

## Synthesis, Pharmacological Evaluation and Hydrolytic Behaviour of Ethylenediamine and Benzathine Conjugates of Naproxen: Diamide Derivatives as Potential Prodrugs

N.R. CHATTERJEE\*, S.S. THUBE, A.A. KULKARNI, B.G. CHANDAK and A.D. DESHPANDE  
Department of Pharmaceutical Chemistry, Pad. Dr. D.Y. Patil College of Pharmacy,  
Akurdi, Pune-411 048, India  
E-mail: nitharc@rediffmail.com

The carboxylic acid group of naproxen was masked by synthesizing its diamide conjugates with ethylenediamine and benzathine (**4a** and **4b**) by carbodiimide assisted coupling method. *In vitro* hydrolysis of conjugates showed that they were stable in HCl buffer (pH 1.2) indicating that the conjugates did not break in stomach and therefore naproxen did not release at gastric pH; whereas in phosphate buffer (pH 7.4) significant hydrolysis following first order kinetics was observed releasing naproxen in adequate amount. The synthesized compounds **4a** and **4b** retained antiinflammatory activity intact and exhibited better analgesic activity along with much reduced ulcerogenicity on comparison with the parent drug. These findings suggested that both the conjugates are better in action and possessed less gastrointestinal side effects compared to the parent drug. However, **4b** showed better analgesic activity and longer action ( $t_{1/2}$ ) than **4a** and hence it could be considered as a more suitable candidate to act as prodrug among the two.

**Key Words:** NSAIDs, Naproxen diamides, Dialkylamines, *in vitro* hydrolysis, Analgesic, Ulcerogenicity.

### INTRODUCTION

Gastrointestinal (GI) side effects constitute the most frequent of all the adverse reactions of nonsteroidal antiinflammatory drugs (NSAIDs) and literature is abundant with gastric and other side effects of naproxen, because of the presence of free carboxylic acid group<sup>1</sup>. These adverse reactions range in both severity and frequency leading to GI bleeding, ulceration and hemorrhage<sup>2,3</sup>. 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<sup>4</sup>. Furthermore GI lesions produced by NSAIDs are the result of two different mechanisms *i.e.*, a direct contact effect and a generalized systemic effect, which may be manifested after absorption following intravenous dosing<sup>5</sup>. This type of damage could be prevented or reduced if the carboxylic acid functionality be masked through prodrug approach in order to decrease GI toxicity due to the direct contact effect. Some amide conjugates and a few ester

derivatives of naproxen with reduced ulcerogenic tendency have been reported<sup>6-12</sup> but the search for a better prodrug with reduced side effects still continues.

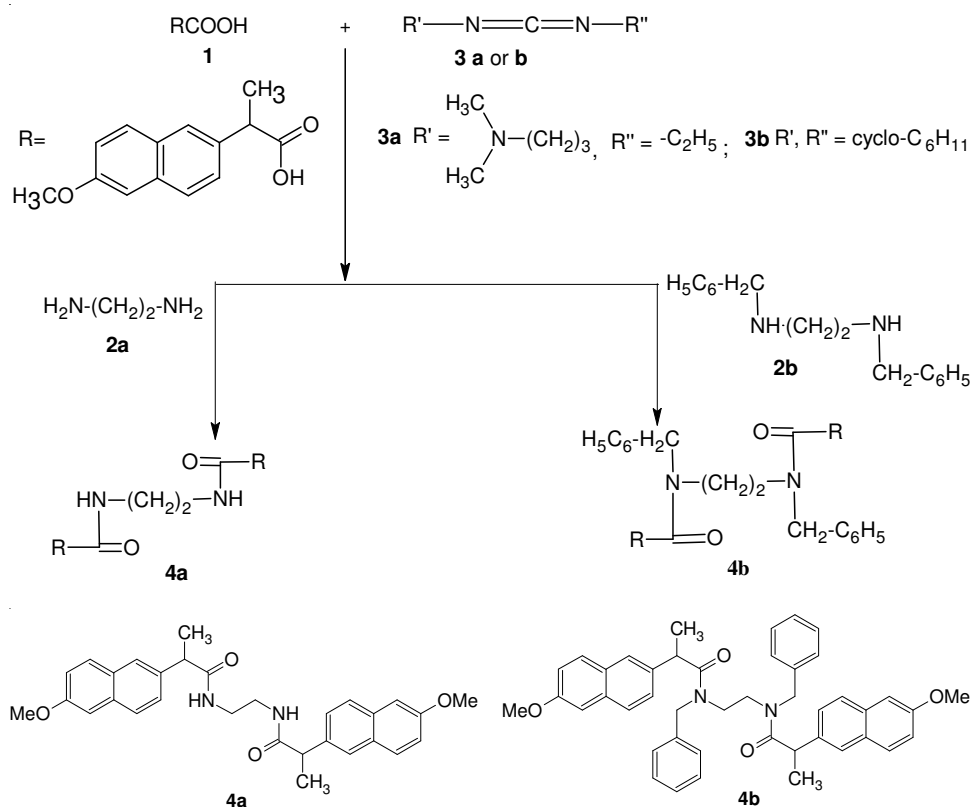
The purpose of this investigation is to synthesize the diamide conjugates of naproxen with ethylenediamine and benzathine (N,N'-dibenzylethylenediamine) through N,N'-1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) or N,N'-dicyclohexyl carbodiimide (DCC) assisted coupling method and their characterization by physicochemical, spectral (UV, IR, NMR and MS) and elemental analysis. Finally, preliminary pharmacological screening of the said conjugates were undertaken along with their *in vitro* hydrolytic behaviour for determination of half life ( $t_{1/2}$ ) in order to evaluate their biological activity and thus indicating the usefulness of the diamines deployed 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 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<sup>13</sup>. 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 to date<sup>14-16</sup>.

