyl)-propionamide **4b**

To a cooled stirring solution of **1** (2.88 g, 14 mmol) in 50 ml chloroform was slowly added **3a** (14 mmol) in DMF (8 ml) and the mixture was kept stirring for 2 h at 0–2 °C. Benzathine base (prepared from its diacetate 3.75 g by treatment with dil. NaOH followed by extraction with $CHCl_3$ and usual work-up) was added to the above stirring solution at 0–2 °C and stirring continued for 3 h more followed by 5% $NaHCO_3$ and water wash. After usual work-up as mentioned in the earlier experiment, the product was obtained as semisolid mass, which on trituration with hexane was kept overnight in freezer to afford off-white solid in 58% yield. A portion of the above solid was recrystallized from acetone and ethanol to give **4b** as white solid melting at 89–91 °C; R_f: 0.50 in benzene:ethyl acetate (2:1). UV ($CHCl_3$): λ_{max} at 260 nm (log ε 2.94). IR (KBr): 2960, 1637, 1460, 1282, 875 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.9 (s, 12H, $4CH_3$), 1.2 (d, 6H, $2CH_3$), 1.8 (m, 2H, 2C–H), 2.3 (d, 4H, $2CH_2$), 2.4 (s, 4H), 3.5 (br s, 2H, 2CO–CH), 2.6 (t, 4H), 6.9–7.4 (m, 18H, Ar-H) ppm; MS (EI): *m/z* 617 (m + 1), 526, 435, 246, 161, 91 (100%), 57, 28; calc. for $C_{42}H_{52}N_2O_2$: C-81.78%, H-8.50% and N-4.54%, found: C-81.70%, H-8.45% and N-4.50%.

6.4. Hydrolysis study in aqueous buffers

The hydrolysis kinetics of diamide conjugates **4a** and **4b** was studied at pH 1.2 and 7.4 using hydrochloric acid buffers and phosphate buffers. The total buffer concentration was 0.05 M and constant ionic strength of 0.5 M for each sample was maintained by adding KCl. The total buffer volume was 900 ml. The mixture was equilibrated at 37 °C for 1 h. To this mixture 100 mg of each sample dissolved in alcohol was added separately and the mixture agitated by an overhead stirrer. At selected time intervals of 15, 30, 45, 60, 75, 90, 105 and 120 min, 5 ml of the solution (at pH 1.2) was removed and transferred to a separating funnel containing chloroform. Free parent drug supposed to be released after hydrolysis was estimated by UV from the chloroform extract by the following usual procedure. The samples were withdrawn at time intervals of 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 h at pH 7.4 and free drug released was estimated. The rate of reaction and hydrolysis constant were calculated with the help of the equation, $k = 2.303/t \times \log(a/a - x)$, where, '$k$' is the hydrolysis constant, 't' is time in minutes, 'a' is initial concentration of the drug, 'x' is the amount of drug hydrolyzed and '$a - x$' is the amount of drug remaining.

The initial concentration of each of the diamide conjugates was 100 mg/900 ml. The half life was calculated using the formula, $t_{1/2} = 0.693/k$ for first order reaction.

6.5. Anti-inflammatory activity

The anti-inflammatory activity was evaluated using carrageenan-induced paw edema method on rat [14]. Wistar rats (150–200 g) were divided into four groups of six animals each. Group I served control group without using the drug, group II received ibuprofen (20 mg kg^{-1}), groups III and IV received diamide conjugates **4a** and **4b** (21.15 and 29.98 mg kg^{-1}, respectively) where the dose was molecularly equivalent to ibuprofen. A stock solution of 4, 4.23 and 5.99 mg ml^{-1} was prepared as a homogeneous suspension in aqueous solution of sodium CMC (0.5% w/v) and each animal received 0.75–1.0 ml of the respective drugs orally. After 30 min of administration of drug, each rat received a sub planter injection of 0.1 ml of 1% carrageenan solution in its left hind paw. The measurement of the hind paw volume was carried out using an Ugo Basile Plethysmometer before any treatment (V_0) and in any interval (V_t) after the administration of the drugs. All the results are expressed as mean ± S.E.M. Statistical evaluation was performed using analysis of variance followed by *t*-test for sub group comparison [18].

6.6. Analgesic activity

Analgesic activity was carried out by using acetic acid induced writhing method [15] in Swiss albino mice (25–30 g) of either sex. A 1% v/v solution of acetic acid was used to induce writhing. Test compounds were administered orally 3 h prior to acetic acid injection. Number of writhings for 10 min in control and test compounds were counted and compared. Analgesic activity was measured as percent decrease in writhings in comparison to control. Mice were divided into four groups of six animals each. Group I served as a control group receiving vehicle 5 ml kg^{-1}, while group II received ibuprofen

(20 mg kg^{-1}), groups III and IV received diamide conjugates **4a** and **4b** (21.15 and 29.98 mg kg^{-1}, respectively), where the dose of conjugate was molecularly equivalent to ibuprofen. A stock solution of 1, 1.05 and 1.49 mg ml^{-1} was prepared as a homogeneous suspension in aqueous solution of sodium CMC (0.5% w/v) and each animal received 0.5–0.6 ml of the respective drugs orally. Acetic acid was administered intraperitoneally 1 ml/100 g body weight of the animal. All the results are expressed as mean ± S.E.M. Statistical evaluation was performed using analysis of variance followed by *t*-test for sub group comparison (level of significance is $P < 0.001$).

6.7. Ulcerogenicity

Gastrointestinal toxicity was determined by the method of Cioli et al. [16]. The studies were carried out on adult Wistar rats (150–200 g) at the dose of 100 mg kg^{-1}. The animals were divided into four groups of six each, group I served as control and received vehicle only. Group II received pure ibuprofen 100 mg kg^{-1}. Group III received test compound **4a** and group IV received test compound **4b** in a dose molecularly equivalent to 100 mg kg^{-1} of ibuprofen. The animals were fasted 8 h prior to administration of each of the control and test compounds. The animals were sacrificed 17 h after drug administration and food and water were available ad libitum. The gastric mucosa of the rats was examined by means of a 4× binocular magnifier. The lesions were counted and divided into large (greater than 2 mm in diameter), small (1–2 mm in diameter) and punctiform (less than 1 mm in diameter). For each stomach, the severity of mucosal damage was assessed according to the following scoring system:

- 0 – no lesions or upto five punctiform lesions;
- 1 – more than five punctiform lesions;
- 2 – 1–5 small ulcers;
- 3 – more than five small ulcers or one large ulcer;
- 4 – more than one large ulcer.

The mean score of each treated group minus the mean score of the control group was considered as the 'severity index' of gastric damage (level of significance is $P < 0.05$ with respect to control).

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