## EXPERIMENTAL

Melting points were determined in open capillary tubes on Veego digital automatic heated melting point apparatus and are uncorrected. UV measurements were done on Shimadzu 1700 UV-visible spectrometer and IR spectra were recorded from KBr pellets on Shimadzu 8400 FT-IR spectrometer. <sup>1</sup>H NMR spectra were run in CDCl<sub>3</sub> at 300 MHz on Shimadzu FT-NMR spectrometer with TMS as an internal standard (chemical shift in  $\delta$ ) and mass spectra were recorded on Bruker (Ultraflex TOF) spectrophotometer using electron impact technique at 70 eV and only the relevant and prominent mass fragments were considered.

**Synthesis of N,N'-bis-[2-(6-methoxynaphthyl)-ethyl]ethane-1,2-diamide (4a):** A solution of N,N'-1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) (2.63 g) in 8 mL dimethylformamide was added to a cooled stirring solution of naproxen (2.41 g) in 150 mL chloroform at 0-2 °C and kept stirring for 2 h. Ethylenediamine (0.46 mL) was then added drop wise to the above cooled solution maintaining its temperature below 2 °C. The reaction mixture was stirred for 3 h more for completion of reaction (TLC) followed by 5 % NaHCO<sub>3</sub> (15 mL  $\times$  2) and water (15 mL  $\times$  3) washes. Subsequently the organic layer was dried over anhydrous sodium sulphate, concentrated under vacuum to get semisolid mass, which on trituration with petroleum ether gave white crystalline solid product (3.4 g; 74 %) and recrystallized twice from methanol-acetone to give pure **4a** m.p. 168-70 °C; R<sub>f</sub>: 0.72;  $\lambda_{max}$  235 nm (log  $\epsilon$ : 4.0); IR (KBr,  $\nu_{max}$ , cm<sup>-1</sup>): 3326 (N-H), 3050, 2960 (C-H), 1637 (C=O amide I), 1605, 1460 (C=O, amide II), 1540, 1465, 1280, 810. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub> + DMSO-*d*<sub>6</sub>) 1.54 (d, 6H, -CH<sub>3</sub>), 1.56 (q, 2H, -CH-CO), 3.42 (t, 4H, -CH<sub>2</sub>-CH<sub>2</sub>),

3.87 (s, 6H, -OCH<sub>3</sub>), 4.36 (s, 2H, CO-NH), 7.08-7.66 (m, 12H, Ar-H). MS: m/z 485 (M<sup>+</sup>+1), 271, 213, 157, 58; Anal. calcd. (%). for C<sub>30</sub>H<sub>32</sub>N<sub>2</sub>O<sub>4</sub>: C, 74.36; H, 6.66; N, 5.78. Found (%): C, 74.01; H, 6.26; N, 5.29.



**Synthesis of N,N'-bis-[(2-(6-methoxynaphthyl)ethyl)-benzyl]ethane-1,2-diamide (4b):** To a cooled stirring solution of EDAC (2.67 g) in 8 mL dimethylformamide was added a solution of naproxen (3.22 g) in 50 mL chloroform and kept stirring for 2 h at 0-2 °C. To the above stirring solution was then added benzathine base (prepared from benzathine diacetate 3.6 g by dilute alkali treatment followed by extraction with chloroform and usual work up) by maintaining the temperature at 0-2 °C. The reaction mixture was then stirred for 2.5 h. After completion of reaction (TLC), the work-up was done following the earlier mentioned procedure to afford a semi-solid mass. It was triturated with *n*-hexane to give crude solid product in 70 % yield. A portion of the above product was recrystallized thrice from alcohol and acetone (1:9) to give off-white solid of the desired product (4b), m.p. 148-50 °C; R<sub>f</sub>: 0.75; λ<sub>max</sub>: 235 (log ε: 4.176); IR (KBr, ν<sub>max</sub>, cm<sup>-1</sup>): 3050, 2960 (C-H), 1680 (C=O), 1610, 1460, 1282 (C-N), 875. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>

+ DMSO-*d*<sub>6</sub>) 1.54 (d, 6H, -CH<sub>3</sub>), 1.56 (q, 2H, -CH-CO), 2.45 (s, 4H, Ar-CH<sub>2</sub>), 3.42 (t, 4H, -CH<sub>2</sub>-CH<sub>2</sub>), 3.86 (s, 6H, -OCH<sub>3</sub>), 7.08-7.66 (m, 22H, Ar-H) ppm. MS: m/z 665 (M<sup>+</sup>+1), 451, 213, 157, 91; Anal. calcd. (%) for C<sub>44</sub>H<sub>44</sub>N<sub>2</sub>O<sub>4</sub>: C, 79.49; H, 6.67; N, 4.21. Found (%): C, 79.03; H, 6.28; N, 4.01.

***in vitro* Hydrolysis kinetics:** The kinetics of hydrolysis cleavage of the conjugates **4a-b** was studied at 37 °C in aqueous buffer solution of pH 1.2 and pH 7.4. The total buffer concentration was around 0.05 M and ionic strength ( $\mu$ ) of 0.05 was maintained for each buffer by adding a calculated amount of potassium chloride. The reaction was monitored by measuring UV absorption for the release of free drug with time interval and thereby the order of reaction and half-life (*t*<sub>1/2</sub>) were calculated by using standard method<sup>17</sup>.

**Hydrolysis in 0.05 M phosphate acid buffer (pH 7.4):** In a stoppered 1 L volumetric flask, 800 mL of phosphate buffer (pH 7.4) was taken and each conjugate (**4a**) and (**4b**) (10 mg) dissolved in 100 mL of alcohol was added for individual experiment in the basket. It was then kept in a constant temperature bath at 37 ± 1 °C. As the equilibrium was attained, the solution in the volumetric flask was added to the buffer. The solution was occasionally stirred and 5 mL aliquot portion were withdrawn at various time intervals upto 6 h and were transferred to a separating funnel containing chloroform (10 mL). Free naproxen supposed to be released after hydrolysis was extracted twice with chloroform. The drug content in the combined chloroform layer after washing with water (10 mL) was estimated on UV spectrophotometer at 264 nm by following the standard procedure. The rate of hydrolysis for each conjugate in phosphate buffer (pH 7.4) was calculated using the equation,  $K = (2.303/t) \log (a/a-x)$ , wherein K is hydrolysis constant, t is time in min, a represents initial concentration of prodrug conjugate and (a-x) is the amount of prodrug remaining after hydrolysis period. The kinetic studies as mentioned above were carried out in triplicate for **4a** and **4b**. The K value was calculated separately from the plots in the usual manner leading to average K and hence standard deviations were also calculated. The results of the same are summarized in Table-1.

TABLE-1  
HYDROLYSIS OF ETHYLENEDIAMINE AND BENZATHINE  
CONJUGATE OF NAPROXEN

Compound	K* ± SD Phosphate buffer (pH 7.4)	<i>t</i> <sub>1/2</sub> (min)	
		Hydrochloric acid buffer (pH 1.2)	Phosphate buffer (pH 7.4)
4a	0.00081 ± 0.007	–	858.0
4b	0.00067 ± 0.005	–	1034.40

\*Mean of three sets of experiment.

**Hydrolysis in 0.05 M hydrochloric buffer (pH 1.2):** A similar experiment as described above was carried out for each of the conjugates **4a** and **4b** wherein instead of phosphate buffer (pH 7.4), HCl buffer pH (1.2) was used and no significant

hydrolysis was observed upto 6 h indicating that both the conjugates **4a** and **4b** were stable at gastric pH (1.2).

**Pharmacological evaluation:** The pharmacological evaluation of the conjugates was carried out in the department of pharmacology, Dr. D.Y. Patil Institute of Pharmaceutical Science and Research and its animal facility is approved by CPCSEA. The experimental protocols for the same have been approved by the Institutional Animal Ethics Committee. The biological screening of the conjugates was carried out with their homogenized suspension in 1 % sodium carboxymethylcellulose using the parent drug as the standard. Albino rats (Wistar strain) of either sex (150-200 g) were used for screening of all the activities. The test compounds were administered orally to the animal at doses equimolar to standard for each group of six (n = 6).

**Antiinflammatory activity:** Antiinflammatory activity testing was carried out using carrageenan induced rat paw edema method of Winter *et al.*<sup>18</sup> using naproxen as standard. Albino rats were deprived of food but not of water for 24 h before the experiment. The edema was induced by injecting 0.01 mL of 1% carrageenan suspended in 1 % carboxymethylcellulose into the planar surface of the right hind foot of each rat. The volume of paw was measured immediately using a UGO-Basile plethysmometer after carrageenan injection and again after 3 and 24 h. The test compounds and standard were given orally at 1 h prior to carrageenan injection. Mean increase in paw volume and standard error (SE) were calculated and the results were expressed as % inhibition of edema as compared to the control (Table-2).

TABLE-2  
PHARMACOLOGICAL PROFILE OF ETHYLENEDIAMINE AND  
BENZATHINE CONJUGATES OF NAPROXEN

Compound	Dose (mg/kg)	Antiinflammatory activity* (% inhibition of edema)	Analgesic activity (% analgesia)	Ulcer index
Naproxen	10.00	46.81	35.6	14.45 ± 0.42
4a	21.10	45.69	33.5	4.59 ± 0.36
4b	28.88	48.13	34.9	Nil

\*Statistical analysis was performed with ANOVA followed by Dennett's test  $p < 0.01$ .

**Analgesic activity:** Analgesic activity was determined by Eddy's hot plate method<sup>19</sup>. Albino mice were randomly distributed in control and experimental group of six animals. Test compounds were administered orally to the animals at doses equimolar to standard. After 1 h of administration, animals were placed gently on the hot plate, which was preset at 550 °C. The response of licking and/or jumping latency was recorded in seconds. The animals were removed from the hot plate soon after they exhibited jumping, cut-off time being 20 s.

**Ulcerogenic activity:** The ulcerogenic activity was determined by the cold stress method of Rainsford<sup>20</sup>. The test compounds and standard were administered orally at a dose of 10 times that of usual dose. After oral administration, animals were stressed by exposure to cold (-15 °C for 1 h). After 2 h of drug administration, the animals were sacrificed. The stomach was opened along the greater curvature

and the number of lesions was examined by means of a magnifying lens. Ulcers larger than 0.5 mm were counted. The ulcer was scored according to the method by Cioli *et al.*<sup>21</sup>. The number of lesions observed for **4b** was negligible in comparison with the controls receiving the parent drug while **4a** showed more number of lesion than **4b** but significantly lesser than the control.

## RESULTS AND DISCUSSION

The diamide conjugates of naproxen **4a** and **4b** were synthesized by carbodiimide assisted coupling method employing either N,N'-1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDAC) (**3a**) or N,N'-dicyclohexyl carbodiimide (DCC) (**3b**) and assignment of their structures was done through their satisfactory spectral (UV, IR, <sup>1</sup>H NMR and mass) data as well as elemental analysis. The said coupling reaction of naproxen with diamines using **3a** afforded cleaner product yielding about 5 % more compared to identical reaction using **3b** in place of **3a** and hence not separately described in experimental section. This could possibly be accounted due to facile formation of reactive O-acyl urea intermediate along with a soluble urea by-product in the former case; whereas dicyclohexyl urea formed as byproduct needed to be filtered out from the reaction medium in the latter case. However, the conjugates **4a** and **4b** (representing the major enantiomer) obtained by using either **3a** or **3b** were identical in all respects (m.p. and spectral data) and attempts to isolate any other enantiomer from the mother liquor by fractional crystallization proved to be abortive. The identity of **4a** and **4b** was also checked by HPLC (C-18 column) using 5 mM phosphate buffer (pH 2.6) contain in 0.9 % CH<sub>3</sub>CN as mobile phase (1 mL/min) showing strong peaks having retention time 10.11 and 9.67 min, respectively.

Preliminary pharmacological screening of the naproxen diamide conjugates (**4a** and **4b**) and their hydrolytic behaviour (at pH 1.2 and 7.4) revealed that both the conjugates were stable at gastric pH and kept antiinflammatory activity of the parent drug in tact (Table-1).

Moreover they displayed better analgesic activity and much reduced ulcerogenic tendency compared to the parent drug (Table-2).

However the benzathine conjugate of naproxen (**4b**) in addition to possessing higher  $t_{1/2}$  than that of the parent compound exhibited better biological action than its ethylenediamine counterpart **4a**. Hence, the effectivity of diamines having -(CH<sub>2</sub>)<sub>2</sub>- as linker for pro-drug approach appeared to be rationally feasible as mentioned earlier. Thus, ulcerogenicity of naproxen could mostly be overcome by conjugation with ethylenediamine or benzathine and the conjugate (**4b**) having longer retention time ( $t_{1/2}$ ) at pH 7.4 than **4a** could well be considered as a promising pro-drug candidate for naproxen.

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SAN DIEGO, CALIFORNIA, USA

*Contact:*

250 First Ave Needham, MA 02494

E-mail: [peted@healthtech.com](mailto:peted@healthtech.com)

<http://www.chi-peptalk.com/